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Francisco Pelegri
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Vertebrate Development

Maternal to Zygotic Control

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Preface

Living systems achieve each generation the truly amazing feat of reinitiating organismal development from a single cell. A first key step in this process is the establishment of the embryonic body plan, with its species-specific, stereotypic arrangement of differing cell types within layers, tissues, and organs. In this early event, the embryo achieves multicellularity through cell division. At the same time, the resulting cells acquire different gene expression programs that will influence cell fate and behavior, often influenced by biases caused by the localization of maternal factors inherited from the egg. The processes leading to multicellularity and cell fate specification are fully integrated. For example, achieving threshold cell numbers results in zygotic gene activation, and patterns of cell division influence the segregation of patterning determinants and the three-dimensional arrangement on which those determinants act. Conversely, inheritance of maternal cell determinants can affect patterns of cell division and the behavior of cells within the overall cellular framework.

This integrated process mediates the transition between the egg as a structure generated by the previous generation and the embryo with an established set of gene expression programs. Processes during this transition, which can last several cell cycles in mammals and significantly longer in amphibians and fish, are driven largely by products produced by the mother and inherited through the egg. The end point of this transition involves the achievement of gene expression from the embryonic genes themselves and the specification of cell types. This volume addresses this transitory yet key developmental period, involving the transfer of maternal to zygotic control during embryonic development.

Our goal was to compile descriptions of various mechanisms involved in this integrated process, focusing on the vertebrate embryo, from egg activation to the initiation of zygotic gene expression and clearance of maternal factors. To achieve this, we employed a comparative approach, based on principles established largely in the primary vertebrate developmental model systems (fish, amphibians, chicken, and mice) while incorporating information from other vertebrate species where available. While the basic body plan of vertebrates is highly conserved, the strategies used by embryos in various vertebrate species to reach that basic body plan can differ. A comparative approach allows us to highlight the diversity between such

varied strategies, which result from differences in reproductive selective pressures. At the same time, comparisons can also identify mechanisms that are similar in multiple lineages, perhaps even throughout all vertebrates, and which may in turn reflect fundamental cellular and developmental mechanisms conserved through evolutionary time. Both differences and similarities are informative and essential for the modern developmental biologist to understand.

The chapters in this volume address the basic mechanisms as developmental themes and are roughly arranged following the temporal order in which the related processes are implemented during embryogenesis. Chapter One addresses changes that occur as the egg becomes fertilized, which will prevent polyspermy and initiate cascades of events that initiate embryonic development. Chapter Two describes mechanisms involved in the initiation of a primary cascade of events: the regulation of maternally inherited transcripts to produce proteins that will drive embryonic development. Chapter Three discusses mechanisms and regulation of the early embryonic cell cycle as a modified version of the cell cycle in adult cells, caused at least in part to accelerate the process of achieving multicellularity. Chapter Four describes mechanisms by which the outcome of cell division is spatially regulated, which generates the cellular arrangement of the early embryo onto which other developmental processes are implemented. Chapter Five focuses on the initial transfer of patterning information from the egg to the embryo, with particular emphasis on the Balbiani body, a cellular structure conserved throughout vertebrates that helps determine polarity in the egg and which facilitates the transfer of positional information from the egg to the embryo. Chapters Six, Seven, and Eight address further processes of cell fate information transfer to specify, respectively, embryonic axes, cellular layers, and the germ line. Chapter Nine summarizes our knowledge on mechanisms required for the initiation of expression from the zygotic genome at the end of the maternal control period, in the so-called midblastula transition, as well as changes in the cell cycle associated with this transition. Chapter Ten addresses mechanisms used by the embryo to further ensure a precise transition from maternal to zygotic control, involving the degradation of maternal factors. This chapter also describes epigenetic changes in the embryonic chromatin, which both facilitate and reinforce acquired gene expression programs. Interconnections between various developmental mechanistic themes are common and are highlighted throughout the book.

We hope this volume will be useful for the reader to obtain a more comprehensive view of early vertebrate embryogenesis, both within a single species with regard to the integration of various developmental processes and across lineage boundaries with regard to the conservation and divergence of mechanisms involved in early embryonic development. While we feel that the chapters in this volume can convey mechanistic details relevant to these processes, we also hope they can convey those broader principles which stand out for their beauty and elegance and sometimes astounding simplicity. We additionally hope that topics presented in this compilation can not only facilitate ongoing research but also inspire and engage new generations of scientifically educated audiences. Concepts conveyed in this volume are key for our basic understanding of the process of embryonic pattern

formation, with implications for our interpretation and possible prevention of developmental abnormalities and the development of reproductive biology therapies and technologies in animals, including humans. Processes occurring in the fertilized egg and early embryo may also be able to help us understand the reprogramming of cells, harnessed to implement the regeneration of embryos, cell types, and organs useful for applications varying from biomedical research to conservation biology.

As an integral element of the life cycle, the events occurring in the very early embryo represent much more than a set of related mechanistic processes. In a broader sense, these events reflect how organisms can generate pattern out of simplicity; how biological processes are reused, reorganized, and innovated; and how life continues through generations even as it adapts to new conditions. As such, the transition from the maternal to zygotic control is a microcosm that encapsulates the essence of life itself: self-generating, malleable, and enduring.

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Contents

Chapter 1
Egg Activation at Fertilization

Zoltan Machaty, Andrew R. Miller, and Lu Zhang

Abstract Fertilization is the union of gametes to initiate development of a new individual. The female gamete is formed during oogenesis. The process begins when, in the early embryo, primordial germ cells arise and subsequently colonize the genital ridges. They differentiate into oogonia, start meiosis, and become primary oocytes. The cell cycle of the primary oocytes then becomes arrested in mid-meiosis for an extended period of time. Prior to ovulation the oocytes undergo a growth phase and their sizes increase significantly. A hormonal cue then triggers oocyte maturation that involves the resumption of meiosis, the completion of the first meiotic division, and, as a result, the reduction in the diploid chromosome number. The cell cycle then stops again; in vertebrates this arrest occurs at the metaphase stage of the second meiotic division. Meiosis resumes at fertilization, when the sperm activates the egg, i.e., it causes a series of changes that are required for the initiation of embryo development. This is achieved by triggering an elevation in the egg's intracellular free calcium concentration. In response, the fertilized egg completes meiosis and enters the first embryonic cell cycle.

Keywords Fertilization • Oocyte • Egg • Activation • Sperm • Calcium • Signal transduction

1.1 Introduction

Sexual reproduction evolved 1.2 billion years ago (Butterfield 2000). Around that time some organisms stopped simply dividing into two and started to reproduce sexually; fossil records of red algae clearly show traces of specialized sex cells (spores). During sexual reproduction, the genetic material from two individuals come together through the unification of their haploid sex cells, and the arising progeny will have genes from both parents. Sex cell production is associated with

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genetic recombination, i.e., the reshuffling of the genetic information content of the DNA. This is believed to be the evolutionary advantage of sex: increased adaptation to a changing environment and the possibility to reduce the effect of deleterious mutations (Lodé 2012). In fact, among the more than 35 different forms of reproduction, it is the bisexual mode through fertilization that accomplishes this feat most effectively (Tarín and Cano 2000). Initially, sex cells were of equal size; development then led to the formation of two types of gametes that show stark dimorphism: a small, mobile gamete and a large, nonmoving one.

Fertilization is the union of the male and female gametes, a process that creates a bridge between two generations (Hardy 2002). Gamete fusion leads to the formation of a single cell with remarkable developmental potential: the zygote is able to give rise to all cell types of a new organism. The sperm delivers half of the chromosomes to restore the diploid configuration in the resulting embryo. In most species it also brings the centriole necessary for the first mitotic cell division and provides the stimulus to initiate embryo development. The stimulus arrives in the form of a calcium (Ca^{2+}) signal; in response the egg undergoes a series of changes that are collectively known as activation. The events include cortical granule exocytosis to prevent polyspermy, the completion of meiosis, and formation of the female and male pronuclei. The pronuclei migrate toward each other, and the genetic material they carry join to create the blueprint for the newly formed embryo.

In this chapter, we will briefly describe the formation of the primordial germ cells and the transformations they go through until they become mature eggs. We will touch upon the most important steps of fertilization, the chain of events that culminate in the creation of the zygote. The major emphasis will be on egg activation, the process that guarantees that the developmental program of the egg is successfully stimulated and embryo development is initiated. We will discuss in detail the machinery that is responsible for the meiotic arrest of the egg prior to fertilization and the signal transduction mechanism that stimulates this machinery to induce the resumption of meiosis and entry into the first mitotic cell cycle.

1.2 Oogenesis

Oogenesis is the process by which an egg is created with the ability to undergo fertilization. The definition of an egg requires clarification. In the strictest sense, an oocyte becomes an egg when it completes both rounds of meiotic divisions (Bi et al. 2002). Oocytes of a few species, such as the sea urchin, do finish meiosis by the time of ovulation; these are truly eggs as they await fertilization. In most animals, however, the sperm fertilizes an oocyte that has finished maturation and is arrested at a certain stage of the meiotic cell division; meiosis is completed and the second polar body is extruded only after gamete fusion. Nevertheless, many times these are also called eggs. In this review we will also use the looser terminology and refer to the female gamete as an “oocyte” when it is at a stage prior to maturation and as an “egg” once it has gone through the process of maturation and is released from the ovary.

1.2.1 Establishing the Germline

In multicellular organisms, the cells of the germline are specialized to pass on the genetic information to the progeny. As opposed to somatic lineages that ultimately perish, the germ cells have the potential to form a new individual and progress to the next generation. In the process, they carry the diploid genome through embryogenesis, divide it into two haploid sets during meiosis, and arrange it for the union with another haploid genome at fertilization to start the process over and over again (Lesch and Page 2012). The founder cells of the germline are called primordial germ cells (PGCs).

PGC formation generally begins when the future father or mother is still an embryo, but the mechanism by which PGCs are established is not conserved across species (Schoenwolf 1997). In many animals specification of germ cells occurs by preformation, i.e., through the inheritance of preformed determinants known as the germ plasm (Weissmann 1885). Certain RNAs and proteins synthesized during oogenesis are transported to, and stored at, a specific location of the oocyte. Following fertilization, cells that inherit this material will become PGCs. Experiments on anuran amphibians (frogs and toads) led to the recognition of the role of germ plasm in vertebrate germ cell specification. During oogenesis in *Xenopus laevis*, the germ plasm forms when electron-dense granules become associated with an aggregate of mitochondria, known as the mitochondrial cloud. The structure forms on one side of the cell nucleus and is then transported to the vegetal cortex of the oocyte. It contains specific proteins and RNA, and, after fertilization, it accumulates in the vegetal-most blastomeres within the presumptive endoderm. The descendants of these blastomeres will become PGCs (Whitington and Dixon 1975). It has been demonstrated that the dazl gene, with its RNA localized to the germ plasm, plays a critical role in PGC specification (Houston and King 2000).

Preformation seems to be the common mechanism for germ cell specification in teleosts as well. Zebrafish germ cells arise at the periphery of the developing blastoderm in the animal hemisphere. The embryos contain germ plasm, and although they express dazl, its RNA does not initially localize in the germ plasm but within the vegetal yolk (Maegawa et al. 1999). It accumulates in the germ plasm at a later time, after an animally directed movement (Hashimoto et al. 2004; Theusch et al. 2006). This indicates that the mechanisms of segregation of dazl in relation to the bulk of germ plasm are not conserved between *Xenopus* and zebrafish. Instead, zebrafishes synthesize vasa mRNA during oogenesis (Yoon et al. 1997). It is a component of the germ plasm, which, after fertilization, localizes to the cleavage furrows and ends up in four cells by the 32-cell stage. These four cells will give rise to the PGCs. Preformation might be the mechanism for avian germline segregation as well. A vasa protein has been identified in material associated with the mitochondrial cloud in chicken eggs. The chicken vasa protein localizes in cleavage furrows of the early embryo until it ends up in six to eight cells in the ~300-cell embryo (Tsunekawa et al. 2000).

Alternatively, PGCs form later in development and their determination is not directly dependent on maternal molecules. In these animals, PGCs are selected from multipotent embryonic cells via signals generated by neighboring cells through a process termed induction (Extavour and Akam 2003). In reptiles and mammals, no

germ plasm has been identified that would determine germ cell fate. Mouse PGCs originate from the epiblast at around embryonic day 6.25 (Ohinata et al. 2005). The extraembryonic ectoderm and visceral endoderm produce the signals that instruct a small number of epiblast cells to become PGCs. Their formation in mice is induced as a result of bone morphogenetic protein (BMP) signaling. Bmp4 expressed in the extraembryonic ectoderm has been shown sufficient to induce PGCs in cultured epiblast. Bmp2 can do the same with less efficiency, while Bmp8b is also necessary, probably to restrict inhibitory signals arising from the visceral endoderm (Ohinata et al. 2009). Inductive germ cell specification was also described in urodele amphibians (newts and salamanders). Urodele oocytes lack vegetal pole germ plasm; their PGCs arise in the lateral plate mesoderm where they form as a result of inductive signals from the ventral endoderm (Ikenishi and Nieuwkoop 1978). Following induction, Bmp activates the expression of the transcription factor Blimp1 (Prdm1). Blimp1 plays a critical role in PGC specification, as it is responsible for the repression of their somatic program (Ohinata et al. 2005). Additional proteins involved in PGC specification are Prdm14, whose role is to suppress differentiation markers (Tsuneyoshi et al. 2008), and Tcfap2c that seems to function downstream of Blimp1 to suppress mesodermal differentiation (Weber et al. 2010). At the same time, a network of pluripotency-associated genes including Oct4, Sox2, Stella, and Nanog are upregulated in nascent primordial germ cells.

Germ cells rarely become gametes at the location they first emerge. PGCs form at the perimeter of the embryo proper, and during later development they translocate to their final residence inside the embryo. Their migration is well characterized in mammals, where they move from the epiblast to the yolk sac/allantois and then to the developing hindgut until they finally colonize the genital ridges (Hyldig et al. 2011). Avian PGCs use a different path. In the chicken embryo, they move from the epiblast to the hypoblast and then reach the area known as the germinal crescent. Subsequently, PGCs enter the blood vessels and use the embryonic circulation for transport. They exit the circulation in the vicinity of the genital ridges and are drawn to their final destination by chemotactic attraction (Kuwana et al. 1986). Some reptiles utilize a pattern of PGC development similar to that of mice, while in others PGCs take a route similar to that described in the chicken, i.e., they migrate to the anterior region equivalent to the germinal crescent and travel to the genital ridges via the circulation (for a recent review, see Johnson and Alberio 2015).

Since the genetic information needs to be carried over to the next generation, the genome must remain intact during its passage through the germline. Also, the genome in germ cells must be reset to a basic, totipotent state. This is particularly important in species that do not contain maternal germ plasm, and PGC specification takes place later in development. In the mouse, for example, PGC specification is deferred until after implantation. However, the epiblast adopts somatic epigenetic features rapidly after implantation: DNA methylation levels of the epiblast in the embryonic day 6.5 embryo are more similar to somatic tissues than to the inner cell mass of the blastocyst (Popp et al. 2010). Pluripotency genes such as Oct4 and Nanog, as well as germline-specific genes, are repressed in the epiblast cells by DNA methylation; this prevents their activation, which would be detrimental at this point (reviewed by Messerschmidt et al. 2014). Due to these reasons, PGCs must undergo extensive epigenetic

reprogramming that involves DNA demethylation and histone modifications; as a result they are steered away from the path of differentiation and begin expressing major pluripotency genes. (The reader is referred to Chap. 8 of this book for additional details on germ plasm inheritance and PGC induction in the early embryo.)

1.2.2 Follicle Assembly

Once the PGCs colonize the genital ridges, they continue to proliferate. In the mouse, they proliferate until about embryonic day 13.5 when their number reaches ~25,000 (Hilscher et al. 1974). Subsequently, they undergo differentiation either toward an oogenic (female) or spermatogenic (male) pathway; in female embryos they become oogonia. In many vertebrate species, including zebrafish, *Xenopus*, chicken, and various mammals, oogonia divide to form clusters of cells connected by intercellular bridges. The founder cell in a cluster is called a cytoblast, while the cluster itself is referred to as a cyst. Oogonia in *Xenopus* develop synchronously in cysts (in this species the cyst is referred to as nest) consisting of 16 pear-shaped interconnected cells (Coggins 1973). Zebrafish oogonia also develop in nests, and although their development is synchronous, they are not connected by intercellular bridges (Selman et al. 1993). Clusters in birds and mammals do not have a fixed number of cells (Ukeshima and Fujimoto 1991; Pepling and Spradling 1998). Cyst formation is a conserved and widespread event that seems to offer certain advantages to the developing germ cells.

Oogonia in a cyst enter meiosis simultaneously at which point they become primary oocytes. This occurs in the developing embryo; in humans it takes place at the tenth week after fertilization (Gondos et al. 1986). In the pachytene stage of the first prophase, the cysts break down, and the intercellular bridges disappear. Crossing over takes place between non-sister chromatids of homologous chromosomes, which results in the recombination of the genetic information. The cell cycle then moves forward to the diplotene stage where it comes to a sudden halt (Speed 1982). This suspended state is also referred to as the dictyate stage. The block is caused by low activity of the M-phase-promoting factor (also known as maturation-promoting factor or MPF), which is essential for driving the cell cycle from prophase to metaphase. MPF is a complex of two subunits: cyclin-dependent kinase I (CDK1, also known as $p34^{cdc2}$) and its regulatory partner, cyclin B1. Due to low CDK1 and cyclin B1 levels in the oocytes and because CDK1 activity is blocked by phosphorylation at this point, the cell cycle is arrested at prophase (Kanatsu-Shinohara et al. 2000). This first meiotic arrest, which begins in fetal life and ends after the animal reaches puberty, can last for days or years depending on the species.

Somatic cells in the developing gonads envelope germ cells to create an ensemble called a follicle. Follicles provide an environment that facilitates oocyte growth and the accumulation of nutrients in the ooplasm. Somatic cells (presumptive granulosa cells) in vertebrate gonads generate processes that extend toward, and adhere to, the germ cells (Ukeshima and Fujimoto 1991). The processes invaginate from the granulosa cells and establish gap junctional contacts with the oocyte plasma

membrane; this tight physical contact between the oocyte and the granulosa cells is lost just before ovulation. In *Xenopus*, the earliest follicles contain an oocyte surrounded by a single layer of squamous follicular cells (Dumont 1972). As the oocyte begins to grow, the follicular cells become more cuboidal. Right before ovulation the follicular cells become flat and most cytoplasmic projections disappear. In the zebrafish ovary, the layer of prefollicle cells initially envelopes the entire nest of oocytes. Later, after germ cells cellularize, individual oocytes become surrounded by the somatic cells whose shape undergoes changes similar to those seen in the *Xenopus* ovary (Selman et al. 1993). Some follicular cells in the fish ovary have specialized function: a single cell called the micropylar cell participates in the formation of the micropyle that will serve as an entry point for sperm at fertilization (Hart 1990).

Follicle assembly in mammals has multiple steps. Somatic cells first envelop clusters of oocytes; in these polyovular follicles, oocytes are still connected by intercellular bridges (Weakley 1967). The intercellular bridges then disappear and the somatic cells end up encapsulating individual oocytes. The follicle at this point is called a primordial follicle, and it consists of a small oocyte surrounded by a single layer of flattened granulosa cells. The granulosa cells and their associated basement membrane completely envelop the oocyte. At the same time, a heterogeneous layer of thecal cells is added over the basement membrane, thereby creating the basic structure of the ovarian follicle (Tokarz 1978).

1.2.3 Oocyte Growth

Females of most vertebrate animals are born with primary oocytes in their ovaries; the oocytes remain dormant until the animal reaches puberty. Some increase in the size of the oocyte may occur in the prepubertal period; in fish and amphibians, the growth at this time is due to ribosomal RNA production by the nucleoli, lipid deposition, and the synthesis of a large amount of glycoproteins incorporated into the cortical alveoli (Wallace and Selman 1990). The major size increase, however, takes place after puberty, when gonadotropins produced in the pituitary gland induce oocyte growth. In nonmammalian vertebrates the oocyte grows mainly due to the accumulation of yolk (also known as vitellus). In salmonids, increased follicle-stimulating hormone (FSH) levels have been shown to stimulate estrogen production in the follicles, which in turn induce hepatic vitellogenesis (Specker and Sullivan 1994). In chickens, it is also the increased FSH level that causes vitellogenin production in the liver (Schoenwolf 1997).

During vitellogenesis a large amount of yolk is accumulated in the oocyte. The function of yolk is to provide nutrition for the developing embryo. Yolk is composed mainly of lipids and proteins (mostly lipoproteins and phosphoproteins). Vitellogenin, a glycolipophosphoprotein expressed in the females of nearly all oviparous species, is the precursor of the yolk proteins (Robinson 2008). After being synthesized in the liver, it is transported to the ovaries through the blood stream. At the ovaries, vitellogenin leaves the blood vessels, crosses the follicular wall, and, after binding its receptor on

the oolemma, is incorporated into the oocyte by endocytosis (Davail et al. 1998). Proteolytic cleavage of vitellogenin then leads to the generation of yolk proteins that are stored in yolk globules or platelets (depending on the species), throughout the ooplasm. At the time of yolk protein deposition, a significant amount of lipid accumulation also takes place in the growing oocyte of many species. The nucleus and the cytoplasm move to one pole of the oocyte called the animal pole, while the yolk becomes localized to the vegetal pole. In reptiles and birds, the sequestered cytoplasm at the animal pole forms a disklike structure called the blastodisk (or germinal disk) that contains a large nucleus, the germinal vesicle. The ancestral vitellogenin-encoding genes were lost during mammalian evolution in all but the egg-laying monotremes (because the development of placentation replaced yolk-dependent nourishment of the embryo; Brawand et al. 2008). Hence, in mammals, oocyte growth is due mostly to the accumulation of cellular organelles and lipid droplets in the ooplasm.

Concomitant with the onset of oocyte growth, the follicle also begins to develop. In mammals, granulosa cells assume a more cuboidal shape around the oocyte, during the transition from primordial follicle to primary follicle. Then these cells begin to rapidly proliferate and, as a result, enclose the oocyte in several layers creating a secondary follicle. This is followed by the formation of the antrum, a fluid-filled cavity, as the follicle turns into a tertiary (or antral) follicle. The oocyte takes up an acentric position because of the antral cavity, and the granulosa cells form two distinct cell populations: cumulus granulosa cells that immediately surround the oocyte and mural granulosa cells that cover the inner surface of the follicular wall.

Toward the end of oocyte growth, an acellular investment forms around the gamete. This structure is variously called the chorion in fish, vitelline envelope in anuran amphibians, the inner perivitelline layer in birds, and zona pellucida in mammals (Bi et al. 2002). It is made primarily of glycoproteins, and, although its composition differs from species to species, similarities in its structure across all vertebrate species examined so far point at a common evolutionary origin (Prasad et al. 2000). This extracellular matrix plays a vital role in sperm-egg recognition, determination of the sperm entry point (in certain species), the permanent block to polyspermy, and protection of the developing embryo. Fish oocytes have a special structure called the micropyle, located in the extracellular matrix, which is a pore that provides easy access for the sperm during fertilization. In most fish species, there is a single micropyle at the animal pole of the oocyte. In rare cases (such as in sturgeon and paddlefish), there are several micropyles that are also restricted to the animal pole region. The surface of the chorion in many fish species is constructed to guide the sperm toward the micropyle (Iwamatsu 2000).

1.2.4 Oocyte Maturation

Fully grown oocytes in the ovarian follicles are still diploid as they are arrested at prophase of the first meiotic division. The arrest is maintained even as the oocyte grows and its volume increases significantly. When fully grown, it is regarded as meiotically competent and is able to respond to a cue to resume meiosis. The cue

arrives when a surge of luteinizing hormone (LH), produced by the pituitary gland, binds to its receptor on the follicular cells surrounding the oocyte. In teleosts, LH triggers the production of the maturation-inducing hormone (MIH) in the granulosa cells (Patiño and Sullivan 2002). In amphibians, the synthesis of another steroid hormone, progesterone, is stimulated (Elinson 1997), whereas in mammals LH induces the removal of a follicular inhibitor (Dupré et al. 2011). Although the signals are different between animal groups, they all activate signaling pathways in the oocyte that act on the same target: they stimulate a burst in the activity of CDK1. As mentioned above, CDK1 gains activity when it forms a complex with cyclin B1, thereby generating MPF. During the prophase I arrest, MPF activity is low, probably because of limited availability of CDK1 and cyclin B1. Low levels of both subunits are the major determining factors for the maintenance of the first meiotic arrest in large mammals where protein translation is essential for the resumption of meiosis I (Mattioli et al. 1991; Tatemoto and Horiuchi 1995).

Once the oocyte reaches its final size, an additional mechanism helps to maintain the meiotic arrest. Cyclic adenosine monophosphate (cAMP), whose level in the ooplasm is tightly controlled by the granulosa cells, becomes a key regulator of CDK1 activity (Bornslaeger et al. 1986). High concentrations of cAMP are needed for the meiotic arrest; this has been demonstrated in mice, where removing the immature oocyte from the follicle leads to an abrupt reduction in ooplasmic cAMP levels and also to meiotic resumption (Conti et al. 1998). The cumulus cells control ooplasmic cAMP levels by transferring cyclic guanosine monophosphate (cGMP) to the oocyte through cytoplasmic projections across the zona pellucida. cGMP blocks phosphodiesterase 3A, an enzyme that can hydrolyze (and destroy) cAMP (Masciarelli et al. 2004). By inhibiting its hydrolysis, cGMP maintains high cAMP levels in the ooplasm. The connection between the CDK1 and cAMP pathways is provided by a serine/threonine kinase, protein kinase A (PKA). In somatic cells, cAMP can bind PKA, which in turn phosphorylates (and activates) WEE1B and MYT1 kinases. PKA can also phosphorylate the phosphatase CDC25, which as a result becomes inhibited (Kirschner et al. 2009). WEE1B and MYT1 are known to block CDK1, while CDC25 is responsible for CDK1 activation (Morgan 1995). Thus, cAMP-dependent activation of PKA results in CDK1 inhibition through two separate pathways: activation of the CDK1 inhibitors WEE1B and MYT1 and inhibition of the CDK1 activator CDC25. During the meiotic arrest, high levels of cAMP stimulate PKA. Active PKA promotes WEE1B activity and at the same time inhibits the action of CDC25; high WEE1B activity combined with CDC25 inhibition leads to phosphorylated (i.e., inactive) CDK1 and the maintenance of meiotic arrest. In response to the ovulatory LH surge, the amount of cGMP transferred from the cumulus cells drops, partly because of reduced production and partly because of the closure of gap junctions between the cumulus cells and the oocyte. The subsequent increase in phosphodiesterase activity causes a dramatic drop in cAMP levels that results in inactive PKA. In the absence of functional PKA, WEE1B activity drops and CDC25 activity increases. This causes the dephosphorylation and activation of CDK1 that favors meiotic resumption.

Proper timing of meiotic reentry is so critical that the oocyte applies yet another mechanism to keep the cell cycle under arrest. This mechanism limits CDK1 activity by curtailing cyclin B1 levels in the cytoplasm. Protein degradation during the metaphase-anaphase transition is controlled by the anaphase-promoting complex (APC), a multisubunit E3 ligase. APC ubiquitinates protein substrates that marks them for destruction by the 26S proteasome (Peters 2006). For activity, APC requires the binding of a cofactor protein, either CDC20 or FZR1. In GV-stage mouse oocytes, it is FZR1 that activates APC: oocytes lacking FZR1 undergo premature germinal vesicle breakdown (GVBD) due to the absence of APC-mediated cyclin B1 degradation (Holt et al. 2011). FZR1 is positively regulated by the phosphatase CDC14B and inhibited by the early mitotic inhibitor (Emi). It has been demonstrated that overexpression of CDC14B in mouse oocytes causes a delay in meiotic resumption (Schindler and Schultz 2009), whereas microinjection of excess Emi accelerates the reinitiation of meiosis and arrests the oocyte at the first metaphase (Marangos et al. 2007). Thus, oocytes achieve a delicate balance between high and low cyclin B1 levels via APC regulation: they maintain the arrest at the first prophase through APC-/FZR1-mediated cyclin B1 degradation; increasing cyclin B1 synthesis will eventually outweigh destruction by APC, and this leads to elevated CDK1 activity and the induction of GVBD (Holt et al. 2013).

Similar to the presence of multiple control pathways in somatic cell division, yet another mechanism is involved in the regulation of the cell cycle during oocyte maturation. In *Xenopus* oocytes progesterone, the physiological inducer of meiotic divisions, triggers the translation of mRNAs stored in the oocyte. Within 2–3 h of progesterone stimulation, the production of the Mos protein begins and accumulates during maturation. Mos is the product of the proto-oncogene c-mos and functions as a Ser/Thr kinase in a meiosis-specific manner (reviewed by Dupré et al. 2011). Mos appeared early during evolution as an oocyte-expressed kinase and functioned ancestrally in the control of female meiosis (Amiel et al. 2009). It can stimulate mitogen-activated protein kinase (MAPK) by directly phosphorylating and activating MEK, an immediate upstream activator of MAPK. The downstream target of MAPK is $p90^{Rsk}$, and together the activity of the Mos/MEK/MAPK/$p90^{Rsk}$ cascade is known as the cytostatic factor (CSF). At the onset of oocyte maturation in *Xenopus*, Mos production begins before the increase in MPF activity. Based on this observation, it has been suggested that Mos, along with its downstream effectors, is an activator of MPF (Sagata et al. 1988). In the mouse and rat, Mos synthesis is also stimulated in the maturing oocyte, but its accumulation takes place only after MPF is activated (Verlhac et al. 1993; Tan et al. 2001). This implies that Mos is not required for MPF activation. These seemingly conflicting observations can be reconciled in the light of the findings that although Mos synthesis begins soon after progesterone stimulation, the protein remains unstable and cannot stimulate MAPK until MPF is activated. Once activated, MPF stabilizes Mos via phosphorylation at conserved serine residues (Freeman et al. 1992), which prevents it from being recognized by its ubiquitin ligase. Thus, Mos gains activity only after MPF activation in both cases: it needs to be stabilized by MPF despite an early appearance in *Xenopus* oocytes, whereas it is produced only after MPF activation in the mouse

(Dupré et al. 2011). It has also been reported that progesterone can induce MPF activation by a mechanism independent of MAPK (Fisher et al. 1999), which has eventually led to the realization that MPF activation can be mediated by two independent mechanisms: either the Mos/MEK/MAPK/p90^{Rsk} pathway or cyclin B1 synthesis. Therefore, it is now believed that Mos is not essential for the activation of MPF. In both *Xenopus* and mouse, meiotic reentry depends on cyclin B1 synthesis-induced MPF activation; the Mos/MEK/MAPK/p90^{Rsk} cascade will contribute to MPF activation once Mos is stabilized by MPF (Frank-Vaillant et al. 1999).

At the onset of meiotic resumption, just prior to GVBD, the CDK1-cyclin B1 complex translocates to the nucleus, where CDK1 acts primarily on nuclear lamins and DNA histone proteins (Marangos and Carroll 2004). Nuclear lamins are building blocks of the nuclear lamina, a fibrous meshwork underlying the inner nuclear membrane. The dissolution of nuclear lamins by CDK1 causes GVBD and chromatin condensation; high CDK1 activity also drives the formation of the meiotic spindle. Homologous chromosomes (bivalents) then align at the spindle's equator during metaphase. At this point APC activity is held in check by spindle assembly checkpoint (SAC) proteins that also prevent premature separation of the bivalents (Lara-Gonzalez et al. 2012). Once the chromosomes are securely attached to the spindle, APC activity is needed for the cell cycle to move forward to anaphase. Increasing CDK1 activity stimulates the production of CDC20, the other coactivator of APC. Activated APC induces the degradation of cyclin B1, which reduces CDK1 activity allowing the cell cycle to move to anaphase (Jin et al. 2010). Meiosis I is a reductional division: after chromatin segregation the exit from meiosis is marked by half of the chromosomes being extruded in the form of the first polar body. The polar body is a tiny cell, much smaller than the oocyte (which is now haploid and called a secondary oocyte); the difference in size is due to the eccentric location of the spindle apparatus formed after GVBD.

A short interkinesis follows during which the nuclear envelope does not reappear, the DNA does not duplicate, and the oocyte enters directly into the second meiotic division. *Xenopus* oocytes acquire the ability to replicate DNA when, soon after the resumption of meiosis Cdc6, the last factor missing from the DNA synthesis toolkit is synthesized (Lemaître et al. 2002). Since all factors needed for DNA synthesis are present in the maturing oocyte, DNA replication must be inhibited to prevent S-phase entry until after fertilization. In *Xenopus*, this is achieved by Mos; if Mos or MAPK is inhibited, the nuclear envelope reforms and the DNA is replicated (Furuno et al. 1994). Despite these findings, the molecular mechanism through which the Mos/MEK/MAPK/p90^{Rsk} cascade inhibits DNA replication remains unclear. Moreover, this function of Mos does not seem to be universally conserved among vertebrates. In mouse, the ability to replicate DNA only develops after the oocyte reaches the metaphase II stage; therefore, there is no need for Mos (or any other factor) to suppress it (Tachibana et al. 2000).

Soon after the entry into the second round of meiosis, the cell cycle stops again. The role of this block is to prevent parthenogenesis, the entry into the embryonic cell cycles without sperm (Dupré et al. 2011). The cell cycle block prior to fertilization is characteristic of the entire animal kingdom; in vertebrates it occurs at the second metaphase stage of meiosis. It is controlled by CSF whose key component, Mos, is

believed to be responsible for the metaphase II arrest in eggs of all animals. This function of Mos was discovered when extracts obtained from metaphase II-arrested oocytes were found to cause metaphase arrest when injected into blastomeres of 2-cell *Xenopus* embryos (Masui and Markert 1971). It was then demonstrated that Mos-depleted mouse oocytes failed to arrest following maturation (Hashimoto et al. 1994). The metaphase II-arresting ability of Mos is mediated mostly by MAPK: the Mos/MEK/MAPK/$p90^{Rsk}$ pathway functions to sustain MPF activity (Daar et al. 1991). In mature *Xenopus* oocytes, $p90^{Rsk}$ phosphorylates Emi, which promotes its interaction with PP2A, a protein phosphatase. In turn, PP2A dephosphorylates Emi at two clusters of residues, one responsible for stabilizing the protein and the other promoting its binding to APC (Wu et al. 2007). This causes APC inhibition, which saves cyclin B1 from destruction, resulting in high CDK1 activity. MPF is thus stabilized and the cell cycle arrests at the metaphase II stage. In mouse oocytes the situation is slightly different. Although it is firmly established that Mos and MAPK are essential for the maintenance of the cell cycle block prior to fertilization, activated $p90^{Rsk}$ does not play a role in the arrest (Dumont et al. 2005). The exact mechanism by which Mos and MAPK stimulate Emi, inhibit APC, and stabilize the metaphase spindle is yet to be clarified in the mouse (Dupré et al. 2011).

Following the resumption of meiosis, during the course of nuclear maturation, the chromatin progresses to the second metaphase as described above. Cytoplasmic maturation also takes place following meiotic resumption; this enables the oocyte to undergo proper fertilization, pronuclear formation, and normal embryo development. Changes that occur during cytoplasmic maturation include migration of mitochondria from the perinuclear region to the entire cytoplasm, fragmentation and dispersion of the Golgi apparatus, relocalization of the endoplasmic reticulum primarily in the cortical cytoplasm, and an increase in the sensitivity of Ca^{2+} release channels/receptors (for a recent review, see Mao et al. 2014). LH also induces the last step of oogenesis, ovulation, i.e., the release of gametes from the ovary. It is a complex process that involves degradation of follicles, rupture of the follicular wall, and expulsion of the mature egg (Goetz et al. 1991).

1.3 Fertilization

Fertilization can be external or internal. Oviparous animals (most fishes, amphibians, reptiles, and birds) simply deposit eggs that are fertilized externally, outside of the female's body. In the case of internal fertilization, spermatozoa are released into the female body where fertilization takes place. Internal fertilization began about 385 million years ago when a fish called *Microbrachius dicki* began to multiply via copulation instead of reproducing by spawning. Ancient fossils of this primitive bony fish revealed the existence of male genitals, suggesting that *Microbrachius* marks the very point in evolution where internal fertilization in all animals began (Long et al. 2015). After sperm-egg fusion, viviparous animals brood their embryos internally, nourish them directly through the placenta, and give birth to live offspring.

Ovoviviparous animals also brood their embryos internally, but in this case there is no placental connection, and the unborn embryo is nourished by yolk within the egg. This is the situation in some fish and amphibians. Ovulation, as defined by the release of the mature egg from the ovaries, does not follow oocyte maturation in these animals: fertilization and embryo development take place in the ovarian follicles. The embryos then hatch internally and are born as live young (Jalabert 2005).

It is generally the case that the fertilizing sperm stimulates development of an egg into an embryo at the time of fertilization. Interestingly, exceptions exist. Eggs of different types of teleost fishes use different triggers for activation (Iwamatsu 2000). In internally fertilizing fishes, the sperm enters the egg and induces activation in the ovarian follicle. Some fishes with external fertilization conserved the ability to start development upon sperm penetration. Eggs of medaka (Japanese killifish) and stickleback, for example, activate in response to sperm penetration (*Oryzias*-type eggs). However, eggs of the seawater teleost fish *Alcichthys* can undergo sperm penetration and activation by sperm only after exposure to seawater, following spawning (*Alcichthys* type). The most extreme situation can be observed in salmon- and *Carassius*-type eggs, where the function of activating the egg has completely shifted from the sperm to the tonicity of the aqueous medium. These eggs can undergo activation in the absence of sperm, simply by being immersed in hypotonic saline. The identity of the trigger that stimulates activation in the absence of sperm is a matter of debate; mechanical stress, osmotic shock, and changes in the ionic environment or pH have all been suggested as potential causes. Mechanical stress caused by egg laying induces activation in the wasp *Pimpla turionellae* (Went and Krause 1974); stretch-activated channels are expressed in *Xenopus* eggs (Yang and Sachs 1989); and the presence of stretch-activated K^+ channels has been described in the fish *Misgurnus fossilis* (Medina and Bregestovski 1988). Additionally, osmotic shock may occur when during spawning, the eggs are released from the protein-rich ovarian fluid into the freshwater environment. However, the role of these stimuli, if any, during fish egg activation is yet to be demonstrated (Webb and Miller 2013).

Following sperm deposition, the spermatozoa first reach the outer layers of the egg. Fish eggs have a thick and mechanically tough shell, the chorion (vitelline envelope; Iwamatsu 1969). They possess a specialized opening, the micropyle on the chorion above the animal pole; the fertilizing sperm must swim through this hole to gain access to the egg surface (Hart and Donovan 1983). The micropyle also limits the number of sperm reaching the plasma membrane. In amphibians, the general pattern of fertilization is different between anurans and urodeles. Most anuran eggs are inseminated externally after oviposition (Elinson 1975), whereas urodele eggs are generally inseminated in the female's cloaca. Such females receive a spermatophore, a ball of sperm deposited by the male. Females store it in the spermatheca near the cloaca, and the sperm released from the spermatheca fertilizes the egg just before oviposition (Sever and Brizzi 1998). Amphibian eggs are surrounded by a vitelline envelope and 3–6 layers of jelly coat; the sperm must penetrate these investments in order to reach the plasma membrane. Sperm can only penetrate the animal half of the anuran egg but enter both the animal and vegetal halves in urodeles. Finally, only a single sperm fertilizes the egg of most anurans, while most

urodeles show physiological polyspermy, where several sperm enter each egg (see below). In birds the sperm must pass through pores in the inner perivitelline layer of the ovum to gain access to its plasma membrane or oolemma (in birds the term egg is reserved for the calcified hard-shelled product of oogenesis, whereas at the time of ovulation, the follicular oocyte becomes an ovum; Wishart and Horrocks 2000). Mammalian eggs are surrounded by the zona pellucida, and the spermatozoa must digest a penetration slit in the zona matrix in order to reach the plasma membrane.

1.3.1 Electrophysiological Changes

After getting through the egg's extracellular matrix, the fertilizing sperm adheres to, and then fuses with, the oolemma. In many species, the first indication that fertilization is under way is a change in the membrane potential of the egg's plasma membrane. Membrane potential changes during fertilization are well described in invertebrates. The resting potential of the unfertilized sea urchin egg's plasma membrane is about −70 mV. The fertilizing sperm triggers an inward current (carried mainly by an influx of extracellular Na^+ and Ca^{2+} ions) that causes depolarization of the membrane. Depolarization leads to an action potential, taking the membrane potential to positive values (Hagiwara and Jaffe 1979). The initial depolarization has been shown to coincide with gamete fusion, and the inward current that induces the action potential is the consequence of ions flowing into the ooplasm through the plasma membrane of the sperm. This was verified by measuring the electrical capacitance of the egg's plasma membrane (McCulloh and Chambers 1992). An increase in membrane capacitance is due to the addition of the sperm surface area to the oolemma (see below); the results of these experiments indicated that fusion is indeed the first step of fertilization in these species and it takes place before any other notable changes associated with egg activation.

Similar positive-going shift in the membrane potential was also reported in the eggs of certain vertebrate species including medaka (Nuccitelli 1980), lamprey (jawless fishes; Kobayashi and Yamamoto 1994), and frog (Ito 1972). The large positive fertilization potential in lamprey eggs is mediated by the opening of Cl^- channels that reside mostly in the animal pole region. In frog eggs the Ca^{2+} wave that induces activation also triggers a propagative opening of Cl^- channels in the oolemma, causing a positive shift in the membrane potential (Kline and Nuccitelli 1985). In many species, the resulting fertilization potential has an important physiological role: it serves as a fast block to polyspermy. Voltage clamping of the membrane potential of unfertilized *Xenopus* eggs blocks sperm entry, whereas maintaining it below 0 mV under voltage-clamp conditions leads to polyspermy (Charbonneau et al. 1983). The voltage that blocks sperm entry corresponds well to the fertilization potential, indicating that the fertilization potential acts as a fast, electrical block to polyspermy in these species (Jaffe and Cross 1986). The electrical block to polyspermy at the plasma membrane operates in monospermic urodele eggs as well (Iwao 2000).

In other animals such as mammals, there is no indication for an action potential after sperm-egg fusion. Physiologically polyspermic eggs of urodele amphibians lack the ability to mount a positive-going fertilization potential. Additionally, voltage clamping of the plasma membrane to positive levels does not affect sperm entry into such eggs, indicative of an absence of a fast block to polyspermy (Iwao and Jaffe 1989). Mouse and hamster eggs can generate action potentials only under nonphysiological circumstances, after being artificially hyperpolarized to −70 mV (Miyazaki and Igusa 1981; Peres 1986). In the eggs of these species, membrane potential changes of another kind can be measured following gamete fusion. Hamster oocytes show recurring hyperpolarization responses from the original −40 mV to −70 mV. These responses are due to Ca^{2+}-activated K^+ conductance, and they reflect the underlying repetitive elevations in the cytosolic Ca^{2+} levels (Miyazaki and Igusa 1982). In fertilized medaka eggs, the initial depolarization is followed by a large hyperpolarization phase that is associated with increased K^+ permeability (Nuccitelli 1980). Finally, similar hyperpolarizations were reported in mouse, rabbit, and human eggs as well, indicative of underlying Ca^{2+} oscillations in the ooplasm (Jaffe et al. 1983; McCulloh et al. 1983; Homa and Swann 1994).

1.3.2 Increase in the Cytosolic Ca^{2+} Level

Sperm-egg fusion is followed by an interval known as the latent period. The term was coined while studying sea urchin fertilization, and it refers to the time between the sperm-egg membrane fusion and the next observable event of fertilization, the initiation of the cortical reaction (Allen and Griffin 1958). Because the cortical reaction is induced by an increase in cytosolic Ca^{2+} levels, the term latent period is now used to indicate the time between the fusion and the onset of the Ca^{2+} signal that activates the egg (Whitaker and Swann 1993). The most accurate measurements regarding the length of the latent period were performed in sea urchin. Gamete fusion was controlled by holding the eggs at membrane potentials that successively allowed first sperm adhesion only and then gamete fusion (Shen and Steinhardt 1984). This revealed that the time between fusion and initiation of the Ca^{2+} wave was about 15 s. In frog eggs the latent period, defined as the time between the action potential (which indicates gamete fusion) and the Ca^{2+} wave, was found to be longer, at about 1 min (Busa and Nuccitelli 1985). In the absence of an action potential in mammalian eggs, the transfer of a fluorescent dye from the egg into the sperm was used to establish the moment of fusion. The length of the latent period determined this way in mouse eggs was around 1–3 min (Lawrence et al. 1997). In hamster, the cessation of sperm motility was used to characterize the latent period. Sperm tail movement stops when the membranes of the gametes fuse; using this as an indicator, the latent period in hamster eggs was found to be less than 10 s (Miyazaki and Igusa 1981).

The end of the latent period is marked by an increase in the egg's intracellular free Ca^{2+} concentration. In all animals that have been examined so far, the rise in the cytosolic Ca^{2+} levels is the signal the fertilizing sperm use to activate the egg and stimulate

embryo development (Stricker 1999). The elevation of Ca^{2+} level is an evolutionarily conserved mechanism for egg activation and is considered a hallmark of fertilization in all species. The sperm-induced Ca^{2+} increase was observed directly for the first time in the eggs of the medaka fish (Ridgway et al. 1977). Two lines of evidence support the notion that Ca^{2+} is a key second messenger in the stimulation of embryo development. First, artificially increasing Ca^{2+} concentration in the ooplasm triggers early events of egg activation (Whittingham 1980). Second, treatment of eggs with chelators that bind to Ca^{2+} and prevent the rise in its concentration obliterates all changes related to egg activation (Whitaker and Steinhardt 1982). These findings indicate that a Ca^{2+} rise is both necessary and sufficient to induce development at fertilization.

In lower vertebrates, as in most invertebrates, the Ca^{2+} change involves a single transient elevation in the cytosolic Ca^{2+} levels, while mammalian eggs display a series of low-frequency Ca^{2+} oscillations at fertilization (Fig. 1.1). The need for a single versus multiple Ca^{2+} transients for activation seems to depend on the cell cycle stage at which the egg awaits fertilization (eggs of invertebrate animals may arrest at stages other than metaphase II), the time needed for completion of meiosis after sperm-egg fusion, and the extent of changes the intracellular Ca^{2+} stores undergo during oocyte maturation (Jones 1998).

Upon fertilization, eggs of teleost fishes display a transient rise in their intracytoplasmic Ca^{2+} concentration. In medaka eggs the Ca^{2+} transient originates at the site of sperm entry, the micropyle. The elevated Ca^{2+} level then travels as a 0.05–0.1 mm wide band across the yolk-deficient peripheral ooplasm at an average velocity of ~12.5 μm/s, reaching the vegetal pole after about 2 min (Iwamatsu 2000; Fig. 1.2). In zebrafish eggs, the Ca^{2+} signal is made up of two components. First, a Ca^{2+} wave is initiated at the micropyle at the animal pole and propagates through the cortical cytoplasm to the vegetal pole at a velocity of ~9 μm/s (Lee et al. 1999). The cortical Ca^{2+} wave is then followed by a slower elevation of Ca^{2+} in the center of the egg (Sharma and Kinsey 2008).

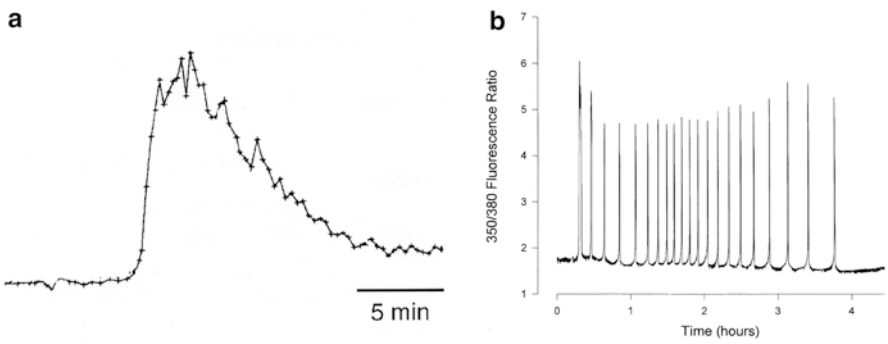

Fig. 1.1 Sperm-induced Ca^{2+} changes at fertilization. In *Xenopus* (and other vertebrates except mammals), a single Ca^{2+} rise is generated at fertilization (**a**). Mammalian eggs, on the other hand, display a series of low-frequency Ca^{2+} oscillations. Shown here is a train of Ca^{2+} spikes detected in a fertilized mouse egg (**b**). The eggs were loaded with a Ca^{2+} indicator dye and inseminated (From Nuccitelli et al. 1993; Swann and Jones 2002; with permission)

In frog eggs there is a local, slow increase in Ca^{2+} concentration at the site of sperm entry that lasts for 20–60 s (Nuccitelli et al. 1993). It is followed by a propagating Ca^{2+} wave that, as in medaka, spreads around the entire egg with a higher wave velocity in the cortex (8.9 μm/s) than in the center (5.7 μm/s) of the egg. During this time the resting Ca^{2+} concentration of ~300 nM rises to about 2.2 μm, with the average peak concentration in the center being ~60 % of that in the cortex. This elevation is maintained for 5–10 min (Fontanilla and Nuccitelli 1998). By contrast, in physiologically polyspermic urodele eggs, only a very small increase in the cytosolic Ca^{2+} level is observed. A representative of this group is the newt *Cynops pyrrhogaster*. Up to 20 sperm enter the newt egg at fertilization (Grandin and Charbonneau 1992). The first sperm that binds to the egg triggers an initial Ca^{2+} spike (Harada et al. 2011). The spike is then followed by a Ca^{2+} wave that starts at the site of sperm entry but travels only partially across the animal hemisphere. Although the exact Ca^{2+} concentration is yet to be determined, the peak level is much lower in *Cynops* than in *Xenopus* eggs. The additional sperm that enter the egg also trigger partially propagating waves so that the Ca^{2+} concentration in the newt ooplasm remains elevated for 30–40 min. The multiple Ca^{2+} waves triggered by the numerous penetrating sperm are probably necessary for complete egg activation. Although physiological polyspermy is a feature in other animals such as cartilaginous fishes, reptiles, and birds, the Ca^{2+} changes that follow gamete interaction remain unknown in these species (Iwao 2012).

Mammalian eggs, on the other hand, show a series of low-frequency Ca^{2+} oscillations at the time of fertilization that persist for several hours (Stricker 1999). The pattern of the Ca^{2+} signal varies across species; the interval between the transients is around 3 min in mice and can be as long as 50 min in cow eggs (Kashir et al. 2013). In all species studied, the first sperm-induced Ca^{2+} transient arises near the site of sperm attachment and propagates as a wave across the entire egg. In mouse and hamster, it travels with a velocity of ~20 μm/s and crosses the ooplasm in about 5 s (Miyazaki et al. 1986; Deguchi et al. 2000). In the mouse, the initial Ca^{2+} elevation lasts longer than subsequent ones, which probably reflects the transition of the ooplasm from a "non-excitable" to an "excitable" state. As the oscillations continue, the initiation site of subsequent waves changes with time; in the mouse it has been shown to translocate from the point of sperm entry to the cortex of the vegetal hemisphere (Deguchi et al. 2000).

1.3.3 The Signaling Cascade

Early observations that the fertilizing sperm is able to generate a propagating Ca^{2+} wave even in the absence of external Ca^{2+} suggested that the source of Ca^{2+} is intracellular (Gilkey et al. 1978). Since then it has been firmly established that Ca^{2+} is mobilized from the intracellular stores that reside in the smooth endoplasmic reticulum. Ca^{2+} is loaded into the store by sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPases (SERCA pumps). Their activity results in a considerable elevation in the Ca^{2+} concentration inside the lumen of the endoplasmic reticulum that can reach approximately

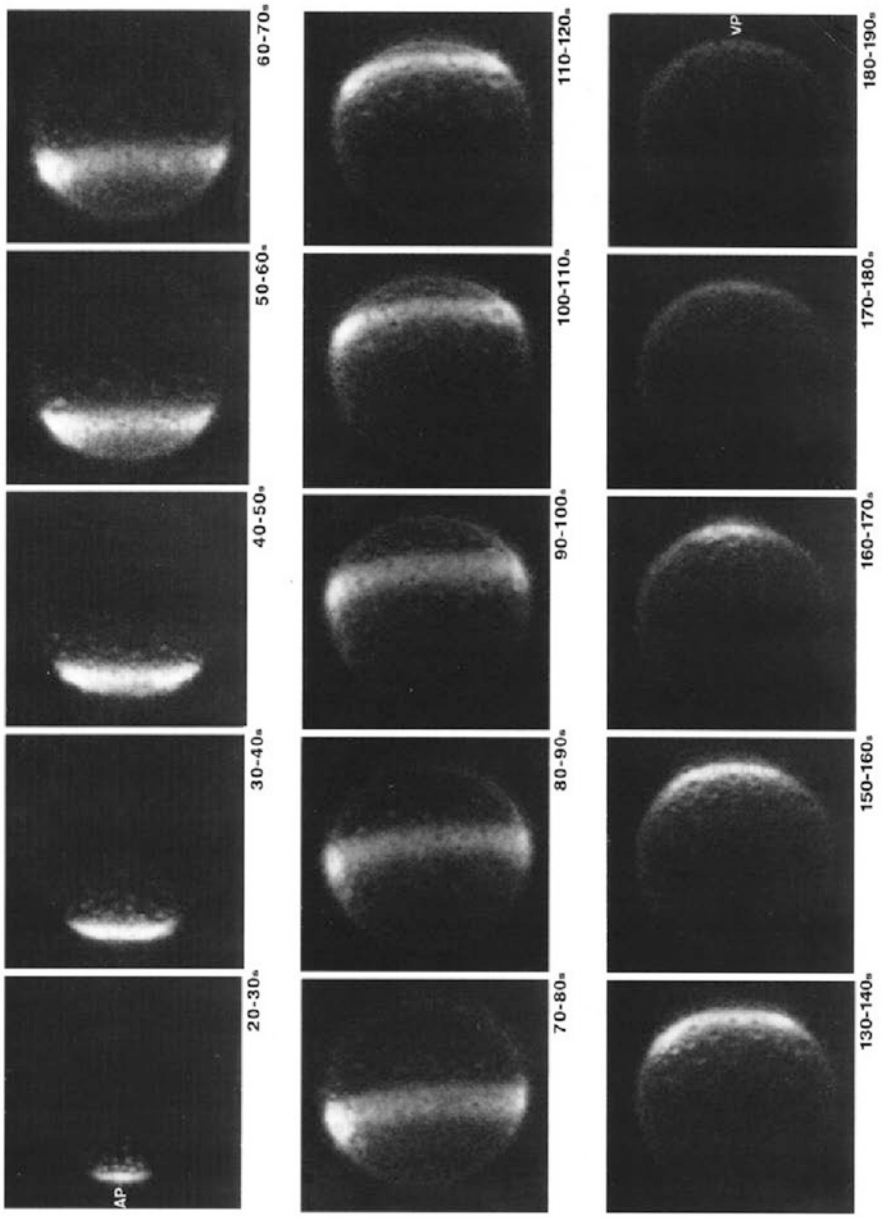

Fig. 1.2 A Ca^{2+} wave triggered by the fertilizing sperm in a medaka egg. The wave was visualized by measuring luminescence of aequorin that was preloaded into the egg's cortical cytoplasm. The photographs were taken at 10-s intervals starting 40 s after insemination. The wave started at the animal pole (AP) and traversed the egg toward the vegetal pole (VP) (From Iwamatsu 2000, with permission)

1 mM. Inside the endoplasmic reticulum, Ca^{2+} is stored through attachment to Ca^{2+}-binding proteins. Such proteins can be classified as either buffers or chaperones. Buffer proteins simply bind Ca^{2+} and thus regulate storage capacity; chaperones are involved in protein processing, while they are also capable of modulating Ca^{2+} signaling (Berridge 2002). The stored Ca^{2+} can be released into the cytoplasm via two types of Ca^{2+} release channels. These channels are massive protein complexes that also serve as receptors; they span the endoplasmic reticulum membrane and mediate the flow of Ca^{2+} into the cytosol during signaling. They are known as the inositol 1,4,5-trisphosphate (IP_3) receptor and the ryanodine receptor. The IP_3 receptor has four subunits and is gated by IP_3 or Ca^{2+} itself (Mikoshiba 1993). The ryanodine receptor is also composed of four tetramers. They open in response to cyclic adenosine diphosphate ribose (cADPR), and their gating is also controlled by Ca^{2+} (Coronado et al. 1994).

In some species, the ryanodine receptor appears to be responsible for mediating the sperm-induced Ca^{2+} increase at fertilization. Microinjection of cADPR, the endogenous activator of the ryanodine receptor into medaka eggs, induces a propagating Ca^{2+} wave in the ooplasm (Fluck et al. 1999). Ryanodine, a pharmacological modulator of the receptor, also causes activation, indicating a ryanodine receptor and a cADPR-sensitive Ca^{2+} release mechanism in medaka eggs. An endogenous supply of cADPR was also demonstrated in the gilt-head sea bream egg, where it was also shown that cADPR causes Ca^{2+} release in egg homogenates in vitro (Polzonetti et al. 2002). More importantly, cADPR levels increased more than 200-fold at fertilization in sea bream eggs. These data implicate the ryanodine receptor as the mediator of the fertilization Ca^{2+} signal in these species.

In most vertebrates, however, Ca^{2+} release is driven by IP_3. During signaling IP_3 is produced when the phosphoinositide-specific phospholipase C (PLC), an ubiquitous cytoplasmic protein, catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) into IP_3 and diacylglycerol (DAG). IP_3 then binds its receptor on the endoplasmic reticulum and induces the release of stored Ca^{2+}, while DAG stimulates protein kinase C (Miyazaki et al. 1993). In zebrafish eggs Fyn kinase, a Src-family tyrosine kinase, is concentrated at the animal pole. Src-family tyrosine kinases are non-receptor tyrosine kinases; the founding member of the family is Src, a proto-oncogene encoding a tyrosine kinase. Fertilization stimulates a Fyn-mediated rise in PLCγ activity leading to increased IP_3 levels and the generation of a Ca^{2+} wave in the cortical ooplasm (Kinsey et al. 2003). Microinjection of the GST-Fyn-SH2 fusion protein, a dominant-negative inhibitor of Fyn kinase, blocks the Ca^{2+} wave in the cortex (interestingly, it has no effect on the Ca^{2+} rise in the central, yolk-rich region of the egg, which is a characteristic feature of the Ca^{2+} signal in zebrafish). In addition hnRNP1, an RNA-binding protein of the heterogeneous nuclear ribonucleoprotein (hnRNP) family, has been suggested to regulate IP_3 levels (Mei et al. 2009). hnRNP1 is defective in the brom bones mutant of zebrafish; however, activation can be rescued by injection of Ca^{2+} or IP_3. The link between hnRNP1 and IP_3 is yet to be determined; however, it has been suggested that hnRNP1 might regulate the production of upstream activators of IP_3 such as a Src-family protein tyrosine kinase or a member of the phospholipase C family. It has also been demonstrated that the Ca^{2+} release in the central cytoplasm can be triggered in the absence

of sperm: a hypotonic shock triggers a Ca^{2+} transient in the central ooplasm (Sharma and Kinsey 2008). This later finding is consistent with the observation that the eggs of zebrafish (and some other teleosts) exhibit pre-fertilization oocyte activation, on contact with water (Sakai et al. 1997).

The sperm-induced Ca^{2+} wave in *Xenopus* eggs also appears to be stimulated via IP_3-dependent signaling (Nuccitelli et al. 1993). The sensitivity of the type I IP_3 receptor to IP_3 increases during oocyte maturation as the oocytes reorganize their endoplasmic reticulum in preparation for fertilization (Machaca 2004). The Ca^{2+} signal is associated with waves of both IP_3 and PKC activation, indicating that the IP_3 increase observed at fertilization is the result of PLC-mediated PIP_2 hydrolysis (Larabell et al. 2004; Wagner et al. 2004). Experimental data indicate that somewhat similar to zebrafish, the fertilizing sperm increases PLCγ activity and IP_3 production in the egg via the stimulation of a Src-family kinase (Sato 2008). When the Src kinase is activated by oolemma-resident uroplakin III (which itself is activated by a sperm protease associated with a surface glycoprotein), it stimulates PLCγ. Ca^{2+} increase at fertilization is not inhibited by the microinjection of recombinant SH2 domains of PLCγ, although the expression of these domains inhibits platelet-derived growth factor (PDGF)-stimulated Ca^{2+} release in eggs with exogenously expressed PDGF receptors (that are known to recruit PLCγ through the binding of its SH2 domains). Thus, the Src-family kinase seems to stimulate PLCγ through a SH2 domain-independent mechanism (Runft et al. 1999). In addition, it has also been suggested that phosphatidylinositol-3-kinase may be the upstream activator of the Src kinase (Mammadova et al. 2009). In *Cynops* eggs the injection of IP_3 triggers a Ca^{2+} transient, whereas the inhibition of the IP_3 receptors with heparin prevents the Ca^{2+} waves at fertilization. This indicates that in *Cynops* the Ca^{2+} signals induced by the fertilizing spermatozoa are probably mediated by the IP_3 receptors (Harada et al. 2011). In mammals, the generation of the fertilization Ca^{2+} transients is also mediated by the phosphoinositide signaling system. The involvement of IP_3 and its receptor in the generation of the sperm-induced Ca^{2+} signal is clearly demonstrated by the fact that a monoclonal antibody raised against the IP_3 receptor or downregulation of the receptor blocked the Ca^{2+} oscillations at fertilization (Miyazaki et al. 1992; Brind et al. 2000). In addition, sustained microinjection of IP_3 or the injection of adenophostin, an IP_3 analog, can also induce regenerative Ca^{2+} rises in mammalian eggs (Swann 1994; Jones and Nixon 2000).

1.3.4 How Does the Sperm Trigger the Ca^{2+} Signal?

Once the major components of the signaling cascade that operates at fertilization were identified, the big unresolved question remained: how does the fertilizing sperm trigger the rise in the cytosolic Ca^{2+} concentration in the egg? There have been a variety of hypotheses proposed to answer this question. One model postulated that the sperm serves as a source of Ca^{2+} entry into the egg. According to the original version, the sperm delivers a "bomb" of Ca^{2+} that eventually sets off a wave of Ca^{2+}-induced

Ca^{2+} release (Jaffe 1983). Theoretically, this mechanism is conceivable in species such as fish, frog, and hamster, where a Ca^{2+} injection indeed triggers a wave of Ca^{2+} in the egg (Gilkey 1983; Busa 1990; Igusa and Miyazaki 1983). However, the sperm volume is rather small and its limited Ca^{2+} content is insufficient to induce Ca^{2+} release. In addition, introducing Ca^{2+} into the ooplasm does not cause a Ca^{2+} wave in sea urchin or mammalian eggs (Swann and Whitaker 1986; Swann 1994), and it is also unable to trigger repetitive elevations of the cytosolic Ca^{2+} level (Swann and Ozil 1994). The hypothesis was later modified to the "conduit" model, where the sperm serves as a Ca^{2+} conduit, channeling Ca^{2+} from the extracellular medium into the egg (Jaffe 1991). The prolonged influx of Ca^{2+} then results in the overloading of the Ca^{2+} stores leading to the release of luminal Ca^{2+}. The Ca^{2+} channel blocker, La^{3+}, inhibited activation of sea urchin eggs after fertilization supporting the idea that in sea urchin an influx of Ca^{2+} is necessary for successful fertilization. In mammals, sustained injection of Ca^{2+} does not trigger regenerative Ca^{2+} rises like those seen at fertilization (Igusa and Miyazaki 1983; Swann 1994). Also, Ca^{2+} entry occurs after, rather than prior to, the first Ca^{2+} elevation after gamete fusion (McGuinness et al. 1996). This implies that the sperm conduit model cannot completely account for Ca^{2+} changes that stimulate embryo development at fertilization.

Another hypothesis proposed that the sperm induces the fertilization Ca^{2+} signal by binding to a receptor on the oolemma. It was suggested that similar to hormone-receptor binding, the interaction between the sperm and the receptor on the egg surface activates PLC in the egg. PLC then generates IP_3, which in turn binds its receptor on the Ca^{2+} stores, resulting in Ca^{2+} release. Increased turnover of polyphosphoinositides has been reported in sea urchin and frog after fertilization (Turner et al. 1984; Snow et al. 1996); IP_3 causes Ca^{2+} release very effectively in many types of eggs (Whitaker and Irvine 1984; Busa 1990; Swann and Whitaker 1986); and sustained injection of IP_3 causes regenerative Ca^{2+} rises in mammalian eggs (Swann et al. 1989). U73122, an inhibitor of PLC activity, blocks the sperm-induced Ca^{2+} transients in mouse eggs (Dupont et al. 1996), while blocking the IP_3 receptors with an antibody or with heparin also inhibits Ca^{2+} oscillations in a number of species (Miyazaki et al. 1992; Fissore and Robl 1994; Fissore et al. 1995). As in many somatic cell types, the PLC might be a β isoform, in which case it would be coupled to membrane receptors via a G protein, or a γ isoform that is linked to receptor tyrosine kinases directly. Injecting GTPγS, a nonhydrolyzable analog of GTP (that activates G proteins), causes activation in sea urchin eggs (Turner et al. 1986) and repetitive Ca^{2+} oscillations in some mammalian eggs (Miyazaki 1988; Swann 1992; Fissore et al. 1995). In addition, overexpression of the G protein-coupled muscarinic receptor in frog, mouse, and pig eggs (Williams et al. 1992; Kline et al. 1988; Machaty et al. 1997) leads to activation after exposure to the receptor's ligand. This seems to implicate the pathway that includes a G protein-coupled receptor and a PLCβ in the generation of Ca^{2+} transients. The other signaling cascade that was suggested to be involved is that mediated by receptor tyrosine kinases and the associated PLCγ enzyme. The finding that overexpression of such receptors in frog and mouse eggs leads to activation after receptor stimulation (Yim et al. 1994; Mehlmann et al. 1998) seems to support this idea. However, although recombinant SH2

domains of PLCγ inhibit PLCγ activation by the receptor, they are unable to block Ca^{2+} release at fertilization (Mehlmann et al. 1998; Runft et al. 1999). Finally, when the phosphatidylinositol signaling system is artificially activated in mammalian eggs (even if using nonhydrolyzable analogs of IP_3 or GTPγS), the resultant Ca^{2+} signal shows poor correlation with that found at fertilization (Miyazaki et al. 1990; Swann and Ozil 1994; Galione et al. 1994). Altogether, these data suggest that eggs contain the signaling machinery that is normally associated with cell surface receptors, but do not necessarily mean that the fertilizing sperm actually use these signaling cascades to trigger embryo development.

Xenopus eggs have been shown to activate in response to external application of sperm components (Iwao et al. 1994), and peptides containing an RGD sequence (an integrin-binding site found in a sperm-associated protein) cause a Ca^{2+} increase (Iwao and Fujimura 1996). In addition, *Xenopus* sperm contain a protein, xMDC16, that is a member of the metalloprotease-/disintegrin-/cysteine-rich protein family (Shilling et al. 1997). Peptides containing a sequence of the disintegrin domain of xMDC16 trigger a Ca^{2+} increase and activation when applied near the egg surface (Shilling et al. 1998). Such peptides are able to bind and stimulate potential integrin-like egg surface receptors (Foltz and Shilling 1993). These results suggest that in *Xenopus* the fertilization Ca^{2+} signal is in fact generated via surface membrane interactions between the gametes (Iwao 2000). However, up to now no receptor has been identified for either the RGD-containing sequence or the xMDC16. Incubation in the presence of a soluble RGD peptide also induces Ca^{2+} increase in bovine eggs (Campbell et al. 2000), but again, the receptors that have been identified on mammalian eggs so far seem to be involved in gamete binding and fusion, rather than the stimulation of Ca^{2+} release (Wassarman et al. 2005).

The sperm content hypothesis claims that the fertilization Ca^{2+} signal is triggered by a substance in the sperm that diffuses into the ooplasm following sperm-egg fusion (Fig. 1.3). In mouse eggs, gamete fusion precedes the Ca^{2+} oscillations by 1–3 min, which is consistent with the idea that a factor diffuses into the ooplasm and induces Ca^{2+} release (Lawrence et al. 1997). Microinjection of a crude extract from the sperm head into mammalian eggs was found to stimulate a series of low-frequency Ca^{2+} oscillations identical to those seen at fertilization (Swann 1990; Wu et al. 1997; Machaty et al. 2000). Initially, it was suggested that the extract's active factor might be IP_3 (Tosti et al. 1993); later, in a number of invertebrate species, nitric oxide (NO; Kuo et al. 2000) and nicotinic acid adenine dinucleotide phosphate (NAADP; Lim et al. 2001) were also proposed. Heat or trypsin treatment of the mammalian sperm extract abolished its Ca^{2+}-inducing activity suggesting that the mammalian sperm factor was a protein, and adding the extract to the eggs did not cause Ca^{2+} release either indicating that the factor was specific to the sperm cytosol (Swann 1990). The activity of the sperm factor is a general phenomenon in mammalian species (Swann et al. 1998), and furthermore, extracts isolated from frog or chicken sperm cause Ca^{2+} oscillations in mouse eggs (Dong et al. 2000). The success of intracytoplasmic sperm injection in mammals, where membrane interaction between the gametes is bypassed by the direct injection of the sperm into the ooplasm, also supports the notion that it is a factor in the sperm head that stimulates Ca^{2+} changes at fertilization.

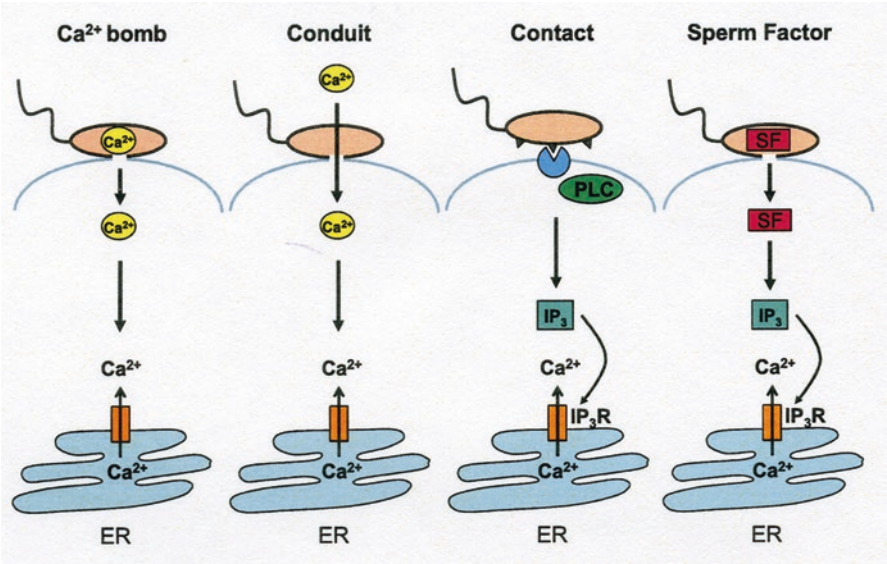

Fig. 1.3 Different hypotheses describing alternative mechanisms by which the fertilizing sperm stimulates the release of Ca^{2+} from the endoplasmic reticulum (*ER* endoplasmic reticulum, *IP*$_3$ inositol 1,4,5-trisphosphate, *PLC* phospholipase C, *SF* sperm factor; from Nomikos et al. 2011, with permission)

One candidate for the sperm factor was "oscillin," a protein identified in hamster sperm. Oscillin was isolated by serial chromatographic purification, and when injected into mouse eggs, it was able to induce a series of Ca^{2+} oscillations (Parrington et al. 1996). It is located in the equatorial segment region of the sperm head, which is the expected location of a signaling molecule that is to trigger the fertilization Ca^{2+} signal after gamete fusion. Its amino acid sequence showed 53 % identity to a bacterial glucosamine-6-phosphate deaminase (Shevchenko et al. 1998). However, recombinant oscillin, while exhibiting deaminase activity, was unable to induce Ca^{2+} oscillations after being injected into mammalian eggs (Wolosker et al. 1998). This indicated that a sperm extract factor other than oscillin was the much sought-after molecule that caused activation.

In vitro assays demonstrated that mammalian sperm extracts had high PLC enzyme activity, even at relatively low Ca^{2+} concentrations typical of mammalian eggs awaiting fertilization (Rice et al. 2000). This suggested that the active factor in sperm might be a PLC isoform. In fact, mammalian spermatozoa express several known PLC isoforms (Fukami 2002). However, recombinant forms of these proteins failed to trigger Ca^{2+} oscillations when injected into eggs (Parrington et al. 2002) or did so only at very high, nonphysiological concentrations (Mehlmann et al. 2001). In addition, chromatographic fractionation of sperm extracts showed that none of the known PLC isoforms were present in the fraction that possessed the

ability to trigger regenerative Ca^{2+} oscillations (Parrington et al. 2002). These data suggested that the sperm factor might be a novel PLC isoform.

The quest for new PLC varieties led to the identification of a set of sequences in a mouse expressed sequence tag (EST) database; this was followed by the amplification of a novel PLC from a mouse spermatid cDNA library (Saunders et al. 2002). The new isoform was named PLCζ (PLCzeta). It was found to be smaller than all other known mammalian PLC isoforms, and Northern blot analysis revealed that it was expressed exclusively in the testis. PLCζ orthologues have also been identified in the sperm of other mammalian species such as hamster, pig, horse, monkey, and human (reviewed by Nomikos et al. 2013). Complementary RNA (cRNA) of the mouse, cynomolgus monkey, and human PLCζ or the recombinant protein is able to recapitulate the sperm-induced Ca^{2+} oscillations in mouse eggs (Saunders et al. 2002; Cox et al. 2002; Kouchi et al. 2004). In human and pig eggs, the PLCζ cRNA triggers embryo development to the blastocyst stage (Rogers et al. 2004; Yoneda et al. 2006), and immunodepletion of sperm extracts using an anti-PLCζ antibody abolishes the extract's ability to induce the oscillations (Saunders et al. 2002). PLCζ protein in mouse sperm resides in the postacrosomal region of the perinuclear theca, a condensed layer of cytosolic proteins that covers the nucleus (Young et al. 2009); in bull sperm it localizes in the equatorial region (Yoon and Fissore 2007). This localization is consistent with the expectation that the Ca^{2+} release-inducing factor must gain rapid access to the egg cytoplasm after gamete fusion (Lawrence et al. 1997). Approximately 40 fg PLCζ in the ooplasm is sufficient to induce Ca^{2+} oscillations; this amount correlates with the range of PLCζ estimated to be present in a single mouse sperm (Saunders et al. 2002). Finally, transgenic mice have been created that showed reduced expression of PLCζ; when spermatozoa of such animals were used for in vitro fertilization, the induced Ca^{2+} oscillations terminated prematurely in the eggs (Knott et al. 2005). PLCζ-deficient male mice have also been generated (Ito et al. 2010); unfortunately, such animals fail to make sperm so the ultimate experiment to demonstrate the critical role of PLCζ during fertilization is yet to be conducted. Nevertheless, the data listed above strongly support the notion that mammalian sperm generate the Ca^{2+} signal that triggers embryo development by means of PLCζ.

The PLCζ enzymes identified in different mammals are all similar in size (Swann et al. 2006). Interestingly, the enzyme lacks an N-terminal pleckstrin homology (PH) domain that is present in other PLC isoforms and instead contains two pairs of EF hand domains at the N-terminus, followed by the X-Y catalytic domain found in all mammalian PLC enzymes and a PKC-homology type II (C2) domain at its C-terminus. The X-Y catalytic domain is the most highly conserved region of the molecule and is responsible for its enzymatic activity. A point mutation in this domain leads to a complete loss of the enzyme's ability to hydrolyze PIP_2 in vitro and, hence, to induce Ca^{2+} oscillations in mouse eggs (Nomikos et al. 2011). The EF hands possess Ca^{2+}-binding residues similar to those found in other Ca^{2+}-binding proteins (Kouchi et al. 2005). They confer the enzyme high Ca^{2+} sensitivity: PLCζ is 100-fold more sensitive to Ca^{2+} than PLCδ1, the isoform it shares the greatest homology with (Nomikos et al. 2005). PH domains can bind phosphoinositides in cellular membranes; most proteins with a PH domain need to associate with the membrane to

function. The fact that PLCζ lacks such a domain is consistent with the finding that PLCζ does not specifically localize in the oolemma following its release from the sperm but allocates throughout the entire cytoplasm (Yoda et al. 2004). This implies that the enzyme either lacks a targeting mechanism or, alternatively, is recruited to a pool of PIP_2 that does not reside in the plasma membrane. In fact, PIP_2 in mouse eggs seems to localize not in the plasma membrane (as it does in most other cell types) but in vesicles in the cytoplasm. Immunocytochemical experiments have indicated that PLCζ also accumulates in such vesicles after gamete fusion (Yu et al. 2012).

The C2 domain is generally found in proteins that can bind to phospholipids (Nalefski and Falke 1996). Most C2 domains bind to Ca^{2+} (although there are some that do not), and Ca^{2+} binding to the C2 domain is generally crucial for enzyme activity (Zheng et al. 2000). Deletion of this domain obliterated the ability of PLCζ to induce Ca^{2+} oscillations without affecting enzyme activity (Nomikos et al. 2005), indicating that the C2 domain is essential for proper PLCζ function. The X-Y linker, the segment that joins together the X and Y sections of the catalytic domain, may also be involved in binding PLCζ to biological membranes. It was proposed that a cluster of positively charged residues within this region might be responsible for membrane targeting, possibly via electrostatic interactions with negatively charged PIP_2 (Nomikos et al. 2007). The successive reduction of the net positive charge within the X-Y linker (Nomikos et al. 2011a) or the deletion of the entire X-Y linker region (Nomikos et al. 2011b) led to a significant decrease in the enzyme's ability to interact with PIP_2 under in vitro conditions and to induce Ca^{2+} oscillations. In addition, the X-Y linker shows marked differences between species, being the shortest in humans and longest in the cynomolgus monkey (Swann et al. 2006). This variation might be partially responsible for the diverse potency of PLCζ of different species to catalyze PIP_2 hydrolysis and generate Ca^{2+} transients (Saunders et al. 2007).

In mice, the sperm-induced Ca^{2+} oscillations stop around the time the male and female pronuclei are formed, possibly because the forming pronuclei sequester PLCζ that halts the signal. This idea is supported by the results of immunocytochemical analyses indicating that recombinant mouse PLCζ accumulates in the pronuclei upon the cessation of the oscillations (Larman et al. 2004). The observation that the basic residues in the X-Y linker region constitute a nuclear localization sequence is consistent with this finding (Ito et al. 2008). Mutational studies have indicated that replacing the basic residues with acidic ones in the nuclear localization signal results in a loss of the nuclear translocation ability and the oscillations do not terminate at the time of pronuclear formation. Although putative nuclear localization sequences have been predicted in the PLCζ of other species, medaka, rat, and human PLCζ are unable to localize in mouse pronuclei (Ito et al. 2008). In addition, rat PLCζ does not accumulate in rat pronuclei although the mouse PLCζ does. This implies that unlike in mice, PLCζ of other species may not be sequestered by the newly formed pronuclei.

Finally, clinical reports that linked deficiency in human PLCζ to male infertility provided further evidence to support the central role of PLCζ in the generation of

the Ca^{2+} signal during mammalian fertilization. Intracytoplasmic sperm injection (ICSI) is a powerful technique used in assisted reproduction to improve conditions of male infertility. It has been found that sperm of a number of patients, which repeatedly failed to stimulate embryo development after ICSI, were unable to trigger Ca^{2+} oscillations and showed reduced or complete absence of PLCζ expression (Yoon et al. 2008; Heytens et al. 2009). In addition, patients with a history of failed ICSI were shown to have a point mutation in the X or Y domain of the PLCζ gene (Kashir et al. 2012). Injection of spermatozoa from these patients or cRNA encoding the mutant PLCζ was unable to induce Ca^{2+} oscillation in mouse eggs indicating the pivotal role of PLCζ in the generation of the Ca^{2+} signal.

The situation in other vertebrate groups is far less clear. Supernatants of fish sperm homogenates trigger activation in medaka eggs after microinjection indicating the presence of an egg-activating factor (Iwamatsu and Ota 1974). Sperm extracts from tilapia (Coward et al. 2003) and recombinant medaka PLCζ (Ito et al. 2008) induce repetitive Ca^{2+} transients after injection into mouse eggs. Intracytoplasmic injection of sperm into eggs leads to normal fertilization and the formation of healthy embryos in medaka suggesting that the fertilization Ca^{2+} signal is triggered by a diffusible factor in the sperm rather than the stimulation of a surface receptor located on the egg (Otani et al. 2009). Similarly, extracts prepared from chicken sperm induce repetitive Ca^{2+} transients after injection into mouse eggs (Dong et al. 2000), and recombinant chicken PLCζ has the same effect (Coward et al. 2005). Also, the developmental potential of quail embryos produced by intracytoplasmic sperm injection improves when PLCζ is co-injected into the eggs (Mizushima et al. 2008), and microinjection of extracts from newt sperm causes a wavelike Ca^{2+} increase in newt eggs (Yamamoto et al. 2001). These findings seem to support the sperm factor model in nonmammalian vertebrates; whether the factor is used to stimulate egg activation under physiological conditions is a different question. *Xenopus* sperm extracts cause regenerative Ca^{2+} rises in mouse eggs (Dong et al. 2000), but interestingly, they are unable to induce activation in *Xenopus* eggs (Harada et al. 2011). In addition, *Xenopus* eggs are insensitive to newt sperm extracts as well (Harada et al. 2011) indicating that these eggs do not contain the signaling cascade required for stimulation by a diffusible sperm factor, at least those present in *Xenopus* and newt spermatozoa. This finding seems to be consistent with the observations that in *Xenopus*, egg activation is triggered as a result of membrane interactions between the gametes.

A number of additional molecules have also been proposed to serve as a sperm-resident activating factor in vertebrate species. Isoforms of PLCζ were found to be present in pufferfish (Coward et al. 2011); unexpectedly, it is expressed in the ovary and brain instead of the testis. Injection of its cRNA does not cause Ca^{2+} oscillations in mouse eggs potentially indicating that pufferfish PLCζ has no role in Ca^{2+} mobilization. Alternatively, this may also suggest that in the pufferfish PLCζ is expressed in the egg rather than the sperm and it needs interaction with other molecules localized in the male or female gamete to become capable of inducing Ca^{2+} release. In the newt, a 45-kDa protein termed citrate synthase is present in

spermatozoa, which, after injected into eggs, is able to fully replicate the Ca^{2+} fingerprint of the sperm. The Ca^{2+} elevation in newt eggs is also stimulated by the microinjection of porcine citrate synthase and by the injection of citrate synthase mRNA as well (Harada et al. 2007). Inhibition of the enzyme's activity prevents egg activation triggered not only by a crude sperm extract but also by the fertilizing sperm (Harada et al. 2011). It seems that citrate synthase elevates PLC activity in the egg through a yet unknown mechanism. The enzyme normally produces citrate from acetyl-CoA and oxaloacetate in the mitochondrial tricarboxylic acid (TCA) cycle, but it can also inversely cleave citrate into acetyl-CoA and oxaloacetate, molecules that are able to activate newt eggs. Although it is not clear how these citrate-derived products trigger the Ca^{2+} signal at fertilization, in other cell types, acetyl-CoA has been shown to sensitize IP_3 receptors (Missiaen et al. 1997), and oxaloacetate is able to stimulate Ca^{2+} release from mitochondria (Leikin et al. 1993). Most citrate synthase is localized in the neck and midpiece region of the sperm cell, outside the mitochondria. Because all sperm components, including the tail, are incorporated into the egg at fertilization, citrate synthase is exposed to the ooplasm soon after sperm-egg fusion (Iwao 2012).

Another proposed sperm factor has been tr-kit, a truncated form of the c-kit receptor, which is known to play an important role in primordial germ cell migration (Sette et al. 1997). In mouse eggs, tr-kit causes activation, supposedly by stimulating the enzyme PLCγ1 through a Src-like kinase, Fyn (Sette et al. 2002). Tr-kit-induced egg activation is blocked by a PLCγ SH3 construct, but interestingly, the same SH3 construct does not interfere with fertilization (Mehlmann et al. 1998). In addition, tr-kit has never been shown to induce repetitive Ca^{2+} transients in mammalian eggs, which is an expected characteristic of a bona fide sperm factor. Finally, the postacrosomal sheath WW domain-binding protein (PAWP) has also been listed as a candidate sperm factor (Wu et al. 2007). The protein resides in the postacrosomal sheath subcompartment of the sperm perinuclear theca (a localization consistent with that of a potential sperm factor), and it is released into the ooplasm at fertilization. Microinjection of recombinant PAWP into mature frog and porcine eggs stimulates Ca^{2+} release and egg activation (Wu et al. 2007; Aarabi et al. 2010), and the recombinant protein or its complementary RNA (cRNA) is also able to elicit Ca^{2+} oscillations and pronuclear formation in human and mouse eggs (Aarabi et al. 2014). In addition, the Ca^{2+} transients can be blocked by co-injection of an inhibitory peptide derived from the WW domain-binding motif of PAWP, and this same peptide is also able to inhibit sperm-induced Ca^{2+} oscillations suggesting that PAWP has a role in egg activation during fertilization. However, it is unclear what pathway PAWP might use to generate the Ca^{2+} signal, and its ability to induce Ca^{2+} oscillations could not be confirmed by other groups (Nomikos et al. 2014, 2015). Recently, PAWP null mice have been produced, and although the sperm of these animals lacked PAWP protein, they were able to fertilize eggs and induce Ca^{2+} oscillations (Satouh et al. 2015). Thus, the recognition of PAWP as a true sperm-borne oocyte-activating factor requires further verification.

1.3.5 Repetitive Ca^{2+} Oscillations

It is not entirely clear what makes the Ca^{2+} signal oscillate. Most signals that are generated by Ca^{2+} mobilization from intracellular stores have a predisposition for oscillation (Berridge and Galione 1988). Both IP_3 and ryanodine receptors show Ca^{2+}-induced Ca^{2+} release that is the basis of oscillatory activity in many cell types, and high cytosolic Ca^{2+} inhibits further Ca^{2+} release through both receptor types. These features are sufficient to elicit the oscillatory pattern. Nevertheless, store depletion adds an additional negative feedback constituent, and because PLCζ shows very high sensitivity to Ca^{2+} (Kouchi et al. 2004), the Ca^{2+}-promoted production of IP_3 provides one more positive feedback for the IP_3 receptor.

The oscillations are probably controlled by the basic feedback properties of the IP_3 receptor (Adkins and Taylor 1999). According to one popular model of Ca^{2+} oscillations, Ca^{2+}-dependent IP_3 production by PLCζ (i.e., the nonlinear feedback loop of Ca^{2+} on PLCζ activity) leads to oscillating IP_3 levels, which accounts for the repetitive nature of the fertilization Ca^{2+} signal (Dupont and Dumollard 2004). However, injection of the mammalian sperm factor into frog eggs causes only one Ca^{2+} transient (Wu et al. 2001), indicating that the feedback of Ca^{2+} on PLCζ cannot in itself explain repetitiveness. Another plausible model argues that instead of controlling PLCζ activity, Ca^{2+} may act directly on the IP_3 receptors (De Young and Keizer 1992). In this version, IP_3 concentrations do not oscillate, but instead IP_3 at a constant level provides continuous stimulation to its receptor. The receptor opens when intracellular Ca^{2+} is low and closes when Ca^{2+} concentration at the receptor rises above a threshold level. Observations that IP_3 levels do not oscillate in HeLa cells during the repetitive Ca^{2+} signal induced by metabotropic glutamate receptor stimulation (Matsu-ura et al. 2006) and that providing a sustained IP_3 supply can trigger Ca^{2+} oscillations in mouse eggs (Jones and Nixon 2000) support this model. Measuring IP_3 at fertilization could help in distinguishing between the different models; unfortunately, due to inherent difficulties, the data available can be interpreted in various ways (Shirakawa et al. 2006). It has also been suggested that in mammalian eggs the two mechanisms may coexist. In unfertilized eggs, the IP_3 receptor alone is responsible for the Ca^{2+} oscillations seen after sustained injection of IP_3. Then at gamete fusion, the sperm introduces PLCζ into the ooplasm after which a new mechanism, regenerative IP_3 production, regulates the oscillatory Ca^{2+} signal (Swann and Yu 2008).

The Ca^{2+} oscillations also seem to be dependent on a Ca^{2+} influx across the plasma membrane. In mouse eggs, the sperm-induced Ca^{2+} spikes stop or slow down significantly upon the removal of extracellular Ca^{2+} (Kline and Kline 1992; Shiina et al. 1993). In addition, incubation in the presence of thapsigargin stimulates Ca^{2+} entry in mouse, pig, and human eggs (Kline and Kline 1992; Machaty et al. 2002; Martín-Romero et al. 2008). Thapsigargin is an inhibitor of the SERCA pumps. Ca^{2+} slowly leaks out of the endoplasmic reticulum, the blocked pumps are not able to reload Ca^{2+}, and the stores become depleted. The fact that store depletion triggers extracellular Ca^{2+} influx indicates that a mechanism known as store-oper-

ated Ca^{2+} entry is functional in eggs and it may serve to refill the endoplasmic reticulum. The analysis of the precise link between the Ca^{2+} influx and the series of Ca^{2+} transients in fertilized mouse eggs has revealed that the rising phase of each transient is followed by an increased influx of extracellular Ca^{2+} (McGuinness et al. 1996). The influx is smaller but still detectable between the transients. Another study also found that the repetitive Ca^{2+} oscillations in mouse eggs were associated with a persistent Ca^{2+} entry triggered during the initial Ca^{2+} release (Mohri et al. 2001). The entry seemed to be store operated, and it was thought to be responsible for refilling the stores, facilitating additional Ca^{2+} release cycles, and thus sustaining the Ca^{2+} oscillations.

In somatic cells, store-operated Ca^{2+} entry is mediated by the collaboration of stromal interaction molecule (STIM) and Orai proteins. STIM1 and STIM2 are single-pass transmembrane proteins in the membrane of the endoplasmic reticulum (Liou et al. 2005; Roos et al. 2005). With a canonical EF hand directed toward the lumen, they are able to sense the Ca^{2+} content of the store. When Ca^{2+} is released from the stores, STIM1 moves to the plasma membrane and stimulates Ca^{2+} entry. The influx channels are formed by Orai proteins (Orai1, Orai2, and Orai3). Of the three isoforms, Orai1 seems to be the most potent; it is located in the plasma membrane, and upon interaction with STIM1, it allows extracellular Ca^{2+} to enter the cytosol (Feske et al. 2006; Vig et al. 2006; Zhang et al. 2006). The presence of STIM1 has been shown in frog, mouse, and pig eggs (Koh et al. 2007; Gómez-Fernández et al. 2009; Yu et al. 2009). Upon store depletion STIM1 proteins form small clusters (puncta) that redistribute in regions of the endoplasmic reticulum close to the plasma membrane. In frog eggs that display a single Ca^{2+} transient at fertilization, store-operated Ca^{2+} entry is inactivated during oocyte maturation (Yu et al. 2009). In the pig, however, it remains functional, and the downregulation of STIM1 by means of siRNAs completely abolishes the sperm-induced Ca^{2+} spikes (Lee et al. 2012). The channel component Orai1 has also been shown to be present in eggs. Both indirect immunocytochemistry and overexpression of fluorescently tagged Orai1 have revealed that the protein is present mostly in the cell cortex consistent with plasma membrane localization (Yu et al. 2009; Wang et al. 2012; Gómez-Fernández et al. 2012). In pig eggs, Orai1 knockdown inhibits Ca^{2+} entry following store depletion and, more importantly, abolishes the train of Ca^{2+} spikes following gamete fusion. These data imply that, at least in porcine eggs, Ca^{2+} influx during fertilization is mediated by STIM1 and Orai1 proteins and their interaction is essential to sustain the repetitive Ca^{2+} signal.

Surprisingly, known inhibitors of store-operated Ca^{2+} entry, or the expression of protein fragments that interfere with STIM1-Orai1 interaction, fail to prevent the sperm-induced Ca^{2+} oscillations in mouse eggs (Miao et al. 2012; Takahashi et al. 2013). This seems to indicate that although extracellular Ca^{2+} is essential to maintain the sperm-induced Ca^{2+} spikes, the Ca^{2+} influx during fertilization in mice is not regulated by the store. In mice, a Ca^{2+} influx mechanism has been described that is under the control of protein kinase C (PKC; Colonna et al. 1989). Fluorescently labeled PKC translocates to the plasma membrane repeatedly in fertilized mouse eggs, and the pattern of translocation follows that of the Ca^{2+} transients and, also,

the periodic increases in the rate of Ca^{2+} influx (Halet et al. 2004). PKC activation with phorbol esters such as 12-O-tetradecanoylphorbol-13-acetate (TPA) or phorbol-12-myristate-13-acetate (PMA) causes Ca^{2+} oscillations in mouse eggs (Cuthbertson and Cobbold 1985; Ducibella et al. 1991). In addition, PMA treatment of fertilized eggs during the ongoing Ca^{2+} oscillations markedly increases oscillation frequency, whereas inhibition of PKC with bisindolylmaleimide I suppresses the sperm-induced Ca^{2+} oscillations (Halet et al. 2004). A potential candidate to mediate the PKC-controlled Ca^{2+} influx is the trp protein that is known to function as a Ca^{2+} entry channel in a number of cell types. TRP channels are present in eggs (Petersen et al. 1995; Machaty et al. 2002), and certain TRP isoforms are modulated by PKC (Hardie 2007), which would explain the stimulatory effect of PKC on the sperm-induced Ca^{2+} signal. However, the TRP channel does not seem to be essential during fertilization. Although selective stimulation of TRPV3 channels mediates Ca^{2+} entry that leads to activation, eggs of transgenic mice lacking TRPV3 channels show normal Ca^{2+} oscillations at fertilization (Carvacho et al. 2013). This indicates that TRPV3 is not required for the maintenance of the sperm-induced Ca^{2+} transients. Currently, it is still unclear what type of Ca^{2+} entry operates in mouse eggs at fertilization.

Oscillatory Ca^{2+} signals seem to have physiological advantages over single monotonic Ca^{2+} elevations. The repetitive behavior provides a means to deliver prolonged Ca^{2+} signals to targets without the deleterious effects of sustained Ca^{2+} elevations. The pattern of the sperm-induced Ca^{2+} signal encodes crucial information and has a profound effect not only on the immediate events of egg activation but also on peri-implantation development (Ozil and Huneau 2001). Although a single rise in the intracellular Ca^{2+} concentration can promote parthenogenetic development, freshly ovulated eggs show limited cell cycle progression following activation with such a stimulus. Recruitment of mRNAs is also abnormal in these eggs, and only after aging can a single Ca^{2+} rise stimulate these critical events (Jones 1998; Ozil et al. 2005). By manipulating the number of Ca^{2+} transients in fertilized mouse eggs, it has been demonstrated that the first few Ca^{2+} rises are able to induce development to the blastocyst stage, but the developmental competence of these blastocysts is compromised. Microarray analysis of global gene expression patterns in these embryos has revealed that approximately 20 % of the genes are misregulated, particularly those involved in RNA processing, polymerase II transcription, cell cycle, and cell adhesion (Ozil et al. 2006). It is not clear how exactly the Ca^{2+} oscillations at fertilization have their effects on many cell divisions later. It is possible that an inadequate Ca^{2+} signal does not sufficiently trigger the degradation of cell cycle regulatory proteins that maintain the metaphase II arrest and that the presence of such proteins hampers embryonic cell divisions later during development. Alternatively, it may be that the suboptimal signal is not able to turn on gene expression properly. These two possibilities are not mutually exclusive, however, and they may both contribute to the reduced developmental potential of embryos whose development was stimulated by suboptimal Ca^{2+} signals (Jones 2007).

It is interesting to speculate why multiple Ca^{2+} oscillations became the norm for egg activation in mammals. Physiological polyspermy might have arisen in animals

having large eggs with external fertilization. The Japanese giant salamander, *Andrias japonicus*, has eggs as large as 5–8 mm in diameter. The eggs are inseminated following oviposition, after which they undergo polyspermic fertilization (Iwao 2000). It is possible that polyspermic fertilization became necessary to ensure fertilization of these large eggs because a single sperm did not contain sufficient amount of sperm factor that could generate a Ca^{2+} elevation high enough to induce activation. Multiple sperm were needed to trigger several Ca^{2+} transients. The large volume of the egg came with a bonus though: the extra space in the ooplasm may help to eliminate the accessory sperm nuclei to prevent polyploidy (Iwao 2012). The platypus *Ornithorhynchus anatinus*, a primitive mammal, lays large yolky eggs of about 4 mm in diameter (Hughes and Hall 1998). The eggs are polyspermic and several sperm may enter the blastodisk at fertilization (Gatenby and Hill 1924). Another primitive mammal, the marsupial *Sminthopsis crassicaudata*, has relatively small eggs (about 120 μm in diameter) that contain a yolk mass in the center, and at fertilization some eggs show polyspermy (Breed and Leigh 1990). It seems that the decrease in egg size and yolk content is associated with the switch in the mode of fertilization, from polyspermy to monospermy. The sperm-induced Ca^{2+} changes have not been studied in these primitive mammals, but it is possible that the ancestor of mammals exhibited polyspermic fertilization that was necessary for multiple Ca^{2+} transients and also, for egg activation. Higher eutherians have small eggs without yolk in the ooplasm, but the need for the repetitive Ca^{2+} signal remained for proper activation. The Ca^{2+} oscillations in the relatively small eutherian eggs may function in place of the multiple Ca^{2+} rises induced by more than one sperm in the ancestral polyspermic eggs.

1.3.6 Resumption of Meiosis

The most important function of the sperm-induced Ca^{2+} signal is the stimulation of meiotic resumption. As mentioned above, vertebrate eggs are ovulated while arrested at metaphase of the second meiotic division. The cell cycle arrest is maintained by high MPF activity that is stabilized by CSF (the Mos/MEK/MAPK/$p90^{Rsk}$ cascade). This is achieved via the phosphorylation of Emi by $p90^{Rsk}$. Phosphorylated Emi is bound by the protein phosphatase PP2A, which causes dephosphorylation of Emi and its interaction with APC, leading to APC inhibition. Cyclin B1 is thus protected from degradation, resulting in high CDK1 (and thus MPF) activity. This keeps the chromosomes in a condensed state and stabilizes the meiotic spindle (Whitaker 1996). At this point the cell cycle can proceed only if CDK1 activity decreases. CSF is sensitive to Ca^{2+} (Meyerhof and Masui 1977), and it would seem logical that the fertilizing sperm causes the resumption of meiosis by reducing Mos activity. However, this is not the case. In both *Xenopus* and mouse eggs, the disappearance of Mos begins 30–45 min after the initiation of the sperm-induced Ca^{2+} signal, whereas MPF inactivation occurs about 15 min earlier (Lorca et al. 1991). It seems that Mos degradation is not the cause but rather the consequence of the release from the meiotic arrest. The fertilizing sperm bypasses CSF and acts instead

on the MPF machinery. It was demonstrated that Ca^{2+} mobilized from the endoplasmic reticulum binds calmodulin, a Ca^{2+}-binding messenger protein located in the cytoplasm. Ca^{2+}-calmodulin then stimulates calmodulin-dependent protein kinase II (CaMKII), an important transducer of Ca^{2+} signals (Lorca et al. 1993). In mammalian oocytes, CaMKII activity increases at fertilization: the activity oscillates in synchrony with the repetitive Ca^{2+} signal (Markoulaki et al. 2004). In turn, activated CaMKII phosphorylates Emi. In *Xenopus* it was demonstrated that this creates a docking site for polo-like kinase that further phosphorylates Emi, causing its destruction through ubiquitination by the E3 ligase Skpl-Cullin-F-box protein (Liu and Maller 2005). With the degradation of Emi the APC is relieved from inhibition. Active APC stimulates the ubiquitination and destruction of cyclin B. In mouse eggs, cyclin B1 undergoes proteolysis soon after the initiation of the sperm-induced Ca^{2+} signal (Nixon et al. 2002). In the absence of its regulatory subunit, MPF loses its activity, the cell cycle is resumed, and meiosis progresses to anaphase. The importance of this cascade has been demonstrated in mouse oocytes, where Emi levels drop after parthenogenetic activation (Madgwick et al. 2006), and constitutively active CaMKII is able to stimulate embryo development to the blastocyst stage (Knott et al. 2006). In the metaphase II-arrested *Xenopus* egg, MPF ensures Mos stability through phosphorylation on its Ser3 residues; therefore, the inactivation of MPF at this point leads to the dephosphorylation of Mos and its destruction through a yet unidentified ubiquitin ligase (Castro et al. 2001) (Fig. 1.4).

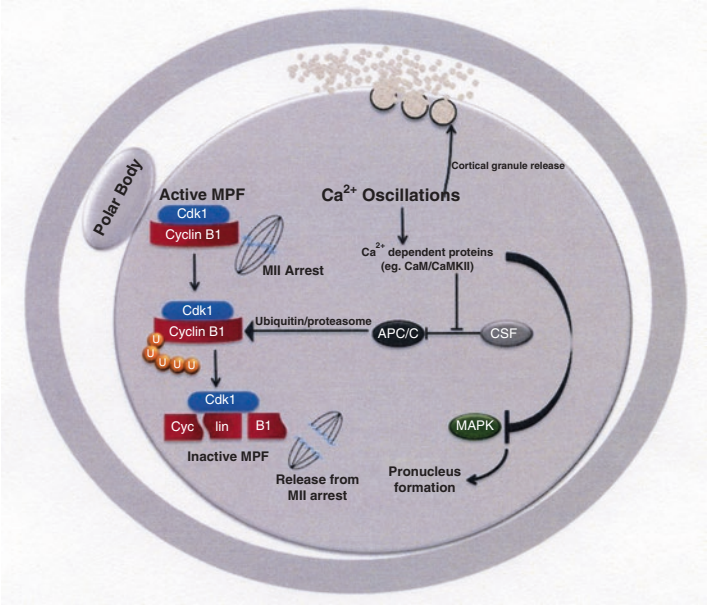

Fig. 1.4 Schematic illustration of the signaling pathway, downstream of the sperm-induced Ca^{2+} release, that triggers meiotic resumption and the initiation of embryo development (From Kashir et al. 2012, with permission)

In most vertebrates, incorporation of the sperm into the ooplasm is followed by the disassembly of the connecting piece of the sperm tail. The sperm generally contributes two centrioles: a proximal centriole located within the connecting piece and a distal centriole that is aligned with the axoneme. Only the proximal centriole has microtubule-organizing capabilities; the distal centriole has undergone partial degeneration during spermiogenesis (reviewed by Schatten and Sun 2009). Eggs, on the other hand, lack centrioles and instead possess a variety of centrosomal proteins. As the proximal sperm centriole becomes exposed to the egg cytoplasm, maternal centrosomal components accumulate around it, and the sperm aster is formed. In most animals the sperm aster is responsible for the migration and eventual union of the male and female pronuclei (Schatten 1994). The sperm nucleus remains in the center of the aster, and as the growth of the aster pushes it radially from the surface, it moves from the egg cortex toward the center. The egg completes meiosis and the second polar body is extruded. The egg chromosomes then form a female pronucleus that migrates toward the sperm nucleus, guided by the sperm aster. The sperm nucleus transforms into a male pronucleus; this transformation is brought upon by disintegration of the nuclear envelope, decondensation of the chromatin, and reformation of a new nuclear envelope. The male and female pronuclei become closely apposed in the center of the egg (Schatten et al. 1985). DNA is replicated; the centrioles also duplicate to form centrosomes on the opposite ends of the mitotic spindle by the time the zygote enters the first cleavage division.

With the appearance of the pronuclei, the Ca^{2+} oscillations stop in mouse eggs; if exit from meiosis is blocked, the oscillation goes on indefinitely (Marangos et al. 2003). In addition, the transfer of nuclei of 1- and 2-cell fertilized mouse embryos to unfertilized eggs triggers Ca^{2+} oscillations that initiate at the time of nuclear envelope breakdown (Kono et al. 1996). These findings led to the conclusion that the train of Ca^{2+} spikes comes to an end when PLCζ, the messenger responsible for the generation of the signal, is sequestered into the pronucleus (Larman et al. 2004). The Ca^{2+} oscillations can be detected again during the first mitotic division of the embryo, probably because PLCζ remains active, and after the nuclear envelopes break down, it again generates IP_3 that causes Ca^{2+} release from the endoplasmic reticulum (Kono et al. 1996). Interestingly, in nucleate and anucleate halves of mouse zygotes obtained by bisection after fertilization, the Ca^{2+} oscillations cease at about the same time irrespective of the presence of the pronuclei (Day et al. 2000). In addition, fertilized bovine and rabbit eggs display oscillations that persist well beyond pronucleus formation (Fissore et al. 1992; Fissore and Robl 1993). These findings indicate species-specific differences and imply that additional factors may also control the cessation of the Ca^{2+} oscillations.

1.4 Concluding Remarks

We have gone a long way toward the understanding of how a sperm activates an egg. A major milestone was the realization that in all animals, an increase in the egg's intracellular free Ca^{2+} concentration is the stimulus that triggers embryo

development. This was followed by the identification of the signaling toolkit that is used in the generation of the Ca^{2+} signal. In nonmammalian vertebrates a single transient elevation is observed at fertilization, while in mammals the sperm induces a series of low-frequency oscillations in the egg's cytosolic Ca^{2+} level. The source of Ca^{2+} is intracellular and it is believed to be the smooth endoplasmic reticulum. The factor that causes activation is best characterized in mammals. It is now widely accepted that the Ca^{2+}-mobilizing molecule is a sperm-specific PLCζ, which, upon sperm-egg fusion, diffuses into the ooplasm and hydrolyzes PIP_2. This leads to the generation of IP_3 that causes the discharge of stored Ca^{2+} from the endoplasmic reticulum. PLCζ isoforms have been identified in nonmammalian species, but the evidence that these are used during fertilization has yet to be reported. Additional sperm-resident proteins such as PAWP might also have a role in the fertilization process. The Ca^{2+} elevation usually takes the form of a Ca^{2+} wave that originates at the site of sperm entry and traverses across the entire cytoplasm. Although Ca^{2+} originates from the intracellular store, a Ca^{2+} influx across the plasma membrane is essential to sustain the train of Ca^{2+} spikes. The Ca^{2+} entry might be operated by the filling status of the stores, although this needs further verifications. A better understanding of the mechanism that regulates egg activation is essential for the improvement of the fertilization process in vivo and for enhancing the efficiency of a great variety of assisted reproductive technologies.

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Chapter 2
Controlling the Messenger: Regulated Translation of Maternal mRNAs in *Xenopus laevis* Development

Michael D. Sheets, Catherine A. Fox, Megan E. Dowdle, Susanne Imboden Blaser, Andy Chung, and Sookhee Park

Abstract The selective translation of maternal mRNAs encoding cell-fate determinants drives the earliest decisions of embryogenesis that establish the vertebrate body plan. This chapter will discuss studies in *Xenopus laevis* that provide insights into mechanisms underlying this translational control. *Xenopus* has been a powerful model organism for many discoveries relevant to the translational control of maternal mRNAs because of the large size of its oocytes and eggs that allow for microinjection of molecules and the relative ease of manipulating the oocyte to egg transition (maturation) and fertilization in culture. Consequently, many key studies have focused on the expression of maternal mRNAs during the oocyte to egg transition (the meiotic cell cycle) and the rapid cell divisions immediately following fertilization. This research has made seminal contributions to our understanding of translational regulatory mechanisms, but while some of the mRNAs under consideration at these stages encode cell-fate determinants, many encode cell cycle regulatory proteins that drive these early cell cycles. In contrast, while maternal mRNAs encoding key *developmental* (i.e., cell-fate) regulators that function after the first cleavage stages may exploit aspects of these foundational mechanisms, studies reveal that these mRNAs must also rely on distinct and, as of yet, incompletely understood mechanisms. These findings are logical because the functions of such developmental regulatory proteins have requirements distinct from cell cycle regulators, including becoming relevant only *after* fertilization and then *only* in specific cells of the embryo. Indeed, key maternal cell-fate determinants must be made available in exquisitely precise amounts (usually low), only at specific times and in specific cells during embryogenesis. To provide an appreciation for the regulation of maternal cell-fate determinant expression, an overview of the maternal phase of *Xenopus* embryogenesis will be presented. This section will be followed by a review of translational mechanisms operating in oocytes, eggs, and early cleavage-stage

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embryos and conclude with a discussion of how the regulation of key maternal cell-fate determinants at the level of translation functions in *Xenopus* embryogenesis. A key theme is that the molecular asymmetries critical for forming the body axes are established and further elaborated upon by the selective temporal and spatial regulation of maternal mRNA translation.

Keywords *Xenopus* • Maternal mRNA • Regulated translation • Embryonic asymmetry

2.1 Introduction

2.1.1 Oogenesis and Oocyte Maturation: Maternal mRNAs Set the Stage

A significant amount of the early development of *Xenopus* is deterministic, meaning that embryonic cells destined to specific cell fates accumulate distinct proteins called maternal determinants (Heasman 2006a; White and Heasman 2008). Maternal determinants, regulatory proteins that function in cell-cell signaling, translational control, mRNA processing, chromatin remodeling, and other processes that can alter cell fates are translated from stored maternal mRNAs that are accumulated in eggs prior to fertilization during oogenesis (Fig. 2.1).

Oocytes form and grow over a period of months to develop into fully mature stage 6 oocytes with a distinctive darkly pigmented animal hemisphere and a lightly pigmented vegetal hemisphere (Fig. 2.1). These pigmentation differences mark the animal-vegetal axis of the oocyte and are carried over into eggs during oocyte maturation and then passed on to embryos after fertilization. The animal and vegetal hemispheres are a striking visual manifestation of the elaborate molecular events that create animal-vegetal distributions of macromolecules that function in cell-fate decisions, particularly localized mRNAs that encode cell-fate determinants (Medioni et al. 2012; King et al. 2005; Houston 2013) (Fig. 2.2a).

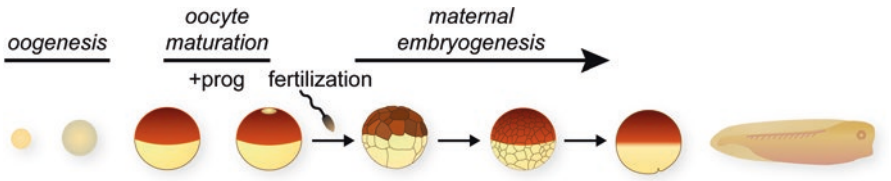

Fig. 2.1 Maternal stages of *Xenopus* development. Summary diagram of oogenesis, oocyte maturation, fertilization, and cleavage stages of embryogenesis. The maternal period of embryonic development begins at fertilization and continues until the maternal mRNAs and proteins are eliminated and replaced by zygotic products during the maternal to zygotic transition

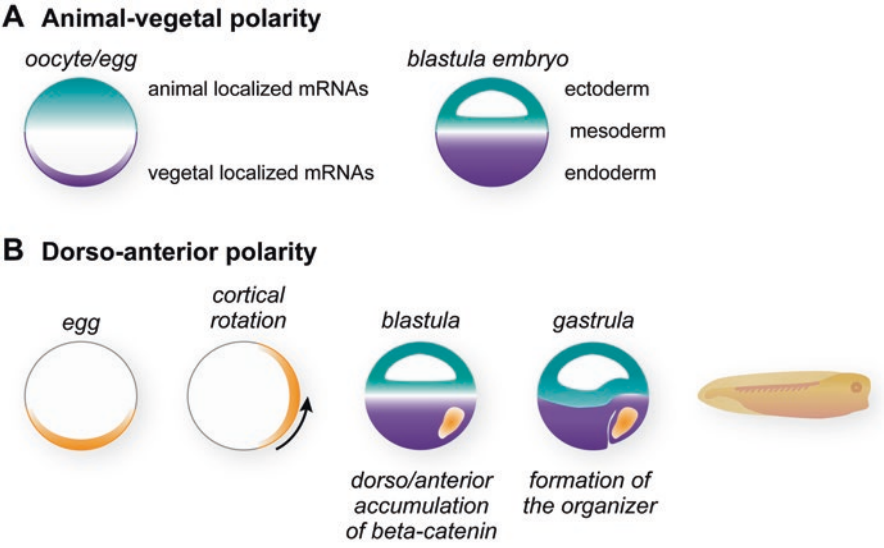

Fig. 2.2 Formation of asymmetries and specific cell types during *Xenopus* early development. (**a**) Animal-vegetal polarity is established during oogenesis by the partitioning of molecules to animal and vegetal hemispheres. These partitioned molecules are exemplified by specific mRNAs that become localized during oogenesis to vegetal cortex and the animal hemisphere (Medioni et al. 2012; King et al. 2005; Houston 2013). After fertilization the cells of the embryo inherit these molecular asymmetries when they contribute to the formation of the ectoderm, the mesoderm, and the endoderm germ layers. (**b**) After fertilization asymmetry in the dorso/anterior to ventral/posterior dimension is established as a result of cortical rotation (Gerhart et al. 1989; Houston 2012). Wnt11 is translocated from the vegetal pole of the egg during cortical rotation to create the embryonic asymmetry in the dorso/anterior to ventral/posterior dimension (Schroeder et al. 1999). Wnt11 activates Wnt signaling to direct the accumulation of beta-catenin protein. The high levels of beta-catenin cause the dorso/anterior cells to induce and give rise to the organizer (Heasman et al. 1994). At the gastrula stage, the organizer produces extracellular signals that influence and pattern the adjacent cells of each germ layer (Gerhart et al. 1991; Harland and Gerhart 1997)

Growing *Xenopus* oocytes are transcriptionally active and accumulate mRNAs as they develop (oogenesis stages 1–6). Oocytes are thus accumulating the maternal mRNA population required to drive the later stages of oogenesis, the initial cell divisions following fertilization, and the subsequent maternal stages of embryonic development. Some maternal mRNAs translated in oocytes generate proteins used by oocytes and eggs, while others are stored in translationally inactive states until later in maternal embryogenesis when their encoded proteins are needed. These stored mRNAs include a specialized cohort that are transported and anchored to the vegetal cortex of the oocyte (Medioni et al. 2012; King et al. 2005; Houston 2013) and another group that are transported and concentrated in the animal hemisphere (Fig. 2.2a). These localized mRNAs encode proteins that help establish animal and vegetal cell identity that defines the animal-vegetal axis of the oocyte. This animal-vegetal axis is carried over into the fertilized egg and early embryo. Thus the com-

plex control of maternal mRNA expression in both space and time that governs embryogenesis and the formation of distinct cell types begins in the oocyte.

At the completion of oogenesis, fully grown stage 6 oocytes arrest in meiosis (Fig. 2.1). In response to the hormone progesterone, they are released from meiotic arrest (oocyte maturation), complete meiosis, and become fertilizable eggs released by the mother. During oocyte maturation some localized mRNAs are released from their storage forms and are translated into proteins (Cragle and MacNicol 2014a; Standart and Minshall 2008). This translation contributes to the conversion of the mRNA asymmetries formed in the oocyte to protein asymmetries present in the egg that will be inherited by cells of the embryo. Some maternal mRNAs encode cell cycle proteins and fundamental cell structural proteins needed to drive the first rapid cell divisions in the fertilized embryo. Thus the regulated translation of maternal mRNAs prepares the embryo for the rapid cell divisions that immediately follow fertilization and generates proteins that guide formation of the vertebrate body plan (Heasman 2006a; Cragle and MacNicol 2014a; Gray and Wickens 1998; Richter and Lasko 2011).

2.1.2 *Embryonic Development: Cortical Rotation Establishes Embryonic Asymmetries*

At fertilization, animal-vegetal asymmetries that began during oogenesis are further elaborated and additional embryonic asymmetries are established (Fig. 2.2). In particular, the first cell division after fertilization is long (90 min) compared to the subsequent divisions (20–30 min). During this elongated first cell cycle, the *Xenopus* egg undergoes a cytoplasmic rearrangement in which the outer cortex rotates with respect to the heavy inner yolk mass. This movement occurs directionally along a longitudinal line centered on the sperm entry point in the animal hemisphere. The result is that the vegetal cortex (probably associated with localized mRNAs and/or proteins) moves upwards 30° toward the animal hemisphere. This process is referred to as cortical rotation (Gerhart et al. 1989; Houston 2012; Vincent and Gerhart 1987) (Fig. 2.2b). Cortical rotation creates new molecular asymmetries in the embryo in the horizontal dimension, the so-called dorsal/ventral axis of the embryo that is perpendicular to the animal-vegetal axis. Cells that arise in the path of the upward displacement will form the Nieuwkoop inducing center, the organizer, and anterior structures of the embryo, while cells along the path of the downward displacement will form posterior structures (Gerhart et al. 1989, 1991; Houston 2012; Heasman 2006b). Thus additional critical decisions about the body plan have already been made during this first cell cycle and involve the asymmetric relocalization of cell-fate regulators.

The identities of the mRNAs and proteins directly transported and/or activated by cortical rotation remain incompletely described (Houston 2012; Heasman 2006b). However, it is clear that the Wnt signaling pathway is activated in blastula

cells along the pathway of cortical rotation (Heasman et al. 1994; Schohl and Fagotto 2002). Wnt signaling stabilizes the beta-catenin protein. Beta-catenin's translocation to the nucleus will activate the transcription of genes that establish the Spemann organizer, a critical-inducing center that forms and functions after zygotic transcription begins (Hikasa and Sokol 2013) (Fig. 2.2b). The organizer in turn emits signals to nearby tissues, driving the cell movements of gastrulation and patterning the adjacent germ layers (Gerhart et al. 1991; De Robertis 2006; Harland and Gerhart 1997). Thus, the organizer has its roots in the earliest events of embryogenesis following fertilization that include maternal mRNA localization events.

2.1.3 Later Embryonic Development: The Organizer and Patterning of Germ Layers

During the blastula and gastrula stages, the embryo in the animal-vegetal dimension is partitioned into groups of cells that will form the three germ layers: the ectoderm, the mesoderm, and the endoderm (Fig. 2.2a) (Gerhart and Keller 1986). Each layer represents a group of progenitor cells whose fate is restricted to derivatives characteristic of that layer. For example, cells of the ectoderm germ layer will give rise to ectoderm and neuroectoderm cell types. Organizer signals promote cells to differentiate as anterior derivatives of each germ layer (Gerhart et al. 1991; De Robertis 2006; Harland and Gerhart 1997). The particular germ layer derivatives that form are a function of proximity to the organizer and its signals. For example, ectodermal cells exposed to organizer signals form anterior neural structures—the anterior parts of the nervous system that include the brain and the anterior spinal cord. In contrast, cells of the ectodermal germ layer that do not receive organizer signals form primitive ectoderm and posterior neural derivatives such as the posterior spinal cord.

2.2 Examining the Biological Functions of Maternal mRNAs in *Xenopus* Embryogenesis

The above sections make clear that the early stages of *Xenopus* embryogenesis rely on the expression of maternal mRNAs. In this section a key method that has allowed distinct functions to be assigned to many maternal mRNAs in *Xenopus* will be discussed. Over the last decade, this method has allowed the list of functional maternal mRNAs in *Xenopus* to grow substantially to the point where knowledge about functional maternal mRNAs in *Xenopus* is fairly equivalent to that in a powerful genetic vertebrate model, zebrafish (Houston 2013).

The challenge of demonstrating that a particular *Xenopus* maternal mRNA encodes a cell-fate regulator has been addressed with the development of methods that eliminate specific maternal mRNAs from eggs prior to their fertilization

(Torpey et al. 1992; Heasman et al. 1991; Olson et al. 2012; Schneider et al. 2010; Hulstrand et al. 2010). In this method, modified antisense oligonucleotides complimentary to the maternal mRNA of interest are microinjected into stage 6 oocytes causing the degradation of the target maternal mRNA. The injected oocytes are marked with a colored dye and then reinserted into an ovulating female frog. The oocytes mature in the female and she then lays eggs that can be fertilized. The eggs containing the depleted mRNA are easily identified and distinguished from the control eggs by the marker dye. Monitoring the embryonic phenotypes of the experimental and control embryos, by classic morphological and molecular techniques, allows the biological function of the maternal mRNA to be determined. For example, the maternal VegT mRNA encodes a T-box transcription factor. Embryos depleted of VegT mRNA lack endoderm, indicating that the VegT functions to regulate processes required for endoderm formation (Zhang et al. 1998). In another example, oligonucleotide-directed depletion of the maternal mRNA encoding the Wnt11 ligand leads to mutant embryos lacking dorso/anterior structures that also fail to express zygotic organizer genes such as Xnr3 (Tao et al. 2005). Thus, Wnt11 signaling is required for organizer formation and function. This depletion method, combined with some knowledge of how the maternal Wnt11 mRNA is controlled, connects the cellular phenomenon of cortical rotation to the molecular localization of a specific maternal mRNA and ultimately to the function of a key regulatory tissue, the organizer, that performs its role later in development after the onset of zygotic transcription (Gerhart et al. 1991; Harland and Gerhart 1997).

2.3 Localized mRNAs in Formation of the Germ Layers and Embryonic Asymmetries

Localized maternal mRNAs and the proteins they encode participate in at least three important processes in *Xenopus* embryos: (1) primordial germ cell formation, (2) germ layer formation, and (3) formation of embryonic asymmetries and polarities. Specific vegetally localized mRNAs, such as the mRNA encoding the Nanos protein, reside in the germplasm that ultimately gives rise to the primordial germ cells (PGCs). The control of germ cell formation and its regulation by maternal mRNAs have been discussed recently (see Chap. 8) (Lai et al. 2012; Lai and King 2013; King 2014).

The establishment of the primary germ layers, the ectoderm, the mesoderm, and the endoderm, is one of the critical processes controlled by localized mRNAs (Medioni et al. 2012; King et al. 2005; Houston 2013) (Fig. 2.2a). The partitioning of different mRNAs to distinct regions of the oocyte and egg establishes polarities and the conditions for creating cell-fate differences during embryogenesis (Heasman 2006a; White and Heasman 2008; Houston 2012). mRNAs localized during oogenesis are released from their cytoskeletal anchors either at later stages of oogenesis or during the completion of meiosis (oocyte maturation to form an egg). Importantly, the released mRNAs and proteins do not diffuse freely through the cytoplasm but instead remain concentrated

near their original sites of localization. After fertilization the new cells that form during the initial cell divisions in cleavage-stage embryos capture these mRNAs and their surrounding cytoplasm. As a consequence, vegetally localized mRNAs become concentrated in vegetal cells that will give rise to endoderm, while animally localized mRNAs become concentrated in animal cells that will give rise to ectoderm.

The importance of localized maternal mRNAs for the formation of the germ layers is supported by loss-of-function experiments that deplete specific maternal mRNAs from developing embryos, as discussed above (see Sect. 2.2). A role for the vegetally localized maternal VegT mRNA was discussed above. Another vegetally localized mRNA, the maternal Vg1 mRNA, encodes a secreted ligand that can activate TGFβ signaling. Vg1-depleted embryos exhibit defects that indicate that Vg1 is required for endoderm and mesoderm formation and anterior cell types (Birsoy et al. 2006). In contrast, the Foxi2 maternal mRNA encodes a transcription factor and is localized to animal cells. Depletion of Foxi2 causes defects in ectoderm formation by disrupting the normal activation of zygotic genes important for the ectoderm (Cha et al. 2012). Thus, specific localized mRNAs provide important links between the asymmetries formed in the oocyte and the establishment of germ layers during embryogenesis.

Finally, localized mRNAs provide the molecular basis for establishing the asymmetries that form during the initial stages of embryogenesis and organize the vertebrate body plan (Houston 2012, 2013). During the earliest steps of *Xenopus* development, there are at least two types of embryonic asymmetries that must form animal-vegetal and dorso/anterior. mRNA localization in the oocyte creates an unequal distribution of molecules in the animal-vegetal dimension that persist after fertilization to contribute to the animal-vegetal axis of the embryo (Fig. 2.2a). The second asymmetries form as a result of cortical rotation and are keys for establishing the early signaling centers of the embryo: the Nieuwkoop center and the Spemann organizer that are located to one side of the embryo (Gerhart et al. 1989, 1991; Houston 2012) (Fig. 2.2b). While the importance of cortical rotation is unequivocal, our understanding in molecular terms of exactly what it accomplishes remains incomplete. The challenge is to explain, at the level of molecular mechanism, how both type asymmetries influence development.

2.4 Signaling Pathways and Their Activation During Maternal *Xenopus* Development

Multiple signal transduction pathways are present in *Xenopus* embryos, and their regulated activation is necessary to guide the normal events of development. These pathways include the fibroblast growth factor (FGF), Wnt, bone morphogenetic protein (BMP), and Vg1/Nodal pathways. Each of these pathways functions in different regions of the embryo to direct the formation and patterning of the embryonic germ layers (Heasman 2006b; Schohl and Fagotto 2002; Harland and

Gerhart 1997). Each pathway includes a similar set of components: extracellular ligands, transmembrane receptor(s), and intracellular signaling proteins that transduce the signal from the cell surface to the nucleus. Most of these components are encoded by maternal mRNAs whose regulated translation helps control pathway function and localization.

The FGF pathway relies upon specific cell surface tyrosine kinase receptors (FGFRs) that are activated upon binding by an FGF ligand (Dorey and Amaya 2010; Goetz and Mohammadi 2013). The activated receptor transduces signals by phosphorylation and activation of cytoplasmic MAPK, PI3K, and PLCγ pathways. Depleting the maternal FGFR1 RNA gives rise to embryos with defects in gastrulation as well as defects in genes associated with the mesoderm (Yokota et al. 2003).

Binding of Wnt ligands to the Frizzled and LRP5/6 receptor proteins activates the Wnt pathway (Hikasa and Sokol 2013). This triggers a series of events that culminate in the stabilization of beta-catenin. Once beta-catenin accumulates, it translocates to the nucleus where, in concert with Tcf transcription factors, it regulates the expression of specific genes, most notably the genes of Spemann's organizer. The Wnt pathway is elaborate, consisting of both activators and inhibitors of signaling to allow for the integration of molecular cues from other signaling pathways. Loss-of-function experiments have provided conclusive evidence for the importance of maternal Wnt signaling. For example, embryos depleted of the mRNAs encoding components needed for pathway activation, such as the Wnt11 ligand, lack or have reduced amounts of organizer cells and exhibit defects in dorso/anterior axis formation (Tao et al. 2005). In contrast, embryos depleted of the mRNAs for pathway inhibitors, such as Axin, have enlarged organizers, ectopically express organizer genes, and give rise to embryos with enlarged head and anterior structures (Kofron et al. 2001).

BMPs and the Vg1/Nodal proteins are members of the transforming growth factor beta (TGFβ) superfamily of ligands (Wu and Hill 2009; Moustakas and Heldin 2009; Ramel and Hill 2012). The different ligands activate specific versions of a core pathway. Each group of ligands binds and activates heteromeric cell surface receptors. The activated serine/threonine kinase of the ligand-bound receptor phosphorylates cytoplasmic Smad proteins, and the modified proteins translocate to the nucleus where, in conjunction with other transcription factors, they active specific genes. This general scheme applies to each type of TGFβ ligand. Distinct signaling outcomes result from the use of different components, usually the receptors and Smad transcription factors used for each type of ligand (Wu and Hill 2009; Ramel and Hill 2012). There are shared components used by multiple pathways, such as the common Smad4 factor. In addition, some receptors may mediate signaling by multiple ligands, and such results have led to some confusing receptor nomenclature.

BMP signaling in *Xenopus* embryos is first activated coincident with the onset of zygotic transcription; however, this activation requires maternal signaling proteins (Faure et al. 2000). Thus controlling the synthesis of maternal BMP pathway components is necessary to create a functional pathway. The BMP pathway functions in the posterior cells and is activated when BMP ligand binds the type I and type II serine/threonine kinase receptors. Activation of the kinase domains

results in phosphorylation of the receptor Smads 1, 5, and/or 8. The phosphorylated Smads function in complex with Smad4 and other transcription factors to activate the transcription of BMP-responsive genes.

The Vg1 mRNA encodes a growth factor ligand of the TGFβ family and is localized to the vegetal cortex of *Xenopus* oocytes (Weeks and Melton 1987; Melton 1987). Loss-of-function experiments demonstrate a critical role for Vg1 in formation of the germ layers; embryos depleted of Vg1 mRNA lack endoderm and have reduced amounts of mesoderm (Birsoy et al. 2006). The Vg1 ligand signals through the same pathway as the Nodal TGFβ ligands. The receptors for these ligands are referred to as activin receptors, and upon ligand binding they phosphorylate the Smad2 protein. While loss-of-function analysis demonstrates a clear maternal requirement for the Vg1 ligand and hence a requirement for a pathway to transduce Vg1 signals, biochemical experiments monitoring the timing of signaling indicate that the active pathway cannot be detected until the blastula stages, coincident with the activation of zygotic transcription (Schohl and Fagotto 2002; Faure et al. 2000; Lee et al. 2001).

In summary, ligands, receptors, signaling proteins, and transcription factors of key signaling pathways are critical for transducing signals that guide the initial steps of development. Yet despite the importance of these proteins, we are only beginning to understand the processes that control their synthesis. Much remains to be learned about how regulated translation of maternal mRNAs impinges on the assembly and function of signaling pathways that guide development.

2.5 Translational Control Mechanisms Operating During *Xenopus* Oocyte Maturation and Early Cleavage Stages of Embryogenesis

While we now know the identity of several maternal mRNAs that encode key cell-fate determinants that drive development, we have relatively little direct knowledge about the specific translational mechanisms that may control their translation. In fact, most of our knowledge about molecular mechanisms that control maternal mRNA translation comes from studies of maternal mRNAs that drive oocyte maturation, the second phase of the meiotic cell cycle (Groppo and Richter 2009; Richter 2007; MacNicol and MacNicol 2010; Weill et al. 2012). While these mRNAs do not encode cell-fate determinants, examination of their regulation has provided important insights into mRNA regulatory mechanisms that serve as a useful foundation for examining maternal mRNAs encoding cell-fate determinants and their possible modes of regulation. In this section, the translational regulation of maternal mRNAs during oocyte maturation will be discussed to serve as context for the subsequent discussions about mRNAs encoding developmentally relevant cell-fate regulators.

The translational state of an mRNA depends upon the sequence elements it contains and the proteins that bind these elements and influence the mRNA's interaction with ribosomes. These complexes of proteins bound to an mRNA (mRNA

ribonucleoprotein particles (mRNPs)) are the determining factors of whether an mRNA engages ribosomes and is actively translated into protein or is stored in a translationally repressed state. Therefore, a key to understanding the behaviors of specific mRNAs with respect to translation is to define their mRNP composition and its dynamics during changes in its translational status. For example, stored *Xenopus* maternal mRNAs are translationally repressed due to their association with general repressor proteins such as FRGY2, XP54 (DDX6), and RAP55 (Colegrove-Otero et al. 2005a; Minshall et al. 2001, 2007; Tanaka et al. 2006, 2014; Tafuri and Wolffe 1993; Ranjan et al. 1993; Deschamps et al. 1992). A comprehensive discussion of the RNA-binding proteins that mediate general translational repression in *Xenopus* oocytes and embryos is beyond the scope of this review (see Cragle and MacNichols for a thorough discussion (Cragle and MacNicol 2014a)). However, the following section will present a brief overview of the mechanisms of translation control that operate during *Xenopus* oocyte maturation and early cleavage stages with an emphasis on what is known about the functional sequence elements and their cognate binding proteins.

2.5.1 *Control of Translation During* Xenopus *Oocyte Maturation: Regulated mRNA Polyadenylation*

The regulated addition of adenylates to the 3′ end of maternal mRNAs, referred to as poly(A) tail lengthening or polyadenylation, is a mechanism used to control the translational activation of specific mRNAs during oocyte maturation (Cragle and MacNicol 2014a; Standart and Minshall 2008; Richter and Lasko 2011). The majority of eukaryotic mRNAs are cleaved and polyadenylated in the nucleus in two coupled reactions that recognize the conserved 5′-AAUAAA-3′ present in their 3′ untranslated regions (UTRs). Once mRNAs enter the cytoplasm, the poly(A) tails of many mRNAs are further subjected to both poly(A) tail lengthening and shortening (deadenylation) (Moore 2005). Depending on cell type, these changes can affect mRNA stability, translational activity, or both. During *Xenopus* oocyte maturation, the vast majority of maternal mRNAs are stable, regardless of poly(A) tail length. However, some mRNAs that have poly(A) tails and are translationally active in oocytes lose these structures during maturation, in a process called deadenylation, and become translationally inactive (Fox and Wickens 1990; Hyman and Wormington 1988). In contrast, other mRNAs with very short poly(A) tails that are translationally inactive in oocytes undergo poly(A) tail lengthening and translational activation during oocyte maturation (Weill et al. 2012; Sheets et al. 1994; Ivshina et al. 2014). In the following sections, two different mechanisms of translational activation of mRNAs coupled to polyadenylation will be discussed.

2.5.2 CPE/CPEB-Dependent Polyadenylation and Translation

Many *Xenopus* mRNAs that are polyadenylated during oocyte maturation contain cytoplasmic polyadenylation elements (CPE) in their 3′UTRs that serve as binding sites for the CPE-binding protein-1 (CPEB1) (Weill et al. 2012; Ivshina et al. 2014; Pique et al. 2008; Fox et al. 1989; McGrew et al. 1989; Hake and Richter 1994). These elements are distinct from the nuclear recognized AAUAAA element and are generally comprised of U-rich elements. CPE-containing mRNAs are repressed in stage 6 oocytes, and this repression is mediated by CPEB via CPEB-interacting proteins, such as Maskin and 4ET (Minshall et al. 2007; Stebbins-Boaz et al. 1999). The Maskin and 4ET repressor proteins are tethered to CPE-containing mRNAs via their interactions with CPEB, and they block translation of their bound mRNAs by preventing the productive assembly of a translation initiation complex on the 5′ cap structure of the mRNA, a necessary step in translational activation. In response to progesterone, the hormone that directs oocyte maturation, CPEB is phosphorylated (Mendez et al. 2000). Phosphorylation of CPEB triggers several important events including the dissociation of the Maskin and 4ET repressor proteins from the CPEB-mRNA complex. In addition, the CPEB-bound Gld2 poly(A) polymerase becomes activated and thus adds a poly(A) tail to CPE-containing mRNAs (Kwak et al. 2004; Barnard et al. 2004). The elongated poly(A) tail recruits poly(A)-binding protein (PABP), and PABP stimulates translational initiation through mechanisms that are not fully understood but probably involve its interactions with the 5′ cap structure (Gray et al. 2000). Thus, CPE-containing mRNAs are translationally activated during maturation by a dual mechanism: the relief from Maskin and 4ET repression combined with the stimulation provided by an elongated poly(A) tail bound by PABP. Other studies suggest that, the mRNA encoding another specificity factor for polyadenylation, CPEB4 is a substrate for CPEB1 polyadenylation (Novoa et al. 2010; Igea and Mendez 2010). Results suggest that these two CPEB proteins function sequentially to mediate temporally distinct polyadenylation events.

2.5.3 Musashi-Dependent Polyadenylation

Some *Xenopus* mRNAs that are polyadenylated during maturation do not contain CPEs but instead contain binding elements for the Musashi protein, referred to as Musashi binding elements or MBEs (Charlesworth et al. 2006; Arumugam et al. 2010). mRNAs containing MBEs are bound by the Musashi protein, contain short poly(A) tails, and are translationally repressed in oocytes by an unknown mechanism. In response to progesterone and oocyte maturation, Musashi is phosphorylated, and the protein directs the polyadenylation of MBE-containing mRNAs, through the recruitment and/or activation of a poly(A) polymerase (Cragle and MacNicol 2014b; Arumugam et al. 2012).

2.6 *Xenopus* Embryo-Specific Translational Control Mechanisms: Poly(A) Removal and Addition

While most studies of translational control in *Xenopus* have focused on oocyte maturation, some have focused on translational control mechanisms in cleavage-stage embryos. For example, the Eg1, Eg2, Eg5, and c-mos mRNAs are all deadenylated and translationally repressed after fertilization (Sheets et al. 1994; Le Guellec et al. 1991). These processes require specific sequence elements in the target mRNAs that recruit the CELF1 protein (also called EDEN and CUGBP1) (Paillard et al. 1998). Conversely, translation of the maternals Cl1, Cl2, and activin receptor mRNAs in *Xenopus* embryos is activated following fertilization, coincident with their polyadenylation (Paris and Philippe 1990; Paris et al. 1988; Simon et al. 1992, 1996; Simon and Richter 1994). The embryo-specific polyadenylation of these mRNAs requires specific 3′UTR sequence elements, termed ***e***mbryonic CPEs (eCPEs) that are distinct from CPEs that direct mRNA polyadenylation during maturation (Charlesworth et al. 2013). Consistent with this distinction between stage-specific regulatory mechanisms, the Cl1, and Cl2 eCPEs are bound by ElrA RNA-binding protein (Good 1995). Embryonic specific polyadenylation also occurs on the maternal mRNA encoding the nuclear lamin B1 protein, coincident with its translation in embryos (Ralle et al. 1999). However, the lamin B1 mRNA does not contain eCPE sequences with obvious similarity to the Cl1 and Cl2 mRNAs, suggesting that translational activation during *Xenopus* embryogenesis may occur by multiple parallel pathways.

The poly(rC)-binding protein αCP2 can recruit cytoplasmic poly(A) polymerase activity to mRNAs in *Xenopus* embryos, and this recruitment relies on C-rich sequences recognized by αCP2 (Vishnu et al. 2011). This mechanism is specific for embryos and is not active in oocytes. The αCP2 protein polyadenylates mRNAs that contain C-rich CPEs in their 3′UTRs in close proximity to the conserved hexanucleotide signal AAUAAA. While it is clear that αCP2 can function in embryos as a specificity factor for a unique form of cytoplasmic polyadenylation, it is unclear what endogenous mRNAs are normally substrates for this protein.

2.7 Translational Control of Cell-Fate Determinants During Maternally Controlled Embryogenesis

Translational control of mRNAs encoding cell-fate determinants involves distinct mechanisms that elaborate on the basic mechanisms discussed above and/or employ unique mechanisms such as mRNA localization and cell-type-specific repression. These specialized mechanisms are probably critical for the precise control required by cell-fate regulators to work properly within the *Xenopus* embryo. This section will discuss specific examples and mechanisms relevant to translational control of mRNAs that encode critical cell-fate determinants.

2.7.1 *Translational Control Mechanisms Related to Localized mRNAs*

As discussed earlier, the localization of specific RNAs to subcellular domains of oocytes and eggs restricts the ultimate cellular destination of these mRNAs and their encoded proteins in the developing embryo (Medioni et al. 2012; King et al. 2005; Houston 2012). However, this mechanism only works if the localized mRNAs are translationally inactive during transport to prevent spatially inappropriate expression of protein. Specific examples will be discussed here.

2.7.1.1 Vg1 mRNA

The Vg1 mRNA encodes a growth factor ligand of the TGFβ family and is localized to the vegetal cortex of *Xenopus* oocytes (Weeks and Melton 1987; Melton 1987). Embryos depleted of Vg1 mRNA lack endoderm and have reduced amounts of mesoderm (Birsoy et al. 2006). Translation of the Vg1 mRNA is repressed until it is localized to the vegetal cortex in stage 6 oocytes. This repression in growing oocytes is mediated through a translational control element (TCE) contained within the 3′UTR of the Vg1 mRNA, located adjacent to the sequences for localization (Otero et al. 2001; Wilhelm et al. 2000). The translational repression operates independently of polyadenylation. The ElrA RNA-binding protein, a member of the ELAV family, has been implicated in mediating this repression, but the precise mechanisms remain under investigation (Colegrove-Otero et al. 2005b).

2.7.1.2 VegT mRNA

The VegT mRNA encodes a T-box transcription factor, and the mRNA is localized to the vegetal cortex of fully grown stage 6 oocytes (Zhang and King 1996; Lustig et al. 1996; Stennard et al. 1996). Embryos depleted of VegT maternal mRNA do not form endoderm, and they exhibit defects in the production of signals needed to induce mesoderm (Zhang et al. 1998). As discussed above, localized mRNAs are subject to specific mechanisms of repression while they are transported during oogenesis (Medioni et al. 2012; King et al. 2005). At subsequent stages of development, the translation of localized mRNAs must be activated, but in general these later activation steps are poorly understood. An exception is the XSeb4R protein that acts as a positive regulator of VegT mRNA translation in embryos (Souopgui et al. 2008). XSeb4R is an RRM-containing RNA-binding protein that exerts its effects on the VegT mRNA by directly binding to sequences within the 3′UTR of the mRNA. The mechanism by which this binding enhances translation or stability is unknown.

2.7.1.3 Wnt11b mRNA

Maternal mRNA depletion also reveals a role for vegetally localized Wnt11 (also called Wnt11b) in *Xenopus* axis formation (Hikasa and Sokol 2013) (see Sect. 2.4). Wnt11 mRNA translation regulation is connected to cortical rotation. In oocytes and eggs, Wnt11 mRNA is closely associated with the vegetal cortex. After fertilization Wnt11 mRNA in embryos is uniformly distributed between dorsal and ventral blastomeres of cleaving embryos, but the Wnt11 mRNA in dorsal cells is polyadenylated more extensively than in ventral cells (Schroeder et al. 1999; Flachsova et al. 2013). This differential polyadenylation is sensitive to treatments that disrupt cortical rotation, such as UV light treatment. In addition, Wnt11 mRNA in dorsal cells is preferentially associated with polyribosomes compared to the mRNA in ventral cells, indicating that it is being actively translated in dorsal cells. These observations suggest a connection between cortical rotation, dorsal cell polyadenylation, and translational activation of Wnt11 mRNA. However, it is worth noting that other studies raise questions about the polyadenylation status of Wnt11 mRNA (Tao et al. 2005). The results of these studies suggest that a significant fraction of the Wnt11 mRNA itself is translocated to the dorsal cells during cortical rotation. This movement of the Wnt11 mRNA followed by its translational activation is sufficient to explain differences in Wnt11 protein expression.

2.7.2 Translational Regulation of the FGFR Signaling Pathway

Xenopus embryos provide many advantages for analyzing signaling mechanisms in a developmental context, and the fibroblast growth factor (FGF) pathway was one of the first investigated (Amaya et al. 1991). This pathway relies upon specific cell surface receptors (FGFRs) that possess cytoplasmic tyrosine kinase domains (Dorey and Amaya 2010; Lea et al. 2009). These receptors are activated to initiate signaling when an FGF ligand binds to the FGF receptor causing it to multimerize and activate its tyrosine kinase. The activated kinase phosphorylates specific cytoplasmic proteins to transduce the signal (Goetz and Mohammadi 2013).

In *Xenopus*, mRNAs encoding FGFRs and several different FGF ligands are present maternally (Dorey and Amaya 2010; Lea et al. 2009). Depletion of the maternal FGFR1 mRNA or expression of a dominant negative FGFR causes specific defects in gastrulation and gene expression (Yokota et al. 2003; Amaya et al. 1991). Antibody staining experiments reveal that translation of the FGFR1 maternal mRNA is highly regulated, with RNA translation repressed in oocytes and only activated during oocyte maturation (Amaya et al. 1991; Musci et al. 1990). This regulation relies upon a sequence element in the 3′UTR of the FGFR1 mRNA, the translational inhibitory element (TIE) that efficiently represses translation in oocytes (Robbie et al. 1995). The proteins that repress by binding the TIE have not

been identified, but relief of TIE repression requires specific signaling events that are activated during oocyte maturation, and these mechanisms function independent of polyadenylation. In embryos the FGFR1 mRNA is also translationally activated, but only in cells of the animal hemisphere. The mechanistic basis of FGFR1 spatially restricted expression is unknown (Cornell et al. 1995).

2.7.3 Translational Regulation of the Bone Morphogenetic Protein (BMP) Signaling Pathway

In vertebrate organisms the BMP signaling pathway is important for multiple aspects of embryonic development and adult organ homeostasis (Moustakas and Heldin 2009; Ramel and Hill 2012; Plouhinec et al. 2011). This pathway consists of a related family of extracellular ligands that signal through heteromeric cell surface receptors. Signaling commences when a BMP ligand binds to the receptor complex and activates its cytoplasmic serine/threonine kinase. The activated kinase phosphorylates the cytoplasmic Smad1/5/8 proteins, and the modified Smads translocate to the nucleus where they guide the transcription of specific genes (Heasman 2006a; Smith 2009).

Interference with BMP signaling during maternally controlled stages of *Xenopus* embryogenesis, by overexpression of wild-type or mutant BMP ligands, receptors, or intracellular Smad effector proteins, severely disrupts the formation of mesoderm and ectoderm/neuroectoderm derivatives and the associated patterns of gene expression (Heasman 2006a; White and Heasman 2008; Kimelman 2006; Kimelman and Pyati 2005). For example, BMP receptor proteins lacking their cytoplasmic domains retain the ability to interact with BMP ligands. But these truncated receptors cannot transduce signals, and they act as "ligand sinks" that reduce normal ligand-dependent signaling. *Xenopus* embryos expressing BMP receptors lacking their cytoplasmic domains develop secondary axes that contain ectopic neural and mesodermal cell types (Graff et al. 1994; Mishina et al. 1995; Suzuki et al. 1994; Maeno et al. 1994; New et al. 1997; Frisch and Wright 1998). These examples, along with other functional studies, demonstrate the importance of BMP signaling for *Xenopus* embryogenesis.

mRNAs encoding the proteins of the BMP pathway are present maternally, but for many components it is not known whether or not the corresponding proteins are expressed (Graff et al. 1994, 1996; Nishimatsu et al. 1992a, b; Hawley et al. 1995; Suzuki et al. 1997). While BMP signaling in embryos is first activated coincident with the onset of zygotic transcription (Faure et al. 2000), this activation requires the maternal signaling proteins. Thus controlling the synthesis of maternal BMP pathway could provide a way to regulate pathway signaling.

Polyribosome association assays reveal that the mRNAs encoding different signaling proteins of the BMP pathway exhibit a diverse set of regulatory behaviors (Fig. 2.3) (Fritz and Sheets 2001). While each of the mRNAs in the BMP pathway is inefficiently associated with polyribosomes in immature oocytes, indicating their

translational inactivity, each is recruited to polyribosomes at a distinct subsequent developmental stage (Fig. 2.3). Specifically, the mRNA encoding the Smad1 transcription factor and the mRNA encoding the BMP receptor, referred to as the

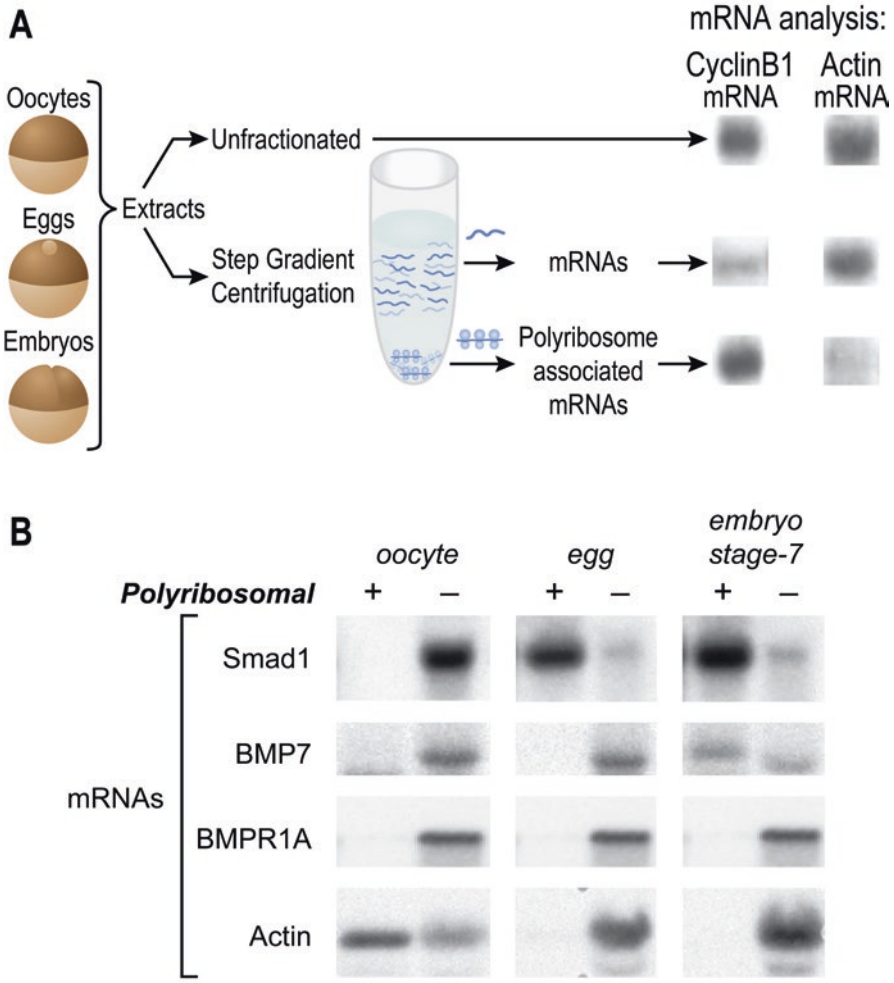

Fig. 2.3 Regulated translation of the mRNAs encoding proteins of the BMP pathway. (**a**) The isolation of polyribosomes from *Xenopus* oocytes, eggs, and embryos (Sheets et al. 2010). At the right, total (unfractionated) RNA from eggs, non-polyribosomal RNA from eggs, and polyribosomal RNA from eggs were analyzed with blot hybridization using probes to the cyclin B1 and cytoskeletal actin mRNAs. (**b**) Polyribosome and non-polyribosome fractions were prepared from *Xenopus laevis* oocytes, eggs, and stage 7 blastula embryos (Fritz and Sheets 2001). Total RNAs isolated from the fractions were analyzed by blot hybridization. Filters were hybridized with probes to detect *Xenopus* mRNAs encoding the Smad1 transcription factor, the BMP7 ligand, BMPR1a receptor, and cytoskeletal actin proteins. A representative RNA blot analyzing each mRNA is shown. Recreated from Fritz, B.R. and M.D. Sheets Developmental Biology, 2001. 236(1): p. 230–243, with permission from Elsevier

activin A receptor, type I (ACVR1a, also called ALK2), are recruited to polyribosomes during oocyte maturation, whereas the mRNAs encoding the BMP7 ligand and the BMP receptor referred to as the activin A receptor, type IIA (ACVR2a, also called XSTK9), are recruited during the early stages of embryogenesis. The BMP receptor1a mRNA (BMPR1a, also referred to as ALK3) is not associated with polyribosomes until after the onset of zygotic transcription. Thus, the translation of the maternal mRNAs of the BMP pathway is highly regulated with different mRNAs exhibiting a distinct pattern of temporal control (Fritz and Sheets 2001) (Fig. 2.3). These distinctions may help coordinate the proper assembly of the entire pathway in space and time in the embryo or may indicate that some pathway components have independent functions.

As predicted from prior work in oocytes , fertilized eggs, and early cleavage-stage embryos, the polyadenylation state of each mRNA coincided with their state of polyribosome recruitment (Fritz and Sheets 2001). For example, the poly(A) tail of Smad1 mRNA is lengthened during oocyte maturation when this message becomes efficiently recruited to polyribosomes. In contrast, the BMP7 mRNA becomes associated with polyribosomes and polyadenylated during embryogenesis. Thus, while the timing of polyribosome association differs for each mRNA, in each case, polyribosome loading is coincident with poly(A) tail elongation. This suggests that the temporal control of polyadenylation governs when and potentially how efficiently each mRNA is translationally activated.

The observation that the timing of polyadenylation differs for each mRNA suggests the involvement of specific regulatory mechanisms. For example, the poly(A) tails of the Smad1 and ACVR1a mRNAs are elongated during oocyte maturation (Fritz and Sheets 2001). This suggests that polyadenylation of these mRNAs is controlled by the CPE/CPEB-dependent mechanism that functions on mRNAs during maturation (Ivshina et al. 2014; Fernandez-Miranda and Mendez 2012). In support of this idea, both the Smad1 and ACVR1a mRNAs contain putative CPE sequence elements for maturation-specific polyadenylation in their 3′UTRs. The *Xenopus* BMP7 mRNA is polyadenylated during the initial cleavage stages that follow fertilization (Fritz and Sheets 2001). Other mRNAs polyadenylated at fertilization contain eCPE sequence elements (see Sect. 2.6) in their 3′UTRs (Charlesworth et al. 2013). However, BMP7 mRNA lacks obvious eCPEs, suggesting that different RNA recognition factors or even distinct mechanisms operate during the blastula stages of developmental.

2.7.4 Translational Control of Cripto-1 mRNA: Cell-Specific Repression

The above examples suggest that differential timing of polyadenylation reflects and/or causes temporal differences in translational activation during embryogenesis. Studies of Cripto-1 mRNA translation reveal that cell-type-specific translational

repression mechanisms can add an additional layer of spatial control on top of this temporal control. Significantly, the capacity for this spatial control of translation later in embryogenesis, mediated by a cell-type-specific repressor named Bicaudal-C (Bic-C), is actually established by early mechanisms that cause the vegetal localization of maternal Bic-C mRNA (Zhang et al. 2013). Thus, asymmetries established early in development are used to establish more refined asymmetries at later stages, as described further in the following sections.

Cripto proteins are secreted co-receptors of the Nodal signaling pathway, a pathway that is critical for normal vertebrate development (Klauzinska et al. 2014). Mutant alleles of Cripto genes cause severe embryonic defects in mouse and zebrafish (Gritsman et al. 1999; Ding et al. 1998). The *Xenopus* Cripto-1 protein (also called xCR1 or FRL1) was discovered as an interaction partner of the fibroblast growth factor receptor (FGFR1) (Kinoshita et al. 1995). Subsequent experiments indicated that Cripto-1 could also bind Wnt ligands and affect their ability to initiate signaling (Tao et al. 2005). These data reveal that Cripto-1's effect on signaling in *Xenopus* could involve other crucial signaling pathways in addition to the Nodal pathway. Regardless, depletion of the maternal Cripto-1 mRNA in *Xenopus* embryos alters cell-fate decisions and severely disrupts axis formation (Tao et al. 2005). These phenotypes are similar to those observed with embryos depleted of the maternal Wnt11 mRNA (Tao et al. 2005), suggesting that Wnt11 and Cripto-1 impact the same developmental events. Thus, while the precise pathways affected by Cripto-1 in *Xenopus* embryos remain to be determined, it is clear that Cripto-1 and the mechanisms that control its maternal expression are critical for *Xenopus* development.

In contrast to some of the cell-fate determinant-encoding mRNAs discussed above, the maternal Cripto-1 mRNA is uniformly distributed throughout the *Xenopus* embryo (Dorey and Hill 2006) (Fig. 2.4). That is, there is no spatial control of Cripto-1 mRNA per se. However, importantly, the Cripto-1 protein accumulates only within the cells of the marginal zone and animal hemisphere (Dorey and Hill 2006) (Fig. 2.4). This effect could be accomplished by differential translation of Cripto-1 mRNA or differential stability of Cripto-1 protein or both mechanisms. However, polyribosome association experiments provide strong evidence for differential translational activity of the Cripto-1 mRNA in animal versus vegetal cells, with translation being more efficient in animal cells (Zhang et al. 2009). Indeed, additional experiments reveal that Cripto-1 mRNA translation is regulated both temporally and spatially in the embryo. In oocytes and eggs, Cripto-1 is translationally repressed, but upon fertilization it is translationally active, but *only* within the cells of the animal hemisphere (Fig. 2.4). When the Cripto-1 mRNA becomes translationally active, it is polyadenylated throughout all cells of the embryo, even though translational activation of Cripto-1 mRNA is confined to animal cells (Zhang et al. 2009).

A luciferase reporter assay designed to measure translational efficiency quantitatively within embryos was used to define the translational control mechanisms responsible for the cell-type-specific expression of Cripto-1 mRNA. Briefly, building on the foundational knowledge from studies in oocytes, eggs, and cleavage-stage embryos, the luciferase-coding region was engineered as a fusion to the 3′UTR of Cripto-1 mRNA. A second luciferase fusion gene was generated fused instead to the

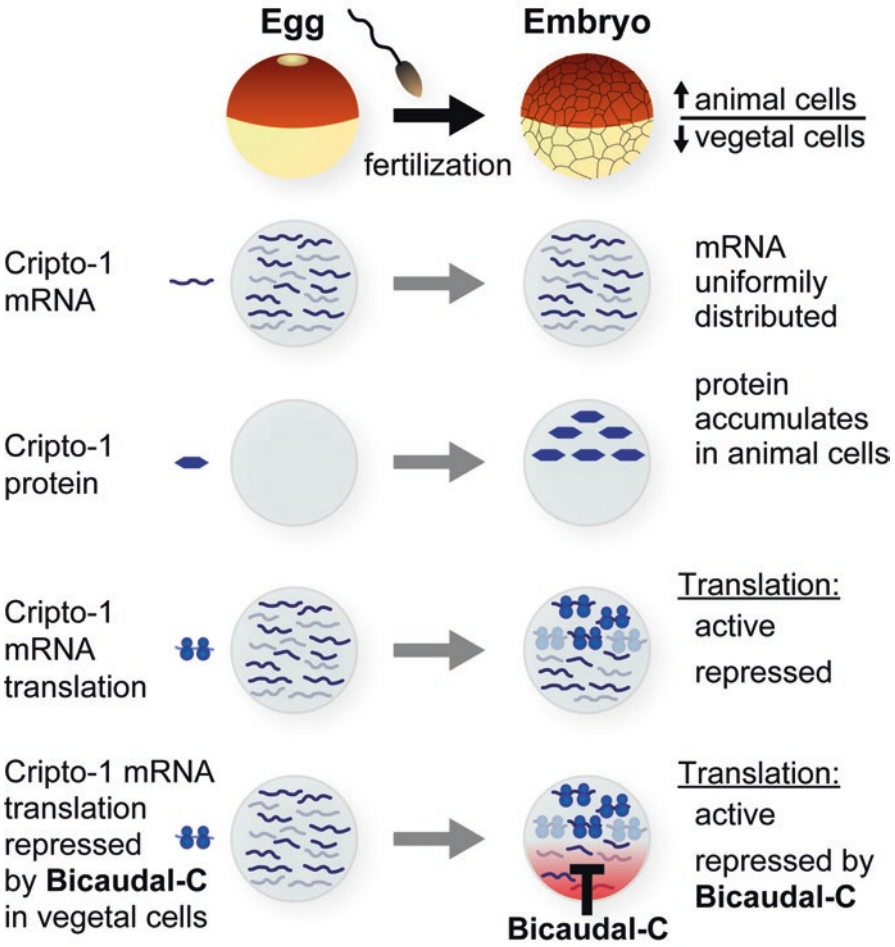

Fig. 2.4 Spatial accumulation of the Cripto-1 protein in *Xenopus* embryos is controlled by regulated translation of the Cripto-1 mRNA. In *Xenopus*, the Cripto-1 mRNA is uniformly distributed throughout the egg and all embryonic cells. The Cripto-1 protein is only produced in animal cells of embryos after fertilization, but not vegetal cells (Cragle and MacNicol 2014a). Differential accumulation of the Cripto-1 protein in *Xenopus* embryos is due to the spatially regulated translation of the Cripto-1 mRNA. Translation is activated in animal cells and repressed in vegetal cells (Heasman et al. 1994; Zhang et al. 2009). The Bicaudal-C (Bic-C) protein is responsible for repression functions in the vegetal cells (Heasman 2006a; Houston 2013; Zhang et al. 2013, 2014). The Bic-C mRNA is localized to the vegetal cortex of developing oocytes, and as a consequence after fertilization, Bic-C is restricted to vegetal cells (Schohl and Fagotto 2002)

3′UTR cyclin B1 mRNA. Cyclin B1, in contrast to Cripto-1 mRNA, is equally associated with polyribosomes in animal and vegetal cells. These reporter mRNAs were independently injected into either the animal or vegetal cells of 4–8 cell embryos that were allowed to develop to the late blastula stage at which point they were harvested

and assayed for luciferase activity. The translation of the luciferase reporter containing the 3′UTR of Cripto-1 mRNA but not the control gene fusion containing the 3′UTR of cyclin B1 mRNA is repressed in the vegetal cell-injected embryos (Zhang et al. 2009). These experiments led to three important conclusions: (1) spatial translational differences in Cripto-1 mRNA translation were caused by a vegetal cell-specific translational repression; (2) repression could be directed by sequences present in the 3′UTR of the Cripto-1 mRNA, nicely following the paradigm established for translational control in *Xenopus* oocytes and in other systems; and (3) spatially controlled translational regulation could be recapitulated in living embryos with a sensitive luciferase assay that would facilitate dissection of the relevant repressive elements and their cognate proteins. Indeed, subsequent deletion-mapping experiments identified a subregion of the Cripto-1 3′UTR that was sufficient for efficient repression, a region referred to as the translational control element (TCE) (Zhang et al. 2009).

2.7.5 Bicaudal-C Is a Vegetal Cell Translational Repressor

The simplest hypothesis for the observations concerning Cripto-1 mRNA regulation is that vegetal cells contain a cell-specific repressor protein(s) that animal cells lack and that this protein(s) binds an element(s) within the TCE identified in the mapping experiments outlined above. The TCE contains binding sites for the repressor proteins pumilio and CUGBP1 (also called CELF1), but while mutational analysis revealed that these binding sites contribute to repression, pumilio and CUGBP1 proteins are uniformly distributed in embryonic cells, suggesting that they are unlikely to be responsible for the cell-type specificity of repression (Zhang et al. 2009). Therefore a targeted functional approach was taken, exploiting what is known about localized mRNAs. This approach identified Bicaudal-C (Bic-C) as the RNA-binding protein responsible for vegetal cell specificity of Cripto-1 translational repression (Zhang et al. 2013) (Figs. 2.4 and 2.5). Bic-C protein is enriched in vegetal cells, or predicted to be, because the Bic-C mRNA is localized to these cells (Wessely and De Robertis 2000). A key experiment involved injecting the mRNA encoding Bic-C into animal cells (where Bic-C is normally not present). It was observed that ectopic expression of Bic-C was sufficient to mediate vegetal cell-specific repression of relevant luciferase reporter mRNAs. Significantly, when Bic-C is expressed in animal cells, it binds to the native Cripto-1 mRNA and to reporter RNAs when they contain the TCE (Zhang et al. 2013). These results strongly support the idea that Bic-C is responsible for the specificity of vegetal cell repression and is largely sufficient for this repression. Importantly, a tethered translation assay also supports a direct and robust role for Bic-C in mRNA translational repression (Zhang et al. 2013) (Figs. 2.4 and 2.5). A reasonable hypothesis for the role CUGBP1 and pumilio proteins in Cripto-1 mRNA repression is that these proteins simply help stabilize Bic-C binding to target mRNAs, though direct biochemical experiments to test this idea remain to be done. Regardless, the ectopic animal cell injection assay provides a powerful tool to further investigate Bic-C protein's

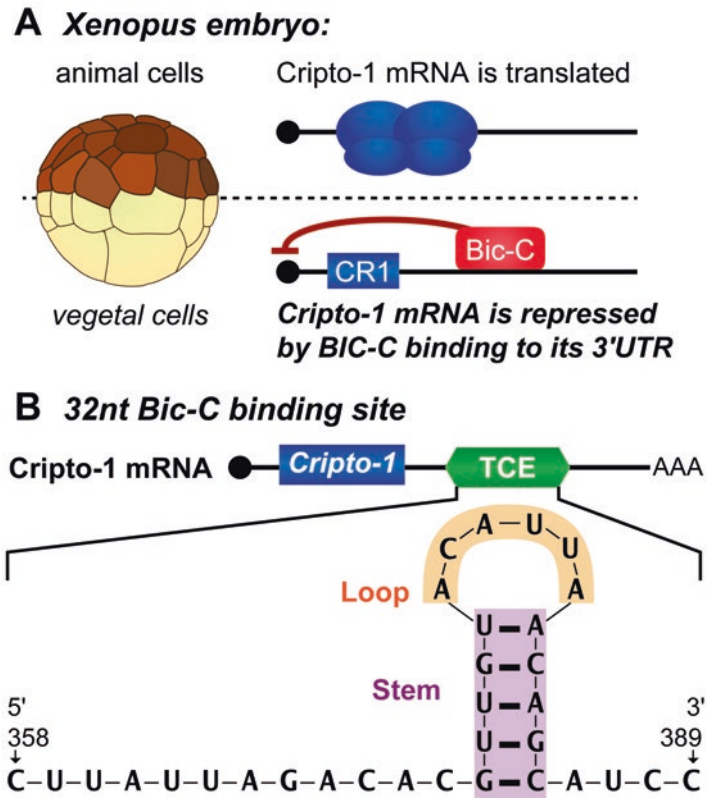

Fig. 2.5 Spatially regulated translation of the Cripto-1 mRNA by the Bic-C repressor. (**a**) Bic-C represses the Cripto-1 mRNA in vegetal cells of *Xenopus* embryos. The Cripto-1 mRNA is translated in animal cells but repressed in vegetal cells. Vegetal cell repression requires Bic-C binding to its recognition element, a sequence within the Cripto-1 mRNA's 3′UTR (Heasman 2006a; Houston 2013; Zhang et al. 2013, 2014). (**b**) The 32nt Bic-C binding site and its predicted stem-loop structure identified from the translational control element (TCE) of the Cripto-1 mRNA 3′UTR (Heasman 2006a; Zhang et al. 2014)

structure and function and offers a platform to advance both biological and medically useful knowledge concerning this important RNA-binding protein.

2.7.6 The Bic-C RNA-Binding Element

The results described above were exploited to define a Bic-C RNA binding site, the first defined for any Bic-C ortholog (Zhang et al. 2014). Bic-C proteins contain multiple heteronucleoprotein K homology (KH) domains that are known to function as sequence-specific RNA-binding modules (Gamberi and Lasko 2012; Hollingworth et al. 2012; Teplova et al. 2011; Nakel et al. 2010; Valverde et al. 2008). The

N-terminal region of *Xenopus* Bic-C containing these domains was sufficient for specific binding to the TCE, indicating, as predicted, that the TCE contains a Bic-C binding element(s) (Zhang et al. 2009, 2013). In vitro RNA-binding and RNA protection experiments using purified Bic-C were used to define a minimal site within TCE that bound Bic-C. The Bic-C target site in the Cripto-1 mRNA's 3′UTR is a 32-nucleotide element that mutational analyses revealed contains a stem-loop structure (Zhang et al. 2014) (Fig. 2.5). While the sequence of the stem is not critical, its structure is. In contrast, the sequence of the loop region is important. In addition, this Bic-C binding element contains a 10-nucleotide region 5′ to the stem loop that may also provide sequence specificity to binding (Fig. 2.5). This 32-nucleotide binding site is *necessary* and *sufficient* for translational repression in the ectopic animal cap assay (Zhang et al. 2014). Together these results support a hypothesis in which Bic-C's role in *Xenopus* maternal development is executed by its direct translational repression of select target mRNAs within the vegetal cells of embryos. The identification of a Bic-C binding element will guide further detailed analysis of the Bic-C RNA interface and ultimately may facilitate the identification of additional Bic-C target mRNAs in embryos and relevant adult tissues.

2.7.7 *Branching Out: Identification of Bic-C Target mRNAs Reveals a Bic-C Regulatory Network that Transforms Bic-C-dependent Repression into Distinct Cell-Fate Programs*

RNA-binding proteins regulate multiple mRNAs to mediate their biological functions. To identify additional mRNA targets, Bic-C was immunoprecipitated from *Xenopus* embryos, and the associated RNAs were analyzed with RNA-Seq. This approach identified 62 new putative Bic-C targets from *Xenopus* embryos (Zhang et al. 2013). Of course, this approach identifies RNAs based on their association with Bic-C in living embryos but does not reveal whether they are indeed functionally repressed by Bic-C. Importantly, several of the 62 mRNA were validated as bona fide Bic-C repression targets using the translational assays initially developed for the study of Cripto-1 mRNA (Zhang et al. 2013).

A critical issue is to determine whether the proteins encoded by these Bic-C target mRNAs do indeed function in embryogenesis and how their repression by Bic-C contributes to their function. Notably, many of the putative Bic-C target mRNAs encode proteins known to function in developmentally relevant pathways. For example, the Dpy30 mRNA encodes a histone methyltransferase important for cell-fate decisions in ES cells (Jiang et al. 2011), while the BCCIP mRNA encodes a protein that guides progenitor cells in neural development (Huang et al. 2012). In addition to Cripto-1, other Bic-C target mRNAs encode proteins implicated in Nodal/TGFβ signaling, including the Smad4b (Chang et al. 2006) and Oct25 transcription factors (Cao et al. 2008), and Coco (referred to as Dand5 in mammals), a secreted signaling antagonist (Bell et al. 2003; Vonica and Brivanlou 2007; Bates et al. 2013). Cripto-1

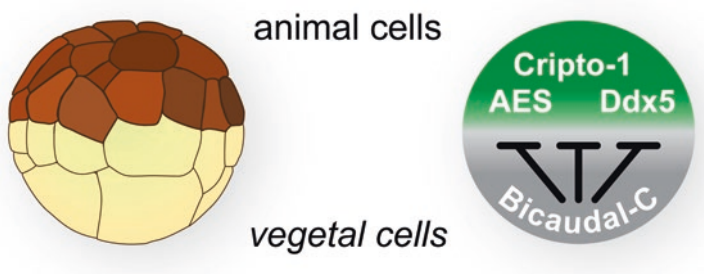

Fig. 2.6 The Bic-C posttranscriptional network (Zhang et al. 2013). The vegetal cells of developing *Xenopus* embryos contain a high concentration of Bic-C that represses the translation of specific targets such as the AES, Cripto-1, and Ddx5 mRNAs. The protein products of Bic-C target mRNAs are potentially concentrated in animal cells due to their repression by Bic-C in vegetal cells

has also been implicated as a regulator of Wnt signaling (Kraus et al. 2012; Maisonneuve et al. 2009), and therefore it is interesting that some Bic-C target mRNAs, such as the AES/GRG5 transcription factor (Costa et al. 2013) and the Ddx5 RNA helicase (Guturi et al. 2014), encode proteins known to affect Wnt signaling.

These observations lead to a model in which Bic-C controls a network of mRNA and many mRNAs in the network encode embryonic cell-fate regulators (Fig. 2.6). This suggests that Bic-C acts as the control point of a posttranscriptional regulatory network that establishes the proper balance of maternal proteins in the embryo essential for normal development. Precedence for this kind of control by RNA-binding proteins has been documented in other systems (Hogan et al. 2008; Gerber et al. 2006; Ule and Darnell 2006; Licatalosi and Darnell 2010; Kershner and Kimble 2010). Future experiments will require a systematic examination of the functions of the Bic-C targets in the embryo with the goal of understanding how different amounts of repression establish the proper balance of cell-fate determinants throughout the embryo to drive normal development.

2.8 Summary: Maternal mRNA Regulation Establishes and Elaborates Molecular Asymmetries in the Embryo

As discussed earlier, the primary animal-vegetal asymmetry in *Xenopus* is established early in embryogenesis by localizing mRNAs and proteins in oogenesis, oocyte maturation, and the early cleavage stages of embryos (Medioni et al. 2012; King et al. 2005; Houston 2012) (Fig. 2.7). The localization of these molecules may establish immediate molecular asymmetries, for example, if the molecule is an active protein or an mRNA that is translated immediately after localization. In addition, this early localization can serve as preparation for a molecular asymmetry that will not become evident until later in development, if, for instance, it involves an

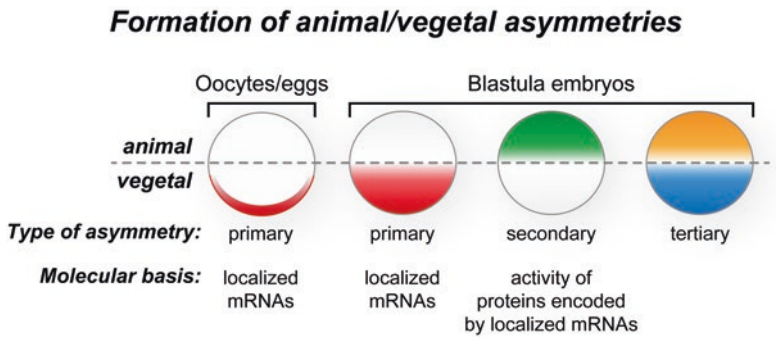

Fig. 2.7 Model for the formation of asymmetries during maternal *Xenopus* development. During oogenesis specific mRNAs are localized to the vegetal cortex, while others are concentrated in the animal hemisphere. These mRNAs and potentially other components (e.g., proteins and metabolites) form the molecular basis of the animal-vegetal polarity of the fully grown stage 6 oocyte. The translation of some localized mRNAs into proteins during oocyte maturation creates additional animal-vegetal asymmetries present in unfertilized eggs. After fertilization the asymmetries of the egg are inherited by specific blastomeres to generate the initial animal-vegetal polarity of the embryo. During embryogenesis the activities of the animal and vegetal localized mRNAs and proteins create additional asymmetries. For example, the Bic-C repressor blocks translation of the Cripto-1 mRNA in vegetal cells, and as a result the Cripto-1 protein accumulated in animal cells. This is an example of a secondary asymmetry that results from the activity of the molecules localized in oocytes and eggs. In this way mRNA localization differences in the animal-vegetal hemispheres that begin in oocytes are carried into the embryo and elaborated upon both temporally and spatially into secondary and tertiary gradients

mRNA that is translationally activated only during later stages of development. In particular, localization of Bic-C mRNA to vegetal cells is predicted to lead to the accumulation of Bic-C protein in this region of the embryo that in turn represses mRNAs, such as Cripto-1, later in embryogenesis (Figs. 2.6 and 2.7). While the Bic-C protein asymmetry is predicted to run from the vegetal to animal hemisphere, the Cripto-1 protein asymmetry runs in the opposite direction (Dorey and Hill 2006) (Figs. 2.6 and 2.7). Given that Bic-C has a number of targets, it is plausible that Bic-C-dependent repression helps establish a number of distinct protein gradients that each differ based on intrinsic translational activation capacities, affinity for Bic-C, and other factors but share with the Cripto-1 protein the same basic directionality.

In this way, mRNA localization differences in the animal-vegetal hemispheres that begin in oocytes are carried into the embryo and reinforced and elaborated upon both temporally and spatially into secondary and tertiary gradients by combining translational repression and temporal modes of translation activation (Fig. 2.7). The animal-vegetal molecular differences are accompanied by molecular asymmetries that run perpendicular to this axis. In particular, during the first cell cycle following fertilization, cortical rotation serves to localize mRNAs and proteins toward one side of the embryo, establishing molecular asymmetries that produce the dorso/anterior-ventral/posterior axis (Gerhart et al. 1989; Houston 2012). The molecular differences formed by the animal-vegetal and dorso/anterior-ventral/posterior axes ultimately impinge

upon the formation of Spemann's organizer that in turn establishes new postzygotic molecular asymmetries (Gerhart et al. 1989, 1991; Harland and Gerhart 1997). While postzygotic embryonic development is complex and highly orchestrated and balanced, it all starts with direct and precise forms of molecular regulation involving the translational control of maternal mRNAs that are beginning to be understood.

2.9 Conclusion

Xenopus laevis has served as an important model for biological research for over 50 years, contributing to fundamental knowledge of cell cycle processes and vertebrate development (Gerhart and Keller 1986; Gurdon 1964, 1977, 1988, 2013; Brown 1967; Dawid 1965; Wu and Gerhart 1980; Scharf and Gerhart 1980; Gurdon et al. 1958; Elsdale et al. 1958). A major advantage of this model was and remains this organism's ability to produce large number of eggs in response to a simple injection of hormone. Moreover, these eggs could be fertilized in petri dishes, producing populations of synchronously developing embryos for observation and experimentation. In addition the relatively large eggs and oocytes were ideally suited for molecular and biochemical analysis, including microinjection of defined molecules and the preparation of cellular extracts that could function in DNA replication, mRNA processing, chromosome segregation, and cell cycle oscillations (Murray et al. 1989; Murray and Kirschner 1989). These advantages have inspired generations of scientists to establish their own colonies of African clawed frogs and embrace the joy and frustrations of amphibian husbandry. While the quest to understand maternally controlled development and particularly the role of maternal mRNAs was and is not confined to *Xenopus*, the success of antisense approaches to target maternal mRNAs in this organism and subsequently follow development of the resulting "mutant" embryos and their control siblings suggests that *Xenopus* can continue to provide critical insights and indeed even lead the way toward a deeper molecular understanding of the maternal stages of animal development and the role of mRNA translational regulation (Olson et al. 2012; Hulstrand et al. 2010). Thus, from the intellectual perspective, it is a truly unique and exciting time for studying *Xenopus* maternal mRNAs and defining their roles in embryonic development. The future of maternal mRNA research in *Xenopus* is no longer limited by major technical challenges. As in many fascinating and important biological fields today, the major limitation this field faces is the availability of sufficient support and resources.

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Chapter 3
Regulation of Cell Division

Andreas Heim, Beata Rymarczyk, Saurav Malhotra, and Thomas U. Mayer

Abstract The challenging task of mitotic cell divisions is to generate two genetically identical daughter cells from a single precursor cell. To accomplish this task, a complex regulatory network evolved, which ensures that all events critical for the duplication of cellular contents and their subsequent segregation occur in the correct order, at specific intervals and with the highest possible fidelity. Transitions between cell cycle stages are triggered by changes in the phosphorylation state and levels of components of the cell cycle machinery. Entry into S-phase and M-phase are mediated by cyclin-dependent kinases (Cdks), serine-threonine kinases that require a regulatory cyclin subunit for their activity. Resetting the system to the interphase state is mediated by protein phosphatases (PPs) that counteract Cdks by dephosphorylating their substrates. To avoid futile cycles of phosphorylation and dephosphorylation, Cdks and PPs must be regulated in a manner such that their activities are mutually exclusive.

Keywords Cell cycle regulation • Cyclin-dependent kinase 1 (Cdk1) • Type 2A protein phosphatase (PP2A) • Type 1 protein phosphatase (PP1) • Anaphase-promoting complex/cyclosome (APC/C)

Abbreviations

AGC-type	Protein kinase A, G and C families
Anp32	Acidic nuclear phosphoprotein 32 family
APC/C	Anaphase-promoting complex/cyclosome
Arpp19	cAMP-regulated phosphoprotein, 19 kDa
ATP	Adenosine triphosphate
βTRCP	Beta-transducin repeat-containing protein

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Bub3	Budding uninhibited by benzimidazoles 3
BubR1	Budding uninhibited by benzimidazoles related 1
c-TAK1	Cdc25C-associated protein kinase 1
CAK	Cdk-activating kinase
CaMKII	Calcium-calmodulin-dependent kinase II
cAMP	Cyclic adenosine monophosphate
Cdc	Cell division cycle
Cdks	Cyclin-dependent kinases
Chk	Checkpoint kinase
CIP2A	Cancerous inhibitor of PP2A
CK1	Casein kinase 1
CPC	Chromosomal passenger complex
CPE	Cytoplasmic polyadenylation element
CPEB	Cytoplasmic polyadenylation element-binding protein
CTD	C-terminal domain
D-box	Destruction box
DNA	Deoxyribonucleic acid
E2F	E2-factor
E6AP	E6-associated protein
Emi1	Early mitotic inhibitor 1
Ensa	Endosulfine α
ES cells	Embryonic stem cells
FCP1	TFIIF-associating CTD phosphatase-1
FZR	Fizzy related/Cdh1
FZY	Fizzy/Cdc20
G1	Gap phase 1
GSK3β	Glycogen synthase kinase 3 β
Gwl	Greatwall
HECT	Homologous to E6AP C-terminus
I2	Inhibitor-2
ICM	Inner cell mass
Knl1	Kinetochore-null protein 1
KO	Knockout
M-phase	Mitotic phase and Cytokinesis
MII	Meiosis II
Mad2	Mitotic arrest deficient 2
MAPK	Mitogen-activated protein kinase
MBT	Midblastula transition
MCC	Mitotic checkpoint complex
MPF	Maturation-promoting factor
Myc	Myc proto-oncogene protein
Myt1	Membrane-associated tyrosine and threonine kinase 1
NEBD	Nuclear envelope breakdown
PDK1	Phosphoinositide-dependent kinase 1
PIPs	PP1-interacting proteins
PKA	Protein kinase A

Plx1	*Xenopus* Polo-like kinase 1
PP1	Protein phosphatase type 1
PP2A	Protein phosphatase type 2A
PPMs	Metal-dependent protein phosphatases
PPPs	Phosphoprotein phosphatases
PPs	Protein phosphatases
PRC1	Protein regulator of cytokinesis 1
Repo-Man	Recruits PP1 onto mitotic chromatin at anaphase
RING	Really interesting new gene
Rsk2	Ribosomal S6-kinase family member 2
S-phase	Synthesis phase
SAC	Spindle assembly checkpoint
SCF	Skp1, Cullin and F-box protein
SCP	Small CTD phosphatases
Sds22	Suppressor of *dis2*
siRNA	Small-interfering ribonucleic acid
T-loop	Activation loop
Ubc	Ubiquitin-conjugating enzyme
UTR	Untranslated region
UV-light	Ultraviolet light
Wee1	Wee1-like protein kinase
XErp1	*Xenopus* Emi1-related protein 1/Emi2
ZGA	Zygotic genome activation

3.1 Introduction

Entry into and exit from the different cell cycle stages are driven by changes in the phosphorylation state and level of cell cycle proteins (Fig. 3.1). In this book chapter, we primarily focus on the transition from interphase into M-phase and exit from M-phase. Entry into M-phase is mediated by active Cdk1 associated with cyclin-B, two key components of the maturation-promoting factor (MPF) (Masui and Markert 1971). Exit from M-phase requires the inactivation of MPF. For a long time, Cdk1 was in the limelight of cell cycle research and in particular the question of how the switch-like activation of MPF is accomplished. However, to achieve the mitotic phosphorylation state, activation of kinases is not sufficient, but also requires the inactivation of antagonising protein phosphatases (PPs) (Fig. 3.2). Resetting the system to interphase, on the other hand, depends on the reactivation of PPs in addition to MPF inactivation. As outlined here, the MPF and PPs modules are controlled in a reciprocal manner such that the activity of one module suppresses the activity of the other one. The selective destruction of proteins via the ubiquitin-proteasome system presents another regulatory element feeding into the control of MPF and PPs activity. Depending on the developmental stage, the different control mechanisms have varying impact on the transitions between interphase and M-phase.

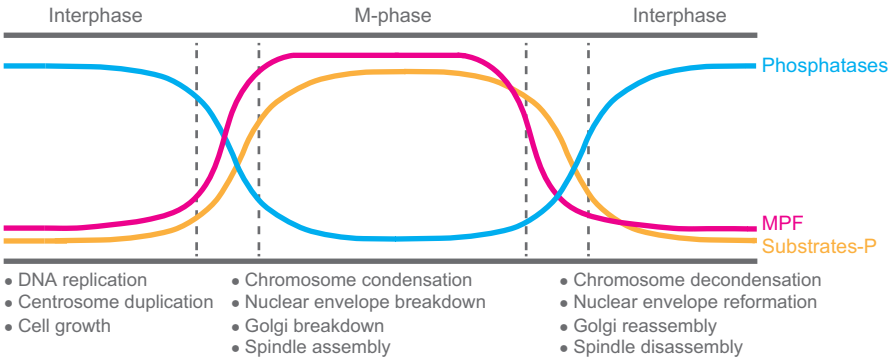

Fig. 3.1 Cell cycle transitions are mediated by changes in the phosphorylation state of the cell division machinery. Key cell cycle events such as chromosome condensation and nuclear envelope breakdown are triggered by the phosphorylation of structural as well as regulatory cell cycle proteins (substrate P). Reversal of these processes depends on the dephosphorylation of mitotic phosphoproteins

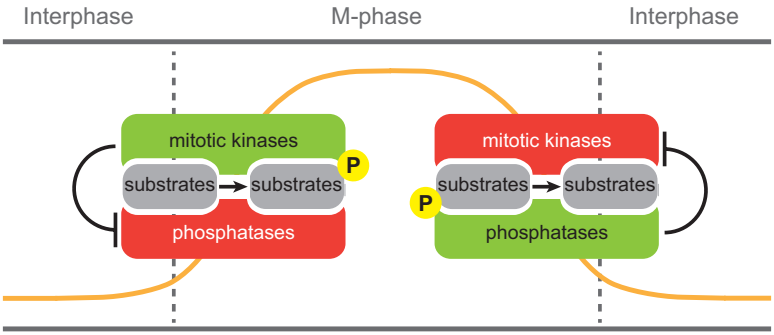

Fig. 3.2 To achieve cell cycle transitions, mitotic kinases and antagonising phosphatases must be regulated in a manner such that their activities are mutually exclusive

3.2 Cyclin-Dependent Kinases (Cdks)

The common feature of all Cdks is that their kinase activity depends on association with a regulatory cyclin subunit. Of the roughly one dozen Cdks identified in mammalian cells, five — Cdk1, Cdk2, Cdk3, Cdk4 and Cdk6 — are directly involved in cell cycle regulation (Malumbres and Barbacid 2005). Notably, mouse knockout studies identified Cdk1, also known as cell division cycle protein 2 (Cdc2), as the only cell cycle Cdk that is essential for cell division, i.e. mice lacking functional Cdk1 did not develop beyond the two-cell stage, whereas, for example, $Cdk2^{-/-}$ mice were viable, but sterile (Santamaria et al. 2007). Structural studies on human Cdk2 provided the molecular explanation for the requirement of cyclin binding for kinase activity. Cdk2 shares with other Ser/Thr protein kinases a two-lobed structure where the catalytic site that binds ATP is sandwiched between a short amino-terminal and a

larger carboxy-terminal lobe (De Bondt et al. 1993). However, Cdk2 distinguishes itself from other kinases by two modifications which keep it inactive when cyclin is not bound. First, the T-loop (activation loop) blocks access of substrates to the active-site cleft, and second, residues critical for the correct positioning of ATP are displaced. Binding of cyclin-A relieves Cdk2 from its autoinhibitory state yielding partially active kinase. Full activation of Cdk2/cyclin-A requires phosphorylation of a threonine residue (threonine-160 in human Cdk2) within the T-loop of Cdk2 by the Cdk-activating kinase (CAK), also known as Cdk7 (Russo et al. 1996). CAK is constitutively active, and therefore T-loop phosphorylation of Cdk2, as well as of Cdk1, Cdk4 and Cdk6, occurs in an unregulated manner (Tassan et al. 1994). In contrast, inhibitory phosphorylations occurring on threonine-14 (T14) and tyrosine-15 (Y15) of Cdks are strictly regulated. Phosphorylation of these residues interferes with the correct positioning of ATP at the active site. In vertebrates, T14 phosphorylation is mediated by the kinase Myt1 (membrane-associated tyrosine and threonine kinase), whereas the phosphorylation of Y15 can be catalysed by either Wee1 (Wee1-like protein kinase, in the Scottish dialect 'wee' means small referring to the fact that Wee1 mutants divide at half the wild-type size) or Myt1 (Mueller et al. 1995; Nurse and Thuriaux 1980). Dephosphorylation of both residues is mediated by the dual-specific phosphatase Cdc25 (Russell and Nurse 1986).

3.2.1 The Cdk1 Autoamplification Loop

High Cdk1/cyclin-B activity triggers entry into mitosis. Precise regulation of this dimeric complex at the levels of protein synthesis, posttranslational modifications and protein destruction is therefore essential to ensure timely progression through the cell cycle (Fig. 3.3). Continuous synthesis of cyclin-B results in the accumulation of cyclin-B as cells progress towards M-phase. However, activation of Cdk1/cyclin-B does not occur in a linear but in a switch-like manner. In interphase, MPF activity is suppressed by inhibitory T14/Y15 phosphorylation of Cdk1. Critical for the activation of MPF in an all-or-none fashion at the transition to M-phase is the autoamplification loop composed of Cdk1/cyclin-B, the inhibitory kinases Wee1/Myt1 and the activating phosphatase Cdc25 (Fig. 3.4). *Xenopus* Myt1 seems to be ubiquitously expressed, whereas Wee1 is present in mature oocytes and during embryonic divisions and absent in immature oocytes, which instead express Wee2 (Wee1B) (Han et al. 2005; Nakajo et al. 2000). In mammalian cells, the Cdc25 family of dual-specificity phosphatases contains the three members Cdc25A, Cdc25B and Cdc25C. In mice, Cdc25A is the only one that is essential for early embryonic development (Lee et al. 2009), whereas Cdc25B and Cdc25C are dispensable for embryonic development (Ferguson et al. 2005). Following cyclin-B accumulation, a small pool of Cdk1/cyclin-B becomes active and phosphorylates Wee1/Myt1 resulting in the inactivation of these inhibitory kinases. At the same time, Cdk1/cyclin-B phosphorylates and activates its activator Cdc25 (Hoffmann et al. 1993). The positive feedback loop of Cdc25 activation and the double-negative loop of Wee1/Myt1 inactivation create a bistable system where Cdk1/cyclin-B cannot exist

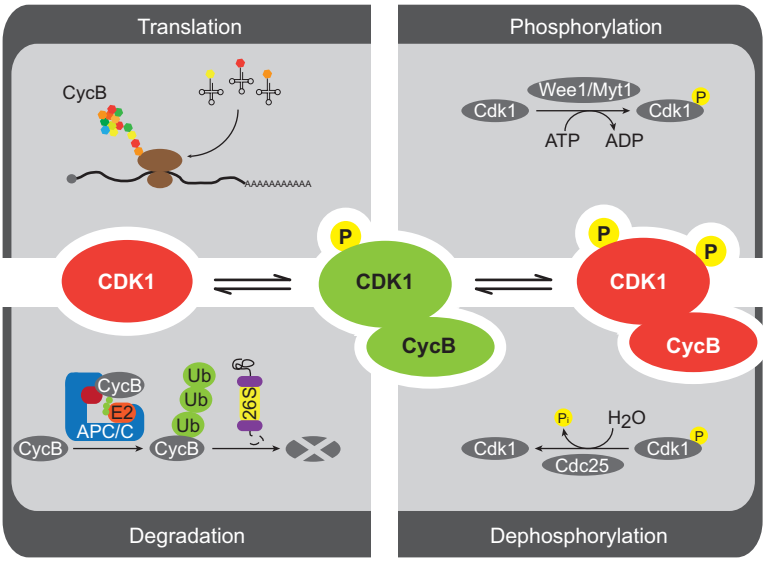

Fig. 3.3 Cdk1 is the master regulator of cell cycle progression. The activity of Cdk1/cyclin-B is regulated at the levels of translation and degradation, phosphorylation and dephosphorylation (active and inactive factors are shown in green and red, respectively. This also applies to Figs. 3.4–6)

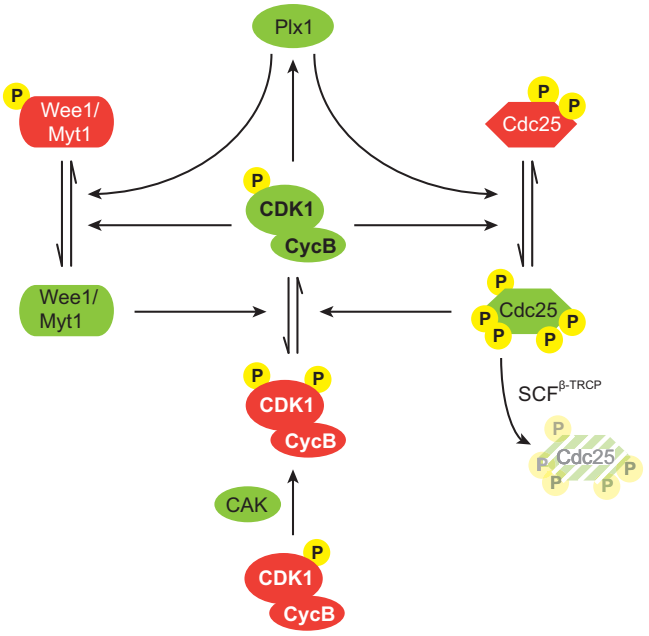

Fig. 3.4 The Cdk1/cyclin-B amplification loop. Cdk1/cyclin-B negatively regulates the activities of Wee1/Myt1 that act inhibitory on Cdk1 by phosphorylating it at T14/Y15. These inhibitory phosphorylations are removed by Cdc25, which is under the positive control of Cdk1/cyclin-B. Cdk1-activating kinase (CAK) constitutively activates Cdk1 by phosphorylating it at the T-loop

in a stable state of intermediate activity, but only in one of the two extreme steady states—'on' or 'off' (Trunnell et al. 2011). Studies in frog egg extracts suggest that Cdk1 stimulates the activity of *Xenopus* Polo-like kinase 1 (Plx1) that in turn amplifies the autoamplification loop by acting in a stimulatory and inhibitory manner on Cdc25 and Myt1, respectively (Abrieu et al. 1998). Cdk1/cyclin-B activation begins in the cytoplasm, most prominently at centrosomes. Yet, to induce nuclear envelope breakdown (NEBD), Cdk1/cyclin-B must accumulate in the nucleus, and this depends on the phosphorylation of cyclin-B at a nuclear export signal sequence resulting in decreased export rates (Yang et al. 1998). Notably, like its activation, nuclear translocation of Cdk1/cyclin-B at late prophase occurs in an abrupt manner. Recent studies identified a spatial positive feedback where nuclear Cdk1/cyclin-B promotes the translocation of Cdk1/cyclin-B from the cytoplasm into the nucleus (Santos et al. 2012). Thus, the irreversible and switch-like onset of M-phase seems to be mediated by positive feedback loops acting on both the activity and localisation of Cdk1/cyclin-B.

3.3 Control of the Cell Cycle Through Ubiquitin-Mediated Proteolysis

Exit from mitosis depends on the inactivation of Cdk1/cyclin-B, which is primarily mediated by the destruction of cyclin-B via the ubiquitin-proteasome pathway. Selective ubiquitylation of cyclin-B in mitosis requires the ubiquitin E3 ligase anaphase-promoting complex/cyclosome (APC/C) (Irniger et al. 1995; King et al. 1995; Sudakin et al. 1995). The APC/C is a multisubunit complex made up of 14 known proteins, including the really interesting new gene (RING) finger protein Apc11 and the Cullin-like subunit Apc2. Unlike homologous to E6AP C-terminus (HECT) ligases, the APC/C does not form a thioester bond with ubiquitin but rather promotes the direct transfer of ubiquitin from the E2 to substrate proteins. Polyubiquitylation of APC/C substrates occurs in a sequential manner using two different E2 enzymes: UbcH10 (*Xenopus*: UbcX) binds to the RING domain of Apc11 and initiates the ubiquitylation of substrate proteins by conjugating ubiquitin to the ε-amino group of a lysine residue in the substrate protein (Brown et al. 2014), whereas Ube2S catalyses chain elongation by assembling Lys11-linked ubiquitin chains (Jin et al. 2008; Kelly et al. 2014). The mitotic APC/C is activated through association with either of the two known activator subunits—cell division cycle 20 (Cdc20 also known as Fizzy (FZY)) and Cdc20 homologue 1 (Cdh1 also known as fizzy related (FZR)) (Visintin et al. 1997). Both activators bind substrates through their WD-40 propeller repeats, which recognise three motifs—the destruction box (D-box) (consensus: RXXLXXXXN) and/or the KEN box (consensus: KENXXXN) and/or the ABBA motif (Di Fiore et al. 2015; Pfleger and Kirschner 2000; Glotzer et al. 1991). The core APC/C subunit, Apc10, together with Cdc20 or Cdh1, forms the bipartite substrate receptor.

3.3.1 Regulation of APC/C Activity

Once in mitosis, active Cdk1/cyclin-B phosphorylates core APC/C subunits, and these modifications enhance its affinity for the coactivator Cdc20 (Kraft et al. 2003; Rudner and Murray 2000; Shteinberg et al. 1999). Active APC/C^{Cdc20} then targets securin and cyclin-B for proteasomal destruction. Thus, Cdk1 triggers its own inactivation by activating APC/C^{Cdc20}. Decreased cyclin-B levels, in turn, result in reduced phosphorylation of the APC/C and, hence, in inactivation of APC/C^{Cdc20}. The destruction of the remaining cyclin-B is mediated by Cdh1 which, in contrast to Cdc20, binds more efficiently to the APC/C in its dephosphorylated form (Kraft et al. 2003; Kramer et al. 2000; Zachariae et al. 1998). Multisite phosphorylation events of the APC/C and inhibitory phosphorylations on Cdc20 seem to be critical for the required time delay between Cdk1/cyclin-B activation and the activation of the APC/C^{Cdc20} (Labit et al. 2012; Yang and Ferrell 2013). To ensure that anaphase onset occurs only once all chromosomes are correctly attached to spindle microtubules, a pathway referred to as the spindle assembly checkpoint (SAC) prevents APC/C activation in the presence of improperly attached chromosomes. Since this pathway does not seem to be active during *Xenopus* early embryo development, we do not describe the mechanism of SAC-mediated APC/C inhibition in detail, but refer to excellent review articles (Hauf 2013; Vleugel et al. 2012; Musacchio 2011; Musacchio and Salmon 2007; Lara-Gonzalez et al. 2012). In brief, unattached kinetochores signal the formation of a complex known as the mitotic checkpoint complex (MCC), which consists of Mad2, Bub3, BubR1 and Cdc20, forming a strong inhibitor of APC/C-mediated poly-ubiquitylation of cyclin-B and securin. Once chromosomes are correctly attached, this checkpoint is turned off resulting in the activation of the APC/C, thereby targeting securin and cyclin-B for destruction. Consequentially, cells separate sister chromatids and exit M-phase. In interphase, APC/C^{Cdh1} is kept inactive by early mitotic inhibitor 1 (Emi1), and this mechanism is important to stabilise cyclin-A, cyclin-B and geminin—a regulator of DNA replication. Destabilisation of these APC/C substrates in Emi1-depleted cells induces DNA re-replication and prevents mitotic entry (Di Fiore and Pines 2007; Machida and Dutta 2007). Emi1 levels are controlled in a cell cycle-dependent manner. At the G1 to S transition, Emi1 is transcriptionally induced by the E2F transcription factor and targeted for destruction shortly before NEBD via the E3 ligase $SCF^{\beta\text{-}TRCP}$ (Skp1, Cullin, F-box protein) (Hansen et al. 2004; Hsu et al. 2002; Moshe et al. 2004). $SCF^{\beta\text{-}TRCP}$-mediated ubiquitylation of Emi1 requires its prior phosphorylation by Polo-like kinase 1 (Plk1) and probably Cdk1. *Xenopus* Emi1-related protein 1 (XErp1)/Emi2 represents another APC/C inhibitor which was initially identified based on its essential function in arresting mature oocytes in metaphase of meiosis II (Schmidt et al. 2005; Shoji et al. 2006; Tung et al. 2005). Upon fertilisation, calcium-calmodulin-dependent kinase II (CaMKII) and Plk1 coordinate to target XErp1 for $SCF^{\beta\text{-}TRCP}$-mediated degradation resulting in exit from meiosis and entry into the first mitotic division (Liu and Maller 2005; Rauh et al. 2005). Notably, XErp1 reaccumulates during the prolonged first division of *Xenopus* embryos, and the presence of XErp1 during the subsequent fast divisions is critical for timely regulation of APC/C activity (see below) (Tischer et al. 2012).

3.4 Serine/Threonine Protein Phosphatases in Cell Cycle Control

As aforementioned, mitotic entry is characterised by massive changes in the phosphorylation state of both structural as well as regulatory components of the cell cycle machinery. These changes trigger key cell cycle events such as nuclear envelope breakdown (NEBD), chromatin condensation and assembly of a bipolar spindle (Olsen et al. 2010; Hegemann et al. 2011). These events mediated by multiple serine/threonine-directed protein kinases are reversed during exit from M-phase by protein phosphatases. There are three families of serine/threonine (Ser/Thr) phosphatases: the phosphoprotein phosphatases (PPPs), the metal-dependent protein phosphatases (PPMs) and the aspartate-based phosphatases FCP/SCP (Shi 2009). In this book chapter, we will focus primarily on the role of protein phosphatases of the PPP type as their function in cell cycle regulation is best understood. Due to the large discrepancy between the number of known Ser/Thr kinases (~400) and phosphatase catalytic core subunits (~30), it was believed for a long time that phosphatases are rather unregulated enzymes with low substrate specificity that work like lawnmowers (trimming phosphates in a relative nonspecific manner from many different proteins) to reset the cell cycle machinery to the dephosphorylated interphase state (Shi 2009; Ceulemans and Bollen 2004). This simplistic view is clearly wrong. In cells, PPPs do not exist as naked catalytic subunits, but in a complex with regulatory subunits that mediate substrate specificity, localisation and activity. Thus, there are many different holoenzymes with specific and distinct functions that share the same catalytic subunit (Shi 2009; Janssens et al. 2008; Virshup and Shenolikar 2009). Within the family of PPPs, type 1 (protein phosphatase 1, PP1) and type 2A (PP2A) phosphatases have been implicated—among other functions in various cellular pathways—in the dephosphorylation of mitotic regulators (Fernandez et al. 1992; Ferrigno et al. 1993; Nelson et al. 1997; Wu et al. 2009; Mochida et al. 2009; Manchado et al. 2010; Schmitz et al. 2010; Cundell et al. 2013). Here, we will explain briefly what is known about the mitotic function and regulation of these phosphatases.

3.5 Type 2A Protein Phosphatases (PP2As)

The quest for the phosphatase that removes mitotic phosphorylations identified PP2A as the major Cdk1 antagonising phosphatase (Ferrigno et al. 1993; Mayer-Jaekel et al. 1994). PP2A is ubiquitously expressed in mammalian cells and can account for ~0.2 % of the total cellular protein content (Ruediger et al. 1991). In cells, PP2A is either present as a dimeric core enzyme or as a trimeric holoenzyme. The core dimer consists of the catalytic C-subunit (two isoforms: PPP2CA and PPP2CB) and the scaffold A-subunit (two isoforms: PPP2R1A and PPP2R1B). The core dimer then associates with a regulatory B-type subunit, which mediates substrate specificity and localisation of the holoenzyme. In the human genome, 15 genes have been identified that encode for B-type subunits belonging to the four

distinct subfamilies B/PPP2R2, B´/PPP2R5, B´´/PPP2R3 and B´´´/Striatins. Alternative splicing of some of the mRNAs gives rise to at least 23 different B-type subunits. Theoretically, the combinatorial assembly of one B-type subunit with one A- and C-subunit would yield more than 90 different PP2A holoenzymes (Sents et al. 2013). Mouse studies revealed that knockout of the PPP2CA gene is embryonic lethal despite the presence of the β-isoform (PPP2CB) which shares 97 % sequence identity with the α-isoform (Gotz et al. 1998). Thus, even the two C-subunit isoforms seem to have nonredundant functions despite their high levels of sequence identity, indicating that the 90 different possible holoenzymes have different substrate specificities, regulations and/or localisations. Notably, PP2As can undergo a complex and highly regulated maturation process. To date, the best understood post-translational modifications of PP2A include C-terminal methylation and phosphorylation of the catalytic subunit. Both modifications are involved in a surveillance mechanism that controls PP2A holoenzyme assembly and prevents premature unspecific activity of the catalytic subunit (Stanevich et al. 2011; Hombauer et al. 2007). An additional mechanism controlling the activity of PP2As involves the binding of inhibitory proteins. CIP2A, SET/I2^{PP2A} and Anp32/I1^{PP2A} are inhibitor proteins that decrease the activity of PP2A against a broad range of substrates (Li et al. 1995; Junttila et al. 2007; Laine et al. 2013). Their importance is highlighted by the fact that upregulation of these inhibitors and the resulting inhibition of PP2A activity are associated with the development of tumours, e.g. by increasing the stability of the oncogene Myc (Junttila et al. 2007; Neviani et al. 2005).

3.5.1 The PP2A-B55 Phosphatase

The complete identity of the phosphatases involved in maintaining the dephosphorylated interphase state and/or reverting the mitotic phosphorylated state to the dephosphorylated state during exit from M-phase and their relative contribution in these processes remains elusive. However, experiments in invertebrates and vertebrates clearly identify PP2A with a regulatory B55 subunit (PP2A-B55) as a key antagonist of Cdk1/cyclin-B (Fig. 3.5) (Mochida et al. 2009; Manchado et al. 2010; Schmitz et al. 2010; Cundell et al. 2013; Mayer-Jaekel et al. 1994). siRNA-mediated knockdown of B55 in cells or immunodepletion of B55 from cytoplasmic extracts blocks dephosphorylation of several Cdk1 model substrates and, with varying efficiency, general dephosphorylation of mitotic phosphoproteins.

3.5.1.1 Regulation of PP2A-B55

Xenopus laevis egg extract studies revealed that the activity of PP2A-B55 is regulated reciprocally to that of Cdk1, i.e. it is downregulated at mitotic entry and reactivated at the beginning of mitotic exit (Mochida et al. 2009). This modulation of PP2A-B55 activity depends on the activity of Cdk1. How is Cdk1 able to inactivate

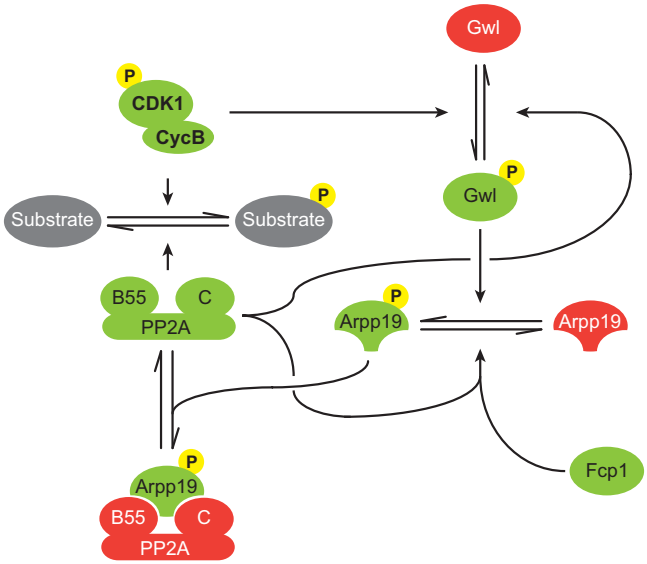

Fig. 3.5 Cdk1/cyclin-B activity results in the inhibition of PP2A-B55. PP2A-B55 is a major Cdk1/cyclin-B-antagonising phosphatase. Active Cdk1/cyclin-B phosphorylates and thereby activates Gwl kinase, which in turn phosphorylates the small protein Arpp19 (Ensa). Phosphorylated Arpp19 is a high-affinity inhibitor of PP2A-B55. The reactivation of PP2A-B55 at mitotic exit involves dephosphorylation of Gwl by PP2A-B55 and Arpp19 by PP2A-B55 and Fcp1

its counteracting phosphatase? The answer to this question started to emerge when the precise role of an AGC-type kinase called Greatwall (Gwl) was examined (Fig. 3.5). Gwl was first identified in a complementation screen for cell cycle regulators in *Drosophila*, and it was shown that a hyperactive Gwl variant called 'Scant' induces mitotic defects (White-Cooper et al. 1996). Almost 10 years later, key insights into the function of Gwl were provided by studies in *Xenopus laevis* egg extract. These studies revealed that Gwl contributes to Cdk1 activation by stimulating the amplification loop (Yu et al. 2006). However, further studies suggested that Gwl does not act directly on activation loop components but rather by inactivating an okadaic acid-sensitive phosphatase that negatively regulates Cdc25 (Yu et al. 2006; Zhao et al. 2008). This phosphatase turned out to be PP2A-B55, and the mechanism of Gwl-mediated PP2A-B55 inactivation—which will be discussed in detail below—affects not only the phosphorylation state of Cdc25 but applies in general for PP2A-B55 substrates (Vigneron et al. 2009; Castilho et al. 2009). Dysfunction of Gwl and, hence, deregulated PP2A-B55 activity affect the phosphorylation state of Cdk1 substrates by two means: first, it alters Cdk1 activity itself by acting on Cdc25, and second, it acts directly on Cdk1 substrates, which ultimately results in severe mitotic defects (Burgess et al. 2010). Cells of Gwl knockout (KO) mice enter mitosis with normal kinetics, but most of them arrest in prometaphase and show severe defects in chromosome condensation and segregation (Alvarez-Fernandez et al. 2013).

3.5.1.2 The AGC-Type Kinase Greatwall (Gwl)

Gwl belongs to the family of AGC-type kinases and shares many functional key residues with other family members, but it also contains an unusual nonconserved insertion between the N- and the C-terminal domains (Vigneron et al. 2011). Gwl activation is initiated by Cdk1-dependent phosphorylation in the conserved activation loop, which results in a conformational change that makes the active site located between the N- and C-terminal lobes accessible (Blake-Hodek et al. 2012). Additional phosphorylation events in the C-terminal domain (turn motif/tail site) are critical for Gwl's kinase activity. One of these C-terminal residues (S875 in human Gwl; S883 in *Xenopus* Gwl) has been characterised best, and there are conflicting reports about the kinase that mediates the phosphorylation of this residue. First, it has been deduced from in vitro activation assays that it is phosphorylated by Cdk1 and Plk1 (Vigneron et al. 2011). However, this result has been challenged by the fact that this residue gets phosphorylated only when active, but not kinase dead, Gwl is incubated with Cdk1. This points to an autophosphorylation mechanism, in which Gwl can phosphorylate itself once the active site is accessible after Cdk1-dependent phosphorylation of the activation loop (Blake-Hodek et al. 2012). Some AGC-type kinases, e.g. PDK1, are only fully activated once they interact with another kinase through a hydrophobic interaction motif. No such interaction has been described for Gwl so far, but it contains a functional hydrophobic pocket. Incubation of Gwl with a phosphorylated hydrophobic peptide of the AGC-kinase Rsk2 stimulates the kinase activity of preactivated Gwl, indicating that such a transactivation mechanism with a so far unknown partner could exist for Gwl (Vigneron et al. 2011). In human cell lines, Gwl is not only regulated at the activity level but also by phosphorylation-dependent localisation. In interphase, Gwl is predominantly localised in the nucleus until it gets phosphorylated in the nonconserved middle region after nuclear import of Cdk1/cyclin-B in late prophase (Alvarez-Fernandez et al. 2013). Upon this phosphorylation event, Gwl is exported from the nucleus and accumulates in the cytoplasm where it mediates the inactivation of PP2A-B55 before the nuclear envelope is disassembled. This mechanism ensures that the mitotic state is not lost when nuclear Cdk1/cyclin-B and cytoplasmic PP2A-B55 intermingle upon nuclear envelope breakdown (Alvarez-Fernandez et al. 2013). In immature *Xenopus laevis* oocytes, which lack significant Cdk1 activity, Gwl localisation is, in contrast, not restricted to the nucleus (Hara et al. 2012). Therefore, it remains to be determined whether the mechanism of Cdk1-mediated nuclear export of Gwl applies only to higher vertebrates or represents a mitosis-specific mechanism.

3.5.1.3 The PP2A-B55 Inhibitors Ensa and Arpp19

In vitro assays failed to detect substantial kinase activity of Gwl towards PP2A-B55, and this observation fuelled the search for the missing link between Gwl and PP2A-B55 (Castilho et al. 2009). Two independent *Xenopus laevis* egg extract studies identified the two closely related proteins Arpp19 (cAMP-regulated phosphoprotein, 19 kDa) and Endosulfine α (Ensa) as the sought-after substrates of Gwl

(Fig. 3.5) (Gharbi-Ayachi et al. 2010; Mochida et al. 2010). Initially, Arpp19 was identified as a cAMP-dependent neuronal phosphoprotein, and Ensa as endogenous ligand for neuronal sulfonylurea receptors (Girault et al. 1988; Virsolvy-Vergine et al. 1992). As shown by the teams of Lorca and Hunt, phosphorylation of Ensa/Arpp19 by Cdk1-activated Gwl turns them into high-affinity inhibitors of PP2A-B55. Thus, the identification of Ensa/Arpp19 as Gwl substrates finally provided the molecular explanation for how Cdk1/cyclin-B activation at mitotic entry results in the inactivation of the antagonising phosphatase PP2A-B55.

3.5.1.4 The Reactivation of PP2A-B55 at Exit from M-Phase

Exit from M-phase requires the reactivation of PP2A-B55 to reset the mitotic phosphorylation state to interphase. Inactivation of the Gwl-Ensa/Arpp19 module after Cdk1/cyclin-B inactivation sets the timing for the reactivation of PP2A-B55 at mitotic exit. The importance of the Gwl-Ensa/Arpp19 module for the correct temporal order during exit from M-phase has been shown recently by mathematical modelling and experiments using the spindle midzone protein PRC1 as model substrate, i.e. uncoupling PP2A-B55 inhibition from Cdk1/cyclin-B activity results in impaired dephosphorylation of PRC1 resulting in premature onset of cytokinesis (Cundell et al. 2013). Studies addressing the underlying mechanism for Gwl-Ensa/Arpp19 inactivation revealed that Gwl-phosphorylated Ensa/Arpp19 is not only an inhibitor of PP2A-B55 but also a substrate. However, compared to other mitotic substrates, phosphorylated Ensa/Arpp19 seems to have a very high affinity for PP2A-B55, but its dephosphorylation by PP2A-B55 is very slow (Williams et al. 2014). Based on these findings, the authors proposed a model of 'inhibition by unfair competition': during early mitosis, most PP2A-B55 holoenzymes are occupied with Ensa/Arpp19, and the few molecules of Ensa/Arpp19 that get dephosphorylated will be replenished by active Gwl and will rebind to PP2A-B55. At exit from M-phase, Gwl gets inactivated and consequently, the pool of phosphorylated Ensa/Arpp19 decreases, allowing PP2A-B55 to focus on other mitotic substrates. An independent study, however, identified RNA polymerase II carboxy-terminal domain phosphatase Fcp1 as the Ensa/Arpp19 phosphatase (Hegarat et al. 2014). While further studies are required to clarify the contributions of PP2A-B55 and Fcp1, it is beyond dispute that PP2A-B55 reactivation requires the dephosphorylation, i.e. inactivation, of both Ensa/Arpp19 and Gwl itself. Studies from the group that identified Fcp1 as the Ensa/Arpp19 phosphatase suggest that PP2A-B55 liberates itself from inhibition by dephosphorylation of Gwl (Hegarat et al. 2014).

3.5.1.5 Additional Mechanisms Regulating PP2A-B55 and Ensa/Arpp19

Arpp19 and Ensa are not only phosphorylated by Gwl but also by Cdk1 and protein kinase A (PKA) (Mochida et al. 2010). The phosphorylation by Cdk1 seems to have an activating effect on Arpp19, thereby enabling a Gwl-independent mode of PP2A-B55 inhibition in mitosis. Yet, the physiological relevance of this mechanism

has not been fully addressed (Mochida 2014; Okumura et al. 2014). In contrast, studies in immature *Xenopus laevis* oocytes revealed that phosphorylation of Arpp19/Ensa by PKA has an inhibitory effect (Mochida 2014; Dupre et al. 2014). Yet, if this mechanism also applies to mitotic divisions remains to be investigated. A recent study suggested that phosphorylation of B55α affects its ability to assemble as a PP2A holoenzyme (Schmitz et al. 2010). The identified phosphorylation site matched the Cdk1 consensus motif suggesting that high Cdk1 activity in mitosis might interfere with the assembly of the PP2A-B55α holoenzyme. However, future studies are required to clarify the physiological relevance and consequence of this event in detail. In accordance with this, there is evidence from fission yeast for a direct inactivation of B55 by mitotic kinases, and this is counteracted by the phosphatase PP1, which binds and reactivates B55 after anaphase onset (Grallert et al. 2015).

3.5.2 The PP2A-B´56 Phosphatase

Several mitotic functions of PP2A holoenzymes with a regulatory B´56 subunit have been revealed since the discovery of this protein class in 1995 (McCright and Virshup 1995). The best understood function of PP2A-B´56 in mitosis is its role in the stabilisation of kinetochore-microtubule attachments and the protection of centromeric cohesion during prophase (Kitajima et al. 2006; Suijkerbuijk et al. 2012; Foley et al. 2011; Nijenhuis et al. 2014; Xu et al. 2014). While both functions are critical for the fidelity of chromosome segregation (Kitajima et al. 2006; Foley et al. 2011; Nijenhuis et al. 2014; Xu et al. 2014), in this book chapter we focus on the role of B´56 as an element of the DNA damage-responsive checkpoint which acts on the Cdk1 amplification loop and its role in controlling the APC/C (Fig. 3.6). To prevent M-phase entry in the presence of DNA damage, Cdc25 is phosphorylated at serine-287 (*Xenopus laevis* Cdc25; serine-216 in human Cdc25C). Several kinases are known to phosphorylate S287, e.g. the DNA damage-activated kinases Chk1 and Chk2 as well as the kinases c-TAK1 and PKA (Furnari et al. 1997; Duckworth et al. 2002; Peng et al. 1998; Zeng et al. 1998; Kumagai et al. 1998; Sanchez et al. 1997), and this phosphorylation promotes binding of the inhibitory protein 14-3-3 (Peng et al. 1997). 14-3-3 binding to Cdc25 is weakened by Cdk2-dependent phosphorylation of Cdc25 at a different site (Guadagno and Newport 1996; Margolis et al. 2006a). Phosphorylation of B´56δ by DNA damage checkpoint kinases leads to an increased incorporation of this subunit into PP2A holoenzymes, which antagonises the phosphorylation of Cdc25 by Cdk2 and thereby prevents the dissociation of 14-3-3 from Cdc25 (Margolis et al. 2006a). Thus, in the presence of DNA damage, checkpoint kinases keep Cdc25 inactive by stimulating the formation of a complex between Cdc25 and 14-3-3 in a PP2A-B´56δ-dependent manner. Once DNA defects are repaired, this mechanism (see below for more details) fades resulting in Cdc25 activation and entry into M-phase (Fig. 3.6).

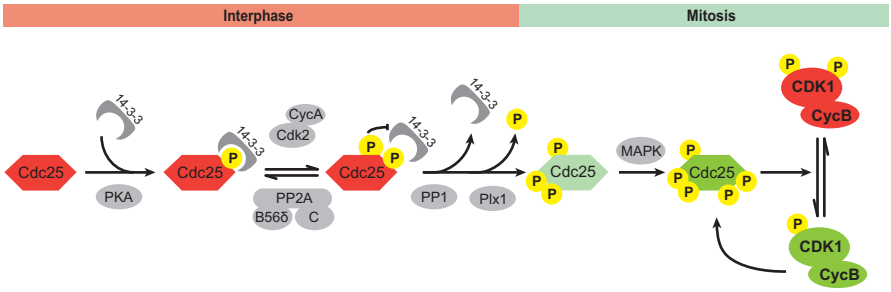

Fig. 3.6 The activity of Cdc25 is controlled by phosphorylation and 14-3-3 binding. Phosphorylation of Cdc25 at *Xenopus* Ser-287 (*human* Ser-216) recruits 14-3-3. Cdk2/cyclin-A phosphorylation of Cdc25 interferes with 14-3-3 binding. This phosphorylation event is antagonised by PP2A-B´56. PP1 promotes M-phase entry by dephosphorylating Ser-287

3.5.2.1 The Link Between PP2A-B´56 and the APC/C

Not surprisingly, the cell cycle regulatory machinery harnesses the interplay of kinases and opposing phosphatases to control the activity of the APC/C as well. An example is PP2A-B´56 and its effect on the evolutionarily conserved APC/C inhibitor XErp1/Emi2. As aforementioned, XErp1 inhibits the APC/C, and this activity is essential to arrest mature oocytes at metaphase of meiosis II (Schmidt et al. 2005; Shoji et al. 2006; Rauh et al. 2005). Phosphorylation of XErp1 by the mitotic kinase Cdk1/cyclin-B primes XErp1 for further phosphorylations by Polo-like kinase 1 (Plk1) and casein kinase 1 (CK1), and these phosphorylations result in the destabilisation and inhibition of XErp1 (Isoda et al. 2011). The inhibitory effect of Cdk1/cyclin-B on the APC/C inhibitory activity of XErp1 is antagonised by PP2A-B´56-mediated dephosphorylation of XErp1. During the MII arrest of *Xenopus* eggs, this mechanism maintains the activity of Cdk1/cyclin-B at constant levels despite ongoing synthesis of cyclin-B (Wu et al. 2007): once cyclin-B levels reach a critical upper threshold, XErp1 is inactivated by Cdk1/cyclin-B resulting in APC/C activation. APC/C activation leads to cyclin-B destruction until PP2A-B´56 prevails over Cdk1/cyclin-B resulting in XErp1 dephosphorylation and the re-inhibition of the APC/C (Isoda et al. 2011). In somatic human cells, PP2A-B´56 has been shown to be important for fine-tuning the APC/C-mediated destruction of securin. Securin is an inhibitory chaperone of separase (Ciosk et al. 1998), the enzyme that triggers sister chromatid disjunction at anaphase onset by cleaving the centromeric cohesin rings that physically tether the chromosomes together. Securin is degraded by the APC/C once the SAC is satisfied by the correct attachment of *all* chromosomes to the mitotic spindle (Cohen-Fix et al. 1996). Ca^{2+}/calmodulin-dependent kinase II (CaMKII) phosphorylation of securin converts it into a better APC/C substrate (Hellmuth et al. 2014). However, a pool of PP2A-B´56 that is associated with separase stabilises separase-bound securin by keeping it dephosphorylated. By ensuring that the large pool of free securin is degraded before the separase-bound fraction, this mechanism contributes to the abruptness and fidelity of sister chromatid segregation in anaphase (Hellmuth et al. 2014).

3.6 Type 1 Protein Phosphatase (PP1)

The Ser/Thr-phosphatase PP1 is a ubiquitous and highly conserved eukaryotic protein that is involved in the regulation of a large variety of cellular processes. There exist four different but highly similar isoforms of the PP1 catalytic subunit, PP1α, PP1β/δ, PP1γ1 and PP1γ2, which show different tissue expression patterns and can have, despite their high similarity, isoform-specific functions (Sasaki et al. 1990; Shima et al. 1993). Since the initial identification of PP1 as a phosphatase that dephosphorylates enzymes of the glycogen metabolism pathway, it has been identified as a regulatory factor in cellular processes as diverse as protein synthesis, DNA replication, mitosis, DNA damage response, apoptosis, energy metabolism, muscle contraction and modulation of signalling processes (Ceulemans and Bollen 2004; Antoniw et al. 1977; Kobayaski et al. 1975). Like for type 2 phosphatases, the diversified skillset of PP1 stems from the combinatorial assembly of a catalytic subunit with a regulatory subunit and, occasionally, the formation of a higher order complex.

3.6.1 PP1-Interacting Proteins (PIPs)

The PP1-interacting proteins (PIPs), of which at least 200 are validated, regulate the substrate specificity, localisation and activity of PP1 (Virshup and Shenolikar 2009; Heroes et al. 2013). PP1 can interact with many different partners—substrates and regulators—because they all use combinations of the same binding motifs, e.g. 90 % of the PIPs contain a 'RVxF' motif that binds to a hydrophobic channel on PP1 (Heroes et al. 2013). Other motifs shared by various PIPs are the 'SILK' and the 'MyPhone' motifs (Heroes et al. 2013; Roy and Cyert 2009), and it is likely that the analysis of the binding mode of PIPs will identify further motifs.

Binding of the PIP Repo-Man (*re*cruits *P*P1 *o*nto *m*itotic chromatin at *an*aphase) targets PP1 to histone H3 where it counteracts the phosphorylation of histone H3 by the kinase Haspin (Qian et al. 2011). Since Aurora-B as part of the chromosomal passenger complex (CPC) is recruited to Haspin-phosphorylated histone H3, the activity of PP1/Repo-Man thus contributes to the regulation of Aurora-B kinase recruitment to centromeres in mitosis. By interacting with other PIPs, PP1 opposes the function of Aurora-B by alternative mechanisms: Sds22 targets PP1 to the kinetochore protein Knl1, where it suppresses the function of Aurora-B by dephosphorylating the kinase itself at its activation loop and substrate proteins of Aurora-B (Eiteneuer et al. 2014; Kim et al. 2010; Liu et al. 2010; Posch et al. 2010). These are just two out of many examples of how the binding of a PIP to PP1 confers specificity for a distinct cellular process to the holoenzyme.

3.6.2 PIPs Regulating PP1 Activity

Some PIPs do not confer substrate selectivity, but act as inhibitors of the PP1 catalytic subunit by masking its active site. Two well-characterised examples of inhibitory PIPs are the heat-stable proteins Inhibitor-1 and Inhibitor-2, both of which play important cell cycle regulatory roles (Huang and Glinsmann 1976). Inhibitor-1 is phosphorylated by the cAMP-activated kinase PKA, which turns it into a potent inhibitor of PP1. In early mitosis, PP1 is phosphorylated and inactivated by Cdk1 (see below), and it was proposed that PKA-phosphorylated Inhibitor-1 helps to prevent PP1 from precocious autodephosphorylation and activation. After Cdk1 is inactivated at the metaphase-to-anaphase transition, PP1 can dephosphorylate both Inhibitor-1 and itself to mediate dephosphorylations of mitotic substrate proteins as cells exit mitosis (Wu et al. 2009). Inhibitor-2 was initially identified as a heat-stable unfolded protein that inhibits the activity of PP1 towards its substrate glycogen phosphorylase a (Huang and Glinsmann 1976). However, its function is more complex in that Inhibitor-2 is also critical for the correct folding of nascent PP1 polypeptides (Alessi et al. 1993). Properly folded PP1 is further kept inactive by Inhibitor-2 and phosphorylation of Inhibitor-2 results in the release of active PP1. Thus, by acting as an inhibitory chaperone, Inhibitor-2 provides a cytosolic reservoir of PP1 that can be activated on demand. Several kinases including GSK3β, Cdk1 and MAPK show in vitro activity towards Inhibitor-2, but their contributions to Inhibitor-2 phosphorylation in cells are not fully understood (Li et al. 2006; Puntoni and Villa-Moruzzi 1995; Leach et al. 2003). In cells, Inhibitor-2 localises to centrosomes, mitotic spindle, midzone and midbody where it seems to be important to balance the interplay between PP1 and Aurora-B activity (Leach et al. 2003; Wang et al. 2008). Interfering with the activity of Inhibitor-2 results in errors in chromosome segregation and cytokinesis (Wang et al. 2008). Notably, apart from its role in modulating the activity of PP1, Inhibitor-2 is directly involved in the regulation of the kinase Aurora-A (Shindo et al. 1998; Kufer et al. 2002; Marumoto et al. 2003). Binding of Inhibitor-2 to Aurora-A, with a domain different to the PP1-binding domain, allosterically activates Aurora-A in vitro, and both proteins seem to form a complex in cells (Satinover et al. 2004).

3.6.3 The Role of PP1 in Regulating the Cell Cycle

As mentioned earlier (see Sect. 3.5.2), the Cdk1-activating phosphatase Cdc25 is inactivated in interphase or after DNA damage by phosphorylation of S287 (in *Xenopus*, S216 in humans), which results in the recruitment of the 14-3-3 protein. At the onset of mitosis, PP1 contributes to the activation of Cdc25 by directly dephosphorylating S287 (Margolis et al. 2003). Once activated, the autoamplification loop starts where Cdc25 dephosphorylates and activates Cdk1/cyclin-B, which in turn phosphorylates Cdc25 at multiple sites resulting in enhanced recruitment of PP1 and increased enzymatic activity of Cdc25 (Margolis et al. 2006b). Thus, in this case PP1—in contrast to PP2A-B´56δ—has a

Cdk1-stimulating and mitosis-promoting effect. Intriguingly, *Xenopus* egg extract studies revealed that PP1 is crucial for the execution of mitotic exit (Wu et al. 2009). PP1 would be an excellent candidate for a mitotic kinase-counteracting phosphatase, because it was shown that the activity of the catalytic PP1 subunit is decreased through direct phosphorylation by Cdk1 (Dohadwala et al. 1994) and inhibitory phosphorylation of PP1 appears in prophase of mitosis and peaks in metaphase, closely resembling the activity pattern of Cdk1/cyclin-B (Kwon et al. 1997). After inactivation of MPF by degradation of cyclin-B, PP1 likely activates itself by autodephosphorylation (Wu et al. 2009). This mechanism ensures that the activities of PP1 and Cdk1/cyclin-B occur in a mutually exclusive manner. The extent to which PP1 directly contributes to the dephosphorylation of mitotic phosphoproteins is still under debate and may also vary between organisms. Data from mammalian and *Drosophila* tissue culture cells indicate that PP1 is directly involved in the decondensation of the chromatin and nuclear envelope reassembly during mitotic exit (Steen et al. 2000; Afonso et al. 2014; Thompson et al. 1997). Studies in fission yeast revealed that PP1 is at the top of a cascade that reactivates mitotically inactivated PP2A-B55 and PP2A-B´56 holoenzymes after the metaphase-to-anaphase transition (Grallert et al. 2015). Thus, PP1 seems to have multiple functions during mitotic exit including the function of an 'initiator' (i.e. activation of other phosphatases) as well as 'executing' (i.e. dephosphorylation of mitotic proteins) phosphatase.

3.7 Basic Plan of the Cell Cycle in Early Development

The development of multicellular organisms begins with a single fertilised egg, the zygote. This zygote undergoes cleavage, which involves multiple rounds of extremely rapid cell division cycles during which the volume of the zygote remains the same but the cell number increases. These cells are called blastomeres. As they divide, they organise into a hollow ball of cells, the blastula. During gastrulation the blastula undergoes dramatic cell movements, followed by cell-fate specification ultimately giving rise to the adult organism. In mammals, the blastula is referred to as a blastocyst whose implantation into the uterus is a crucial step during development. Obviously, the formation of an organism is a challenging process and different organisms employ different strategies to ensure success of the reproductive process. Some vertebrates such as amphibians and most fish follow the strategy of external fertilisation and therefore lay numerous eggs to increase the chances of having a few viable progeny since they are not protected by the parents. Reproduction in mammals, on the other hand, typically involves internal fertilisation and viviparity, i.e. the development of the embryo inside of the female, which results in the birth of only a few offspring. Therefore, cell division mechanisms could have undergone selective evolution to match the requirements of different species. *Xenopus* eggs, once fertilised, undergo one 'long' first cell cycle followed by eleven rapid and synchronous divisions (2–12) to generate blastulae, which contain about 4000 cells (Newport and Kirschner 1984). These rapid divisions lack not only gap phases but also checkpoints such as the SAC and DNA damage checkpoint. In addition, transcription from zygotic chromatin is blocked because of its highly compacted, H3-methylated and hypo-acetylated nature, and all translation events occur from maternal mRNAs

stockpiled into the giant oocyte (Akkers et al. 2009; Hontelez et al. 2015; Thompson et al. 1998). The time when active transcription begins in the zygotic genome (zygotic genome activation, ZGA) varies heavily between different organisms. In *Xenopus* embryos, ZGA and midblastula transition (MBT)—a major developmental transition involving activation of cell motility leading to gastrulation—begin at about the same time at the 13th cycle (Kimelman et al. 1987) (see Chap. 9). Zebrafish embryos also undergo 13 cycles until MBT, but ZGA occurs during the course of the tenth cycle (Tadros and Lipshitz 2009; Kane and Kimmel 1993). In contrast, in mice, ZGA occurs at the two-cell stage (Bolton at al. 1984; Thompson et al. 1998), and it requires three more divisions until embryonic cells undergo compaction, i.e. they activate cell-cell adhesion and form the 16-celled morula, which ultimately differentiates into the blastocyst containing the inner cell mass (ICM) and trophoblast (Thompson et al. 1998). This blastocyst is implanted on the uterine wall and undergoes gastrulation to give rise to the gastrula. Like any type of cell cycle, early embryonic divisions are driven, primarily, by accumulation and destruction of cyclin-B albeit with modulations that enable the special features of the first cell cycle as compared to the following cleavage cycles, which are rapid and synchronous in organisms such as *Xenopus* and most fish and asynchronous in mammals.

3.7.1 The First Mitotic Cell Division

The first mitotic cell cycle in a vertebrate zygote begins with the completion of meiosis II and extrusion of the second polar body. In addition, sperm chromatin has to be incorporated into the egg to allow pronuclear fusion and embryonic development. This is initiated by the breakdown of its nuclear envelope followed by decondensation of chromatin and replacement of sperm protamine by somatic histones (Rodman et al. 1981; MacLay and Clarke 2003). Subsequently, the sperm chromatin recondenses to form the male pronucleus (Kubiak and Ciemerych 2001). The de novo assembly of nucleosomes composed of the canonical histone proteins is an important step to enable the paternal genome to contribute to the formation of the diploid zygote. Following the reassembly of the nuclear envelope, DNA replication initiates (McLay and Clarke 2003). In some vertebrates such as mice, DNA replication and the mitotic cell cycle ensue before the male and female pronuclei fuse (Ciemerych and Czolowska 1993; Mayer et al. 2000), while in others such as Zebrafish and *Xenopus*, the maternal and paternal pronuclei fuse before they undergo mitosis (Ubbels et al. 1983; Dekens et al. 2003). Thus, the first mitotic division has a work programme that is clearly distinct from any other cell division. This characteristic is reflected in the length of the first division. In *Xenopus*, the first cell cycle takes about three times as long as the following fast 30-min cycles (Newport and Kirschner 1982). In mice, the total lengths of the first and second cell cycles are comparable (19–20 h), but the first mitotic M-phase cycle lasts for about 120 min as compared to 70 min in the second division (Artus and Cohen-Tannoudji 2008; Ciemerych et al. 1999; Sikora-Polaszek et al. 2006). Reportedly, cyclin-A2 remains stable during the first

M-phase in mice, a characteristic which might account for the prolonged M-phase (Kubiak and Ciemerych 2001).

In *Xenopus*, the mechanism resulting in an elongated first division seems to be distinct to the one in mice, and one of its most important features is the presence of inhibitory phosphorylations of Cdk1 at T14 and Y15. As aforementioned, the basic feature of these phosphorylations is that they keep Cdk1 inhibited in spite of increasing levels of cyclin-B. The kinases mediating these inhibitory phosphorylations, Wee1 and Myt1, are usually counteracted by the phosphatase Cdc25. But, during the first cell cycle in *Xenopus*, this antagonism via Cdc25 seems to be kept at a minimum due to the action of the proto-oncogenic protein kinase c-Mos, which is active at this stage and triggers the activation of the MEK/MAP kinase (MAPK) signalling cascade (Murakami and Vande Woude 1998). c-Mos is a serine/threonine kinase which was originally identified as the transforming gene of Moloney murine sarcoma virus, causing cellular transformation (Oskarsson et al. 1980). Expression of c-Mos was found to be tissue-specific, e.g. in mice (Paules et al. 1989) and frogs (Sagata et al. 1988), high concentrations of c-Mos transcripts were found in the oocytes in contrast to very low concentrations in the brain and testes. The c-Mos/MEK/MAPK pathway has been reported to have an activating function on Wee1 (Murakami and Vande Woude 1998; Murakami et al. 1999; Walter et al. 2000), and strong MAPK activation can negatively affect Cdc25A function by phosphorylating Cdc25A and thereby targeting it for destruction via the $SCF^{\beta\text{-}TRCP}$ E3 ligase (Fig. 3.4) (Isoda et al. 2009). Accordingly, inhibition of the MAPK cascade by a small molecule inhibitor against MEK (U0126) results in decreased Cdk1 Y15 phosphorylation levels and shortened length of the first cell cycle, proving the importance of the c-Mos/MEK/MAPK cascade in ensuring proper length of the first cell cycle (Tsai et al. 2014). Following fertilisation, c-Mos undergoes selective protein destruction. The destruction of c-Mos within approximately 20–30 min after fertilisation depends on its N-terminal proline residue (Nishizawa et al. 1993). This N-terminal proline was shown to affect the net phosphorylation of serine-2, a modification which slows down c-Mos degradation in oocytes (Nishizawa et al. 1992; Sheng et al. 2002). As the first cell cycle progresses, the levels of c-Mos decline, resulting in a lowering of its ability to antagonise Cdk1/cyclin-B activation. The balance is further tipped by the action of *Xenopus* Polo-like-kinase1 (Plx1), which promotes Cdk1/cyclin-B activation by two mechanisms: first, by activating Cdc25 (Abrieu et al. 1998; Qian et al. 1998; Toyoshima-Morimoto et al. 2002), and second, specifically during embryonic M-phase, by inhibiting Myt1 (Inoue and Sagata 2005).

3.7.2 The Early Embryonic Cell Cycles

After the prolonged first cell cycle, embryos enter a series of specialised rapid early embryonic division cycles. In *Xenopus*, these rapid division cycles are highly synchronous and result in formation of the blastula (Newport and Kirschner 1982, 1984). The length of early embryonic divisions varies drastically between species, from 15 min in Zebrafish, 30 min in *Xenopus*, to around 12 h in mice (Artus and Cohen-Tannoudji 2008). The early divisions in mammalian embryos are characterised not

only by their extended duration but also by their asynchrony resulting in stages with uneven number of cells as compared to the exponential increase in cell number in *Xenopus* and Zebrafish (Kimmel et al. 1995; MacQueen and Johnson 1983; Masui and Wang 1998; Newport and Kirschner 1982). Furthermore, experiments involving UV-light and X-Ray irradiation have shown that preimplantation mouse embryos are sensitive to DNA-damaging agents, suggesting that the DNA damage checkpoint in addition to the SAC is already functional at this developmental stage (Wei et al. 2011; Shimura et al. 2002; Artus and Cohen-Tannoudji 2008). In contrast, *Xenopus* and Zebrafish embryos turn on the aforementioned surveillance mechanisms only during MBT, raising the question of how early embryonic divisions are controlled in these organisms (Clute and Masui 1997; Ikegami et al. 1997).

In contrast to the first prolonged cell cycle in mammals, inhibitory phosphorylations on Cdk1 are barely detectable during the fast cycles of *Xenopus* embryos (Ferrell et al. 1991; Hartley et al. 1996). This could occur because of decreased activity of the inhibitory kinases Wee1/Myt1 or increased activity of the activating phosphatase Cdc25. It has been shown that in *Xenopus*, an embryonic isoform of Cdc25, Cdc25A is expressed in addition to the Cdc25C isoform during the fast cycles (Kim et al. 1999), which might favour the existence of T14/Y15 unphosphorylated, active Cdk1. Recent work by Ferrell and co-workers (Tsai et al. 2014) confirmed the finding that Cdc25C is present at constant levels throughout the fast cycles and, in addition, showed that the amount of Cdc25A increases to half maximal by 75 min after fertilisation. Blocking this increase in Cdc25A increases the length of the second cell cycle by 5 min and leads to a slight increase in Y15 phosphorylation of Cdk1, indicating that indeed a shift in the Wee1/Cdc25 ratio could account for altered cell cycle lengths.

In the absence of inhibitory Cdk1 phosphorylations, mechanisms controlling cyclin-B synthesis and destruction are of key importance to ensure oscillating Cdk1/cyclin-B activity (Tsai et al. 2014). Since, in *Xenopus*, transcription from the zygotic genome does not commence until MBT, the expression of cyclin-B during the rapid early embryonic divisions must be regulated at the posttranscriptional level. For *Xenopus*, it has been shown that distinct sequences present in the 3′-untranslated region (UTR) of maternal mRNAs control their translation in a temporal manner (Mendez and Richter 2001). Cyclin-B mRNAs contain one such element called the cytoplasmic polyadenylation element (CPE) responsible for recruiting the cytoplasmic polyadenylation element-binding protein (CPEB), which mediates polyadenylation and ultimately translation (Groisman et al. 2000; Stebbins-Boaz et al. 1996; Stebbins-Boaz et al. 1999). CPEB is activated in a cell-cycle-dependent manner by the kinase Aurora-A (Mendez et al. 2000), leading to active CPEB and polyadenylation-induced translation of cyclin-B before and during M-phase (Groisman et al. 2002). Aurora-A, CPEB and cyclin-B mRNA have been shown to co-localise with the mitotic spindle and centrosomes in *Xenopus* embryos, and expression of a mutant of CPEB defective in spindle localisation impairs embryo cleavage (Groisman et al. 2000). Similarly, injection of CPEB inhibitory antibodies reduces total levels of cyclin-B and impairs division (Groisman et al. 2000), highlighting the importance of regulating cyclin-B synthesis to maintain the integrity of the fast cycles. Exit from M-phase seems to require translational silencing of

cyclin-B mRNA by deadenylation, and studies in cycling *Xenopus* egg extract identified maskin as an important regulator in this process (Groisman et al. 2002).

To allow cyclin-B to accumulate during M-phase, the APC/C must be kept in an inhibited state. It is known that, in *Xenopus*, inhibitory mechanisms such as the SAC and the inhibitory protein Emi1 are not present till MBT (Ohsumi et al. 2004; Gerhart et al. 1984) raising the question of how APC/C regulation is achieved in such a scenario. Studies in *Xenopus* embryos revealed that the function of XErp1/Emi2 as an APC/C inhibitor is critical for early embryonic divisions (Tischer et al. 2012). Depletion of XErp1 results in slower division cycles and untimely destruction of cyclin-B, ultimately resulting in death at MBT (Tischer et al. 2012; Vinod et al. 2013). Since XErp1 protein levels remain constant until MBT, its activity must be regulated in a posttranslational manner (Tischer et al. 2012; Inoue et al. 2007). As aforementioned (see Sect. 3.5.2), during the metaphase II arrest, the activity of XErp1 is controlled such that increasing concentrations of cyclin-B result in XErp1 phosphorylation and inactivation which leads to APC/C-mediated destruction of cyclin-B until a lower threshold of cyclin-B is reached, which allows the reactivation of XErp1 and, hence, APC/C re-inhibition. The basic concept of Cdk1/cyclin-B-mediated XErp1 inactivation also applies to the fast embryonic cycles with the following modifications. First, since c-Mos is absent during the fast embryonic divisions, the recruitment of XErp1 to PP2A-B´56 which antagonises Cdk1/cyclin-B phosphorylations is not mediated by p90RSK—the downstream component of the MAPK pathway—but by PKA. Second, to ensure oscillating APC/C activity, the system must work as a switch rather than as a thermostat. Based on studies performed in extracts of *Xenopus* embryos, the following model was proposed: During S-phase when Cdk1/cyclin-B activity is low, XErp1 can bind to and inhibit the APC/C resulting in the accumulation of cyclin-B. As cyclin-B levels rise, Cdk1 activity increases and the embryo enters M-phase. At this level of Cdk1/cyclin-B activity, PP2A-B´56 recruited to XErp1 is capable of antagonising the inhibitory phosphorylations of Cdk1/cyclin-B. Once the activity of Cdk1/cyclin-B reaches an upper threshold, it prevails over PP2A-B´56 resulting in the dissociation of XErp1 from the APC/C and hence anaphase onset. While this model nicely explains entry into M-phase and the transition into anaphase, it remains elusive how XErp1 is kept inactive during exit from M-phase despite the fact that Cdk1 activity decreases due to cyclin-B destruction. Such a mechanism has to exist to ensure efficient cyclin-B destruction and avoid futile cycles of XErp1 inactivation and reactivation.

In mice, ZGA occurs at the two-cell stage and is followed by cleavage cycles, which are asynchronous (Gamow and Prescott 1970), giving rise to the blastocyst composed of the ICM and the trophoblast. The ICM contains pluripotent stem cells (ES cells) that will form the embryo proper, whereas the trophoblast will form part of the placenta (Ciemerych and Sicinski 2005). The cell cycle programme of the pluripotent ES cells of the ICM and trophoblast cells is clearly distinct. Trophoblast cells undergo multiple rounds of endoreduplication to amplify their genomes to up to 500-fold (Barlow and Sherman 1972; Varmuza et al. 1988), whereas the stem cells arising from the ICM maintain their diploid state by undergoing rapid mitotic division cycles with short G1 and G2 phases (Fujii-Yamamoto et al. 2005). The striking difference in the cell cycle programme of pluripotent ICM cells and trophoblast cells might be attributed to a differentially regulated activity of the APC/C. Initial studies suggested that cell cycles in

ES cells are characterised by high Cdk2 activity and elevated levels of both cyclins A and E throughout the cell cycle (Fujii-Yamamoto et al. 2005). Increased levels of cyclins in interphase might account for the shortened G1 phase in ES cells and their accelerated cell cycle progression (Fujii-Yamamoto et al. 2005; Nichols et al. 1998; Yang et al. 2011). In pluripotent ES cells of the ICM, Cdk1/cyclin-B seems to be the only Cdk activity that is regulated in a cell cycle-dependent manner (Stead et al. 2002). Interestingly, the levels of the APC/C inhibitor Emi1 (highly homologous to XErp1) are increased in mouse ES cells compared to somatic cells (Ballabeni et al. 2011), and its ability to inhibit the APC/C^{Cdh1} during interphase seems to be important for the shortened G1 phase in ES cells and their pluripotent characteristic. It has been suggested that in addition to muted APC/C activity, high E2F-dependent transcriptional activities in ES cells result in hyperaccumulation of DNA replication-licensing factors such as Cdt1, which promotes rapid entry into S-phase after M-phase exit (Ballabeni et al. 2011). Increased levels of Emi1 in ES cells cause the accumulation of an APC/C^{Cdh1} substrate: geminin. Geminin is well known for its function in limiting DNA replication to once per cell cycle by binding to and inhibiting Cdt1 (Wohlschlegel et al. 2000; Nishitani and Lygerou 2002). Recent studies revealed that geminin not only prevents the reinitiation of DNA replication but also functions as a transcriptional regulator (Del Bene et al. 2004; Luo et al. 2004), and this function seems to be important to maintain the pluripotent state of ES cells (Yang et al. 2011, 2012). Geminin levels in trophoblast cells are significantly lower than in pluripotent ES cells (Gonzalez et al. 2006), and the depletion of Emi1 or geminin results in the loss of stem cell identity of ES cells (Yang et al. 2011). Blocking DNA replication in Emi1-depleted cells did not prevent loss of pluripotency indicating that the effects of geminin on DNA replication and gene transcription are functionally independent. Indeed, geminin seems to prevent the transcriptional repression of Oct4, Sox2 and Nanog, three transcriptional activators essential for the pluripotency of embryonic ES cells (Yang et al. 2011). Thus, subtle changes in the capacity of the APC/C to target substrate proteins for destruction and altered transcriptional activities allow the adaption of cell cycle and differentiation events to the specific requirements of the cells in the ICM and trophoblast.

3.8 Conclusions

The concept of regulating cell cycle progression by alternating activities of Cdk1 and antagonising phosphatases represents the core building block of cell cycle regulation (Fig. 3.7). Yet, within this consensus framework, there is an enormous degree of plasticity to adjust the timing of cell cycle events to the specific requirement of a distinct developmental stage, e.g. first mitotic division vs. early embryonic divisions, or developmental fate of embryonic cells, e.g. ICM versus trophoblast cells. This degree of plasticity is achieved by subtle changes in the impact that the different modules have on the activities of Cdk1 and/or phosphatases. As shown for geminin in mice, equipping an APC/C substrate with two functions, i.e. control of DNA replication and transcription, enables cells to adjust simultaneously two critical embryonic processes by changes in the capacity of the APC/C to target proteins for

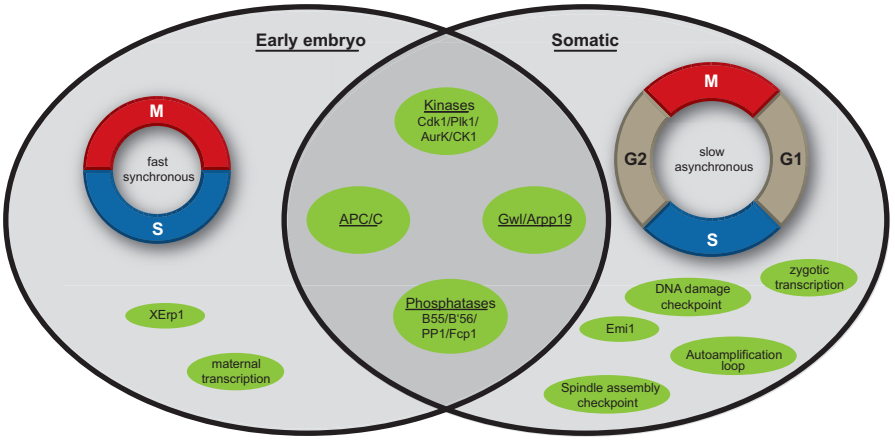

Fig. 3.7 Venn diagram of common and unique regulatory elements of *Xenopus* early embryonic and somatic cell cycles

proteasomal destruction. Most likely, further studies will identify additional cell cycle regulators that are regulated in a similar manner.

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Chapter 4
Vertebrate Embryonic Cleavage Pattern Determination

Andrew Hasley, Shawn Chavez, Michael Danilchik, Martin Wühr, and Francisco Pelegri

Abstract The pattern of the earliest cell divisions in a vertebrate embryo lays the groundwork for later developmental events such as gastrulation, organogenesis, and overall body plan establishment. Understanding these early cleavage patterns and the mechanisms that create them is thus crucial for the study of vertebrate development. This chapter describes the early cleavage stages for species representing ray-finned fish, amphibians, birds, reptiles, mammals, and proto-vertebrate ascidians and summarizes current understanding of the mechanisms that govern these patterns. The nearly universal influence of cell shape on orientation and positioning of spindles and cleavage furrows and the mechanisms that mediate this influence are discussed. We discuss in particular models of aster and spindle centering and orientation in large embryonic blastomeres that rely on asymmetric internal pulling forces generated by the cleavage furrow for the previous cell cycle. Also explored are mechanisms that integrate cell division given the limited supply of cellular building blocks in the egg and several-fold changes of cell size during early development, as well as cytoskeletal specializations specific to early blastomeres

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including processes leading to blastomere cohesion. Finally, we discuss evolutionary conclusions beginning to emerge from the contemporary analysis of the phylogenetic distributions of cleavage patterns. In sum, this chapter seeks to summarize our current understanding of vertebrate early embryonic cleavage patterns and their control and evolution.

Keywords Blastomere • Spindle orientation • Cleavage plane determination • Aster centering • Scaling • Cytoskeleton • Compaction • Cell cleavage type • Evolution

4.1 Introduction

The pattern of early cell divisions in vertebrate embryos varies widely. It is important to understand this patterning and its origin, since, in most organisms, the arrangement of cells resulting from the early cleavages is responsible for generating the earliest features of the embryo's body plan. The cleavage pattern of the early embryo, and the arrangement of cells that results, generates the early embryonic anatomy. During activation of zygotic gene expression at the midblastula transition (MBT), new gene expression initiates new patterns of cell behavior, generating morphogenetic movements that further modify the embryo. However, early cleavage pattern constrains subsequent developmental processes. Moreover, many cellular decisions, including axis induction, germ layer specification, and germ cell formation, occur prior to MBT. The early embryonic anatomy, which originates from the interaction between the initial egg structure and dynamic processes driven by maternally inherited components, must facilitate, or at least be compatible with, such inductive processes.

Various factors, such as embryo size, patterns of yolk deposition (in nonmammalian vertebrates), the symmetry of yolk deposition with respect to oocyte polarity, localization of molecular cues, and cell shape, influence patterns of cell division. The resulting cellular assembly in combination with inductive processes lays the foundation for embryonic morphogenesis. This chapter addresses these cellular and molecular processes, which generate this late blastula architecture, and their relation to the early pattern of the embryo. We explore some of the variety in vertebrate embryonic cleavage patterns and discuss processes involved in their creation. We first examine the two main classes of embryonic cleavage in vertebrates (meroblastic and holoblastic cleavage). Later, we describe cellular mechanisms required for furrow placement during early embryonic development, an essential factor in generating the early embryonic cleavage pattern. We also address additional cellular and developmental mechanisms underlying morphological landmarks of the embryo, such as the formation of specialized cytoskeletal structures in large embryonic cells and cellular compaction, as well as the regulated use of maternal building blocks in cleaving embryonic cells. We also summarize current knowledge on patterns and underlying molecular cues in other vertebrates, including mammals, and in a well-studied proto-vertebrate system.

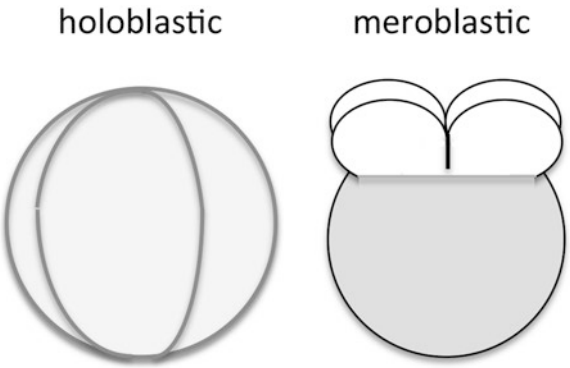

Fig. 4.1 Types of embryonic cleavage. Holoblastic cleavage encompasses the entirety of the embryo, involving meridional planes that cleave through the animal and vegetal poles of the embryo. Meroblastic cleavage involves an early separation between cells at the animal pole and yolk at the vegetal pole of the egg, and cell furrows encompass only the animal-most region of the embryo, leaving the yolk intact

4.2 Main Patterns of Embryonic Cleavage in Vertebrates: Holoblastic Versus Meroblastic

Early embryonic cell division patterns in vertebrates can be broken into two broad categories, holoblastic cleavage (e.g., most amphibians and mammals) and meroblastic cleavage (e.g., birds, reptiles, and teleost fishes) (Fig. 4.1). In holoblastic cleavage, the entire egg undergoes cellularization, and yolk platelets are either absent (e.g., in mammals) or present as cytoplasmic inclusions that partition among cells (e.g., in amphibians). Cleavage furrows encompass the embryo completely, from the animal pole (corresponding to the region containing the meiotic spindle in the oocyte) to the vegetal pole. In meroblastic cleavage, in contrast, cell division does not divide the embryo in its entirety. Instead, embryonic cells divide in the animal pole independently of the vegetally located yolk, with cells typically remaining syncytial to the yolk cell for a period of time that varies among species. We would like to point out that pure holoblastic or meroblastic cleavage patterns are only idealized extremes. We will discuss that many embryos cleave with an intermediate geometry, in which the entire embryo cleaves but the strong asymmetry of yolk leads to a bias of cleavage planes. In any of these cases, dividing cells are called blastomeres regardless of whether they contain yolk.

In all vertebrates, determination of the animal and vegetal poles of the egg develops during oogenesis, with the oocyte typically containing a pre-existing animal region with distinct properties (see Chap. 5). This animal region will contain the nuclear DNA after fertilization and, in embryos with meroblastic cleavage, accumulates yolk-free ooplasm after fertilization. The vegetal region of the egg, in addition to acting as the site for yolk storage in meroblastic species, is also an essential player in early embryogenesis, containing signals involved in early axial patterning

and cell specification that are transmitted to blastomeres (see Chaps. 6–8). In the following sections, we explore the factors that govern holoblastic and meroblastic cleavage patterns, especially the substantial variation observed within these two broad categories.

4.3 Cellular Mechanisms Underlying Cell Cleavage Pattern Determination

The pattern of early cell division in an embryo influences parameters of the forming blastula, such as the number of cells, depth of stacking within a cell layer, and relationship to extraembryonic spaces and structures. The cleavage pattern depends on cell division planes in individual blastomeres. Here, we address cellular mechanisms that influence the furrow plane and which lead to the cellular arrangement of the early embryo.

4.3.1 Induction of Cell Cleavage Plane by Chromatin and Amphiasters

Typically, the cell divides perpendicular to its longest axis into two equal daughter cells. At the core of this process is the spindle apparatus (Fig. 4.2) with the two microtubule asters (amphiasters) emanating from its centrosomes. The spindle apparatus is an arrangement of microtubules that organize around the microtubule-organizing centers (MTOCs) and the DNA during M phase of the cell cycle and is responsible for chromosome segregation during cell division. Kinetochore microtubules connect the spindle pole to chromosomes. The microtubules between the poles that are not connected to the kinetochore are called interpolar microtubules. While we currently know little about microtubule length distribution in the mitotic spindles of early development, in the closely related meiosis II spindles, interpolar microtubules are very short compared to spindle length (Needleman et al. 2010). Another set of spindle-associated microtubules are astral microtubules, which emanate outward from each of the MTOCs in a radial fashion. Typically, microtubule themselves are arranged with their minus ends pointing toward the closest MTOC in the spindle pole. As described below, signals both from chromatin at the spindle midzone and from astral microtubules are involved in furrow induction and therefore cleavage plane determination.

Early observations indicated a correlation between the appearance of the furrow plane and the location of the spindle apparatus, with the furrow forming perpendicular to the spindle at a location equidistant from the spindle poles (Hertwig 1893). This plane normally corresponds to that of aligned chromosomes during the preceding metaphase, as well as the region where astral microtubules overlap.

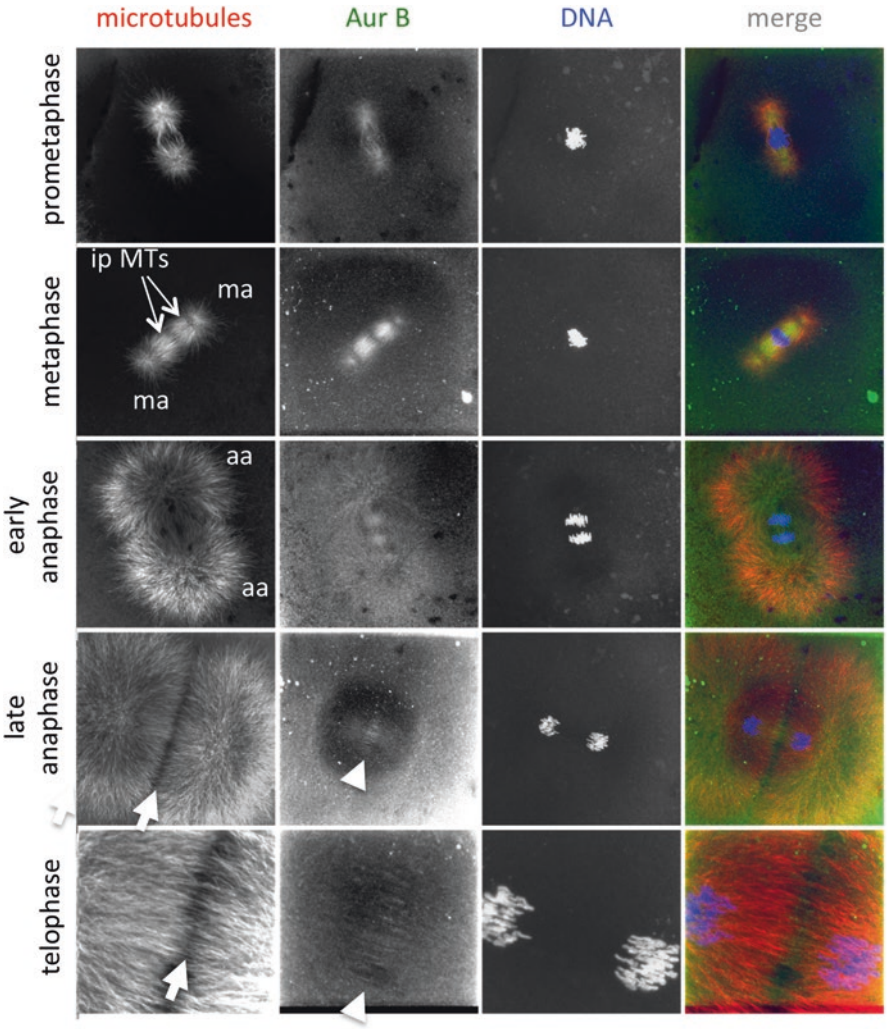

Fig. 4.2 Dynamic changes in spindle morphology during the cell cycle. The spindle apparatus begins to form before metaphase. During metaphase, interpolar microtubules (ipMTs) link the spindle poles, and short metaphase asters (ma) form that are too short to reach the cortex (Wühr et al. 2010). Asters during anaphase appear to be much more developed, reaching a wider radius. A zone of microtubule exclusion, the microtubule interaction zone, forms in the region in which asters meet and which presages the furrow (arrows). AurB, a CPC protein, shows recruitment of this complex to the spindle midzone (arrowhead at late anaphase) and at the tip of FMA tubules in more distal regions of the furrow (arrowhead in telophase; see also Yabe et al. 2009). A zone of microtubule exclusion, the microtubule interaction zone, forms in the region in which asters meet and which presages the furrow (arrows). Microtubules in red, AurB in green, DNA in blue. Figure adapted from Yabe et al. (2009)

Chromosomes therefore can typically be separated equally between the two daughter cells. Analyses of various cell types have shown furrow-inducing activity in midzone microtubules of the spindle (Martineau et al. 1995; Cao and Wang 1996). Studies in amphibians show that neighboring asters induce a cleavage furrow, but only if chromatin is located between them (Brachet 1910), showing a key role for a chromatin-derived factor in furrow induction. However, experimental manipulations of sand dollar embryos by Rappaport, which created a barrier between the poles of a forming spindle to force overlap of normally nonadjacent asters in other regions of the blastomere, showed the induction of an ectopic furrow in the region of overlap in the absence of nearby chromatin, confirming a role for astral microtubules in furrow induction (Rappaport 1961, 1996). These experiments indicate that signals present in both the midzone and astral microtubules contribute to furrow induction (Rappaport 1996; Mishima 2016). The degree to which one of these two mechanisms alone is able to induce a cleavage furrow varies between different species (Brachet 1910; Rappaport 1961; Rappaport and Rappaport 1974; Su et al. 2014; Field et al. 2015). Because the location of the midzone and that of astral microtubule overlap typically occur in the same position, both of these structures act together to establish a robust positioning mechanism to place the furrow at a plane halfway between spindle poles.

The large cells of some embryos like fish or amphibians require some special adaptations related to spindle structure and function. A major adaptation involves aster morphology. In smaller cells, nearly all microtubules' minus ends are believed to be close to the centrosome. However, such an arrangement, due to its radial nature within the volume of the blastomere, would lead to severely reduced microtubule densities near the cortex in very large cells. Instead, large cells such as the zebrafish and *Xenopus* embryonic blastomeres implement an alternative strategy, in which sites of microtubule nucleation are evenly distributed throughout these large asters (Figs. 4.2 and 4.4). The implementation of these internal microtubule nucleation sites results in microtubule density remaining near constant, independent of distance from the aster center (Wühr et al. 2009). The induction of microtubule nucleation sites within the aster can be explained with a chemical trigger wave that relies on microtubule-dependent nucleation (Ishihara et al. 2014).

Another major adaptation of asters which is particularly apparent in very large embryos is the formation of an aster–aster interaction zone, a region depleted of microtubules at the site of overlap between adjacent asters (Wühr et al. 2010; Nguyen et al. 2014; Figs. 4.2 and 4.4). As described below, this interaction zone seems to enable to communicate the proper plane for cell division from the mitotic spindle apparatus to the cell cortex, which can be separated by several hundred micrometers. Furthermore, the interaction zone preempts the barrier of the future cleavage plane allowing the aster to center and orient along the longest axis of the future daughter cell before it actually exists.

Analysis of the function of the Chromosomal Passenger Complex (CPC) component Aurora kinase B (Aur B) in zebrafish embryos provides additional insight into this redundancy as it applies to the large embryonic cells (Yabe et al. 2009). Embryos from females homozygous for a maternal-effect mutant allele in the gene *cellular*

island, which encodes Aur B, exhibit defects in furrow formation. However, in these embryos, cytoskeletal structures associated with the furrow (see below) appear to be induced relatively normally in the center of the blastomere, whereas they are entirely absent in more distal regions of the furrow. As expected, Aur B protein in these embryos is found both at the spindle midzone and the tips of astral microtubules that contact the forming furrow (Fig. 4.2). Genetic analysis of the *Aur B* maternal-effect *cellular island* mutant allele indicates that it retains some functional activity, as homozygotes for complete loss of function alleles are zygotic lethal and do not survive to adulthood, in contrast to homozygotes for the maternal-effect mutant allele. A comparison of the maternal-effect *cellular atoll* phenotype, which allows formation in the medial furrow region, with the effects of a specific Aur B inhibitor, which cause furrow inhibition throughout the furrow, suggests that the partial activity in the maternal-effect *cellular island* allele is provided by Aur B function present in the spindle. Such spindle-provided Aur B may be at a higher concentration or have a higher functional activity than that present in astral microtubule ends. Consistent with this interpretation, embryos maternally mutant for both *futile cycle*, which fails to form spindle structures, and *cellular island* lack furrow-associated structures throughout the length of the furrow, in both medial and distal regions. Altogether, these observations suggest that, in large embryonic blastomeres, CPC activity and potentially other signals from astral microtubules are essential for furrow induction in distal regions of the cell, which are presumably too distant to be influenced by inducing signals from chromatin present at the spindle midzone (Figs. 4.2 and 4.4). Indeed, in several amphibians, early cleavage furrow advance may depend on signals propagated solely through the cortex via the furrow's distal ends (Sawai 1974, 1980; Mabuchi et al. 1988; Sawai and Yomota 1990). These spatial functional specializations of furrow-inducing activity may be a necessary adaptation to the small coverage of the spindle relative to the much larger embryonic blastomeres.

Furrow-inducing activity from the spindle midzone and astral microtubules is concentrated at the point halfway between the spindle poles, and in a plane perpendicular to that of the spindle, thus functionally linking cleavage patterning to the mechanistic determinants of spindle orientation within dividing blastomeres. This linkage will be discussed in the next section.

4.3.2 Centering and Orienting Asters and Spindles

For over 125 years, scientists have known that mitotic spindles tend to align along the longest axis of a dividing cell and that cleavage furrows tend to form perpendicular to the mitotic spindle (Hertwig 1893). The tendency holds even when cell shape is deliberately manipulated to change the orientation of a cell's longest axis. In the original manipulations, artificially elongating frog embryos by compression generated a reorientation of the cleavage plane consistent with realignment of the spindle (Pflüger 1884; Hertwig 1893; Black and Vincent 1988). This phenomenon,

named Hertwig's rule, has been consistently observed in numerous cell types, including both embryonic and somatic cells, in a variety of organisms including vertebrates, invertebrates, and unicellular organisms. The sensitivity of spindle orientation to cell shape underlying Hertwig's rule has been explained by interactions between astral microtubules and the cell cortex, thought to generate forces that become balanced and energetically favorable when the spindles become aligned to the long axis of the cell (Bjerkness 1986; Grill and Hyman 2005; see below).

An important question to understand cleavage plane determination is how astral structures sense cellular geometry. Spindles may sense cell shape through their asters, which extend outward in a radially symmetric manner and are therefore ideally suited to sense intracellular surroundings and the cortex. However, in some large embryos, astral microtubules of the spindle are too short to reach the cortex. In these cells, the task of sensing cellular geometry is performed by the cell-spanning anaphase asters of the preceding cell cycle. For the first cell cycle, the task is performed by the sperm-aster, a mono-aster that forms in the zygote immediately after fertilization (Chambers 1939).

4.3.2.1 The Role of the Sperm-Aster

In most vertebrate lineages after fertilization, centrioles are inherited through the sperm, having been lost during oogenesis. This arrangement is thought to be essential to maintain a constant number of centrioles from one generation to the next (reviewed in Delattre and Gönczy 2004). Surprisingly, however, there are numerous variations on this general theme. Sperm may bring a pair of centrioles, a pair with an incomplete centriole, or a single centriole. In the latter two cases, biogenesis of the second centriole is completed in the zygote after fertilization. Sperm-derived centriolar pairs are thought to act as a template to mediate the reconstitution of a centrosome by nucleating maternally derived centrosome components (Lessman 2012). During interphase of the first cell cycle, this centrosome acts as an MTOC to generate the structure with a single aster, termed the sperm-aster.

The primary function of the sperm-aster is to mediate pronuclear fusion. During fertilization, the maternal pronucleus is formed after reinitiation of meiosis II triggered by egg activation, whereas the paternal pronucleus is introduced by the sperm. After fertilization, the paternal pronucleus remains in close proximity to the centrosome, and the sperm-aster is required for the movement of the maternal pronucleus toward the MTOC and closely associated paternal pronucleus. This movement occurs through the movement of the maternal pronucleus toward the microtubule minus end at the MTOC at the center of the sperm-aster. This movement has been shown in a number of vertebrate species (Chambers 1939; Navara et al. 1994), to be mediated by transport via the minus-directed microtubule-based motor dynein (Reinsch and Karsenti 1997).

In most vertebrates, multiple layers of regulation are in place to inhibit fertilization by more than one sperm (Just 1919). If polyspermy is induced artificially, each sperm produces its own sperm-aster (Brachet 1910). The asters space each other out

in the embryo and multiple mitotic spindles each induce their own cleavage furrow. This condition is lethal for the embryo. However, in most urodeles (newts and salamanders), polyspermy is the natural mode of fertilization (Fankhauser 1932). Although each sperm gives rise to its own sperm-aster, only the one sperm-aster that reaches the female pronucleus becomes dominant to span large parts of the cell. The other sperm-asters disintegrate. It seems necessary that the DNA of the supernumerary asters must also somehow be destroyed later on to prevent polyploidy of subsections of the embryo. The molecular mechanisms underlying this fascinating phenomenon are not understood.

In the zebrafish, the sperm-aster has also been shown to facilitate the multimerization of maternally inherited ribonucleoparticles (RNPs) that confer the germ cell fate, termed the germplasm. This appears to be achieved by the action of sperm-aster ends on an actin-based network associated with these RNPs, where the radial growth of sperm-aster microtubules generates a wave of RNP aggregation (Theusch et al. 2006; Nair et al. 2013), in anticipation of their accumulation at the furrows produced during the early cell divisions (Eno and Pelegri 2013; reviewed in Eno and Pelegri 2016). A similar function for the sperm-aster in germplasm RNP multimerization in other model systems has not yet been shown, although in some systems, such as in frogs and chicken, germplasm RNPs exhibit patterns of accumulation at the furrows that are similar to those occurring in the zebrafish (see Chap. 8).

4.3.2.2 Nuclear–Cytoskeletal Attachment During Nuclear Fusion

In the zebrafish, transport of the paternal pronucleus along the sperm-aster has also been shown to require the function of another maternal-effect gene, *futile cycle/lrmp* (Lindeman and Pelegri 2012). This gene encodes a KASH-domain protein with gene products localized to the nuclear envelope of maternal and paternal pronuclei. This protein is thought to mediate a link between the outer nuclear membrane and the microtubule cytoskeleton. Interestingly, the *futile cycle/lrmp*-dependent nuclear–microtubule connection is also required to maintain the close attachment of the paternal pronucleus to the centrosome at the sperm-aster MTOC (Lindeman and Pelegri 2012). Thus, attachment of the outer nuclear membrane to the cytoskeleton is essential for both long-range transport of the maternal pronucleus toward the MTOC and local attachment of the paternal pronucleus to the MTOC. This coordinated set of processes drives the movement of the maternal pronucleus toward the MTOC while keeping the paternal pronucleus close to this structure, thus mediating the encounter of both pronuclei leading toward their fusion.

Nuclear envelope–cytoskeletal interaction likely continues throughout early embryonic blastomere divisions, as suggested by the highly localized pattern for futile cycle/lrmp proteins at the nuclear membrane–centrosome interphase (Lindeman and Pelegri 2012). This nuclear–centrosomal linkage, coupled to the centering of the (aster-containing) spindle apparatus, guarantees the even distribution of chromatin content among the newly formed blastomeres.

The process of pronuclear fusion is additionally integrated with aster centering. In amphibians, sperm entry occurs at random locations in the animal hemisphere, generating an immediate need for aster centering. Even in zebrafish, where the mature egg contains a sperm entry site at the approximate center of the animal pole that will constitute the future blastodisc area, the aster still likely centers itself along the depth of the forming blastodisc (see below). Below, we describe how aster centering is achieved in a large embryonic cell.

4.3.2.3 Tug-of-War Forces and Aster Centering

Early studies showed growing microtubules can generate pushing forces against an object (Hill and Kirschner 1982), which suggested a mechanism for aster (and bipolar spindle) centering in small cells such as yeast (Tran et al. 2001). However, the larger size of most metazoan cell types necessitates longer astral microtubules in order to contact the cortex, which, due to their greater length and greater tendency to undergo buckling, limit the force that might be transmitted through a pushing mechanism (Dogterom et al. 2005). Instead, in these cells, aster centering has been hypothesized to depend on pulling forces. Indeed, experiments in fertilized sand dollar eggs showed that generating a zone of microtubule polymerization (by localized inactivation of the microtubule inhibitor colcemid) generates movement toward the zone of polymerization (Hamaguchi and Hiramoto 1986), not away, as would have been predicted by a pushing model for aster centering (Fig. 4.3a). This led to a model in which astral microtubules are centered by pulling, rather than pushing forces, a mechanism that was subsequently supported by experiments in *Caenorhabditis elegans* and yeast (Bukarov et al. 2003; Grill et al. 2003; Grill and Hyman 2005). Consistent with the colcemid-inhibition experiments in sand dollar embryos (Hamaguchi and Hiramoto 1986) and a microtubule pulling model, the converse experiment, involving partial aster depolymerization via UV-mediated uncaging of the microtubule inhibitor combretastatin 4A in early zebrafish embryos, results in spindle movement away from the site of depolymerization (Wühr et al. 2010; Fig. 4.3b).

These pulling interactions were assumed to occur between microtubules and the cell cortex (Dogterom et al. 2005; Grill and Hyman 2005; Kunda and Baum 2009). Due to astral microtubule orientation, with minus ends at the aster center and plus ends facing away, such pulling forces could be mediated by the minus end-directed microtubule-based motor dynein (reviewed in Kotak and Gönczy 2013). However, pulling from the cortex under most circumstances will not lead to aster centering but rather MTOC pulling close to the cortex. Grill and Hyman suggested a scenario in which a limited concentration of cortical dynein compared to the number of plus-end microtubules reaching the cortex could lead to stable aster centering (Grill and Hyman 2005).

Nevertheless, studies have suggested that, at least in large cells such as those in early zebrafish and *Xenopus* embryos, dynein is not exerting a force by pulling from the cortex but rather by anchoring of astral microtubules to internal elements of the

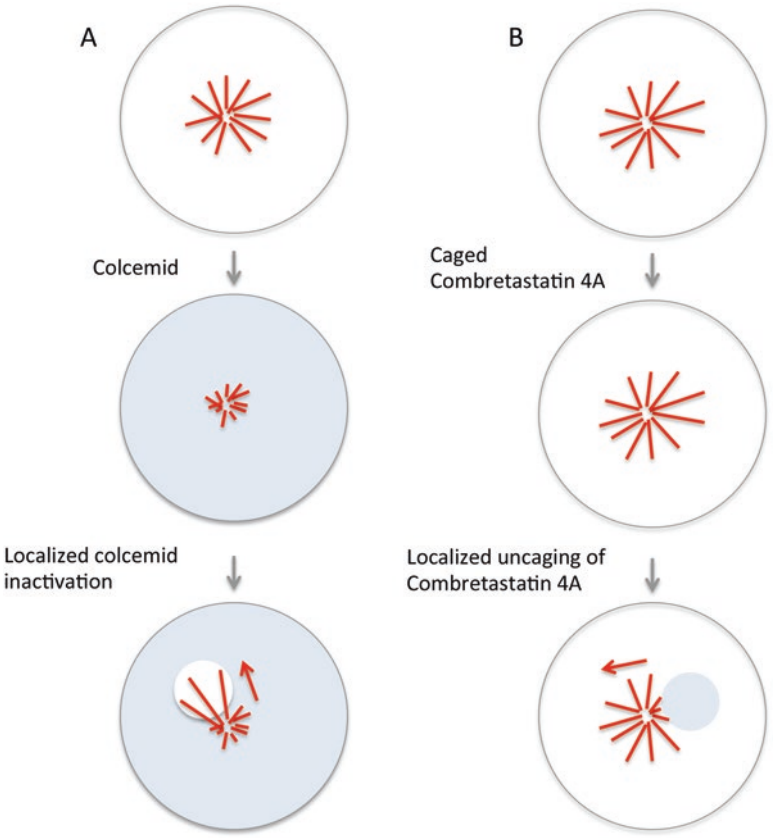

Fig. 4.3 Demonstration that asters are centered by pulling, not pushing, forces. (**a**) Treatment of embryos with colcemid leads to inhibition of microtubule polymerization, and localized inactivation of colcemid with UV light results in the movement of the aster toward the site of microtubule growth (Hamaguchi and Hiramoto 1986). (**b**) Local uncaging of the microtubule inhibiting drug combretastatin 4A results in the movement of the aster away from the site of microtubule inhibition (Wühr et al. 2010). A model in which asters become centered by microtubule pushing forces predicts the opposite effects on aster movement and is ruled out by observations

cytoplasm. This was initially suggested by the observation that both the sperm-aster after fertilization and bipolar spindles during cleavage become centered within the cell before astral microtubules reach the cortex. Similarly, in the abovementioned microtubule inhibitor uncaging experiment (Wühr et al. 2010), spindle movement away from the site of local microtubule depolymerization occurred before the aster reached the cortex. As in cultured cells, interference with dynein function in zebrafish blastomeres (in these experiments through injection of p150-CC1, a dominant-negative form of the dynein partner dynactin) results in spindles that are uncentered as well as misoriented (Wühr et al. 2010). A similar requirement for dynein function was demonstrated for the centering of the sperm-aster immediately after fertiliza-

tion in *Xenopus* eggs (Wühr et al. 2010). These studies suggest that, in zebrafish and *Xenopus*, dynein-dependent pulling forces are required for the centering of astral microtubules during pronuclear fusion and blastomere divisions. Because in these species the centering and experimentally induced movements occur prior to astral microtubules contact with the cortex, the pulling force is unlikely to be generated at the cortex, but is instead generated along presently unknown internal elements of the cytoplasm. Knockdown experiments in *C. elegans* suggest that, in this system, vesicles are likely the anchor for cytoplasmic dynein to generate pulling forces on microtubules (Kimura and Kimura 2011). Distribution of such internal anchors through the cytoplasm could result in a length-dependent force, such that longer microtubules contribute larger pulling forces than shorter ones, resulting in net aster movement. A similar model for microtubule length-dependent pulling depending on cell volume (i.e., internal stores) as opposed to cell surface has been derived through cell shape manipulations and mathematical modeling (Minc et al. 2011). Thus, although pulling from cortical anchors has been documented to orient the spindle in smaller differentiated cells (McNally 2013), large embryonic blastomeres appear to use a tug-of-war pulling mechanism from internal sites, whose consequences for embryonic cleavage patterning are described below.

4.3.3 Mechanisms Underlying Spindle Orientation in Large Embryonic Cells in Fish and Amphibians

Two model systems, zebrafish and *Xenopus*, provide insight on the mechanisms that drive cleavage patterning in early vertebrate embryos with large blastomeres. Embryos from these species exhibit similar behaviors with regard to the spatial arrangement of the spindle and associated DNA in relation to the blastomere center. Due to the small size of the spindle relative to the large size of the blastomere, each of the resulting nuclear masses is at the end of anaphase in a location relatively close to the previous furrow, off-center with respect to the newly formed daughter blastomeres. Thus, in preparation for the next cell division, an important initial requirement is the centering of the forming spindle within the daughter blastomeres. Additionally, the cell cleavage plane in these embryos is known to alternate with each cell division, with each cleavage plane at a 90° angle relative to that of the previous one.

4.3.3.1 Spindle Orientation Based on Cell Geometry Can Be Overridden by Molecular Cues

As described above, aster-mediated forces influence the position of both sperm-asters and amphiasters emanating from bipolar spindles. As will be discussed shortly, in the case of bipolar spindles, stress forces and/or coordinate centering of

astral microtubules at each of the spindle poles is thought to additionally contribute to the alignment of the spindle within the cell. In smaller cells, such alignment is clearly influenced by interactions of the cell boundaries and the metaphase spindle, according to Hertwig's rule of alignment with the longer cell axis. The idea that astral centering occurs by internal pulling forces can be reinterpreted in this context, since forces on MTOCs and the spindle structure will be dependent on the length of astral microtubules emanating from these structures. Since the cortex acts as a barrier to microtubule growth, the long axis of the cell allows for longer microtubule growth and consequently stronger internal pulling forces, which contribute to spindle alignment along this axis. In this manner, cell shape can influence the orientation of the spindle.

The general principle of cells forming a cleavage furrow perpendicular to their longest axis can be overridden by molecular cues, where mechanisms of spindle orientation are influenced by asymmetrically distributed factors (reviewed in Sousa-Nunes and Somers 2013; Williams and Fuchs 2013; Rose and Gönczy 2014; Schweisguth 2015). This mechanism tends to be most common during the division of polarized cells, such as those found in tissue epithelia and cells that are beginning to differentiate into specific lineages. This phenomenon has been observed in some cell fate determination systems that depend on asymmetric cell division mediated by the orientation and/or position of the spindle with regard to intracellular polarity factors, such as in *C. elegans* embryonic development, *Drosophila* neuroblast formation, micromere formation in echinoid embryos, and neural precursor divisions in the vertebrate nervous system. As exemplified in *C. elegans*, off-center positioning of the spindle in addition to its orientation also mediates the formation of different cell sizes of daughter cells. Controlled orientation of spindle and cell cleavage plane has also been observed during extension of the vertebrate axis, with cleavage plane orientation mediating axis elongation.

However, in many vertebrate embryos such as zebrafish and *Xenopus*, early blastomeres are generated through a relatively uniform process in the absence of apparent signals that generate cell asymmetry. Under these conditions, how is the spindle orientation of a sequence of daughter cells determined, and how can this explain the overall cleavage pattern of an early embryo?

4.3.3.2 Transmission of Spindle Orientation Cues During Rapid Cycling

While a cortex-sensing mechanism can explain spindle alignment in smaller cells, studies in zebrafish and *Xenopus* have shown that it cannot explain spindle orientation in the large embryonic blastomeres in those species. In such embryos, spindles become aligned immediately prior to metaphase even before microtubule asters are long enough to contact the cortex (Fig. 4.4). It is only during anaphase, which in these cells coincides with interphase for the following cell cycle, when astral growth becomes extensive enough to reach the cortex. The early commitment of the spindle orientation by the metaphase spindle and refinement of orientation by the telophase astral microtubules has been demonstrated by following the effects of induced cell

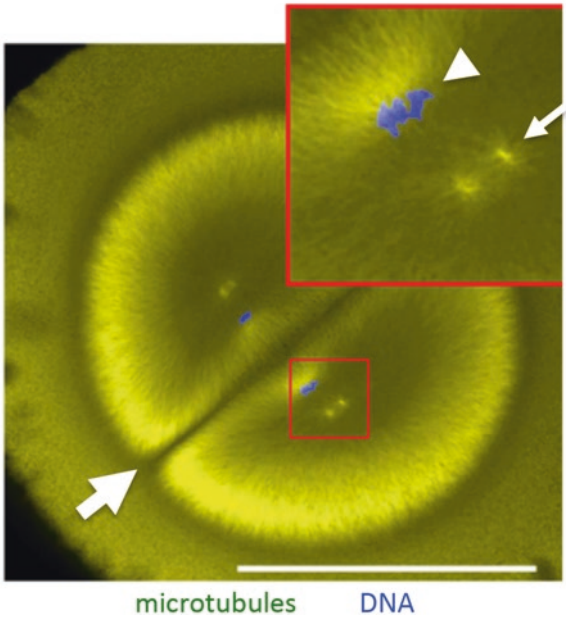

Fig. 4.4 Spindle alignment occurs early in the cell cycle and conforms to an alternating perpendicular pattern. Shown are microtubules (green) and DNA (blue, arrowhead in insert) in a frog embryo shortly before the first cleavage. Alignment of the axis of the incipient spindle is concurrent with telophase for the previous (in this embryo, first) mitotic spindle. Arrow in insert points to the nascent second mitotic spindle indicating the spindle axis. The forming spindle is parallel to the microtubule interaction zone, which indicates the location of the first cleavage furrow (thick arrow in the main panel; Wühr et al. 2010). The second spindle is therefore oriented perpendicular to the spindle of the previous cell cycle. Scale bar corresponds to 500 μm. Figure adapted from Wühr et al. (2010)

shape changes at different time points (Wühr et al. 2010). By applying pressure to *Xenopus* blastomeres to artificially impose a cleavage plane bisecting the elongated cell axis, the authors found that in prophase, prior to nuclear breakdown, the forming spindle (as determined by the inter-centrosomal axis) was already aligned with the long axis of the cell, within about 5° (compared to 45° if randomly oriented). Between anaphase and cytokinesis, when the telophase aster would be expected to have an effect, this alignment improved slightly yet significantly, to 1–2°. These experiments clearly show that (1) the metaphase spindle largely acquires its final orientation at a time when astral microtubules do not reach the cortex and (2) anaphase asters contribute to the fine-tuning of spindle alignment.

The second of these structures—anaphase astral microtubules—appears to provide two functions to the *ongoing* cell cycle. As in the case for the sperm-aster (see above), anaphase astral microtubules experience dramatic growth due to continuous nucleation at internal sites, resulting in microtubules becoming nucleated as they expand outward toward the cortex (Ishihara et al. 2014). This internal priming mechanism may allow astral tips to reach the cortex in spite of the large size of the

blastomeres (see above). For the ongoing cell cycle, a primary function of the anaphase aster appears to be furrow induction at the site of overlap of microtubules from each spindle pole, presumably by providing furrow induction signals such as CPC factors as discussed above. A second function, indicated by the slight improvement in spindle alignment observed in anaphase, seems to be to add precision late in the cell cycle to the orientation of the alignment of the spindle acquired earlier in the cell cycle. The precise mechanistic nature of this late alignment has not been studied, but presumably involves length-dependent internal pulling forces and/or (because anaphase microtubules do reach the cortex) cortex-sensing mechanisms.

The second function, fine-tuning of orientation, can be explained by a cortex-sensing mechanism. But, how does the spindle largely acquire its future orientation early in the cell cycle, even prior to its ability to sense the cortex and possibly cell shape? A key concept for understanding this mechanism of early spindle alignment is that, in the rapidly cycling blastomeres of the early embryo, cytoskeletal structures involved in cell division exhibit a degree of overlap between cell cycles. Thus, the spindle for a given cell cycle is starting to form at a time in which telophase astral microtubules of the previous cell cycle are still carrying out essential cell division functions. In particular, telophase astral microtubules, in addition to executing the above-described functions on furrow induction and spindle alignment fine-tuning for an *ongoing* cell cycle, provide cues for the early orientation of the metaphase spindle for the *following* cell cycle (Wühr et al. 2010).

This influence of furrow orientation from one cycle to the next appears to depend on a zone of microtubule exclusion that forms at the site of anaphase astral microtubule overlap, which develops in the plane in which the future cytokinetic furrow will cleave (Figs. 4.2, 4.4, and 4.5). As mentioned above, metaphase asters are relatively small and do not reach the cortex. On the other hand, anaphase asters grow dramatically to fill in the entire space of blastomeres and contact the cortex, where they provide signals to induce furrowing during cytokinesis. As telophase asters from opposite sides of the spindle reach the midzone, a microtubule-free (microtubule interaction) zone appears which clearly delineates the site of the forming furrow. This zone of microtubule exclusion generates a “dome”-shaped aster, where the sides of the dome correspond to the new spindle’s long axis, aligned parallel to the plane of the forming furrow (Wühr et al. 2010). This dome shape, with microtubules being longer on the side of the spindle opposite the microtubule interaction zone at the furrow (Figs. 4.4 and 4.5), generates an asymmetric force that moves the MTOCs toward the center of the future daughter cells and aligns the nascent spindle along the axis parallel to the forming furrow. The mechanism underlying the formation of the microtubule interaction zone is poorly understood. Embryos mutant for the zebrafish maternal-effect gene *motley*, which encodes an isoform of the CPC component survivin, do not exhibit a microtubule interaction zone at the furrow. In these mutants, anaphase astral microtubules instead cross the furrow boundary from opposite directions to generate a diffuse region of overlap (Nair et al. 2013).

The series of cellular events that result in early spindle alignment in zebrafish and *Xenopus* embryos (and possibly other vertebrates with large blastomeres) can

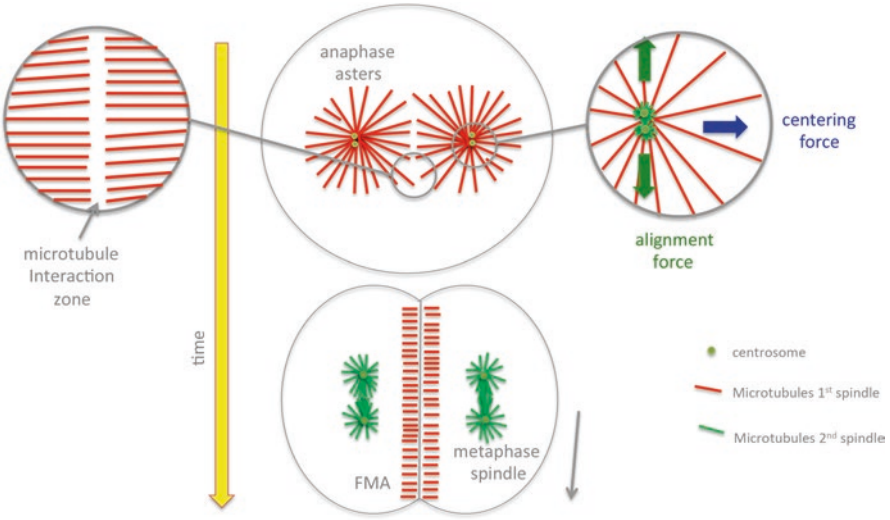

Fig. 4.5 Mechanism of spindle centering and alignment in large embryonic cells. Rapid cell cycling results in temporal overlap between processes corresponding to different cell cycles (see also Fig. 4.4). Anaphase astral microtubules for a given cycle (depicted in red for the first spindle in the top panel) expand all the way to the cortex. The asters form a microtubule exclusion region, the microtubule interaction zone, at the site of aster overlap (left insert). Formation of an incipient spindle for the following cell cycle (depicted in green for the second spindle in the bottom panel and right insert) occurs simultaneous with events associated with the previous cell cycle, including microtubule furrow array (FMA) formation and reorganization (see also Fig. 4.10). The influence of the microtubule interaction zone on the forming spindle for the following cycle results in asymmetric microtubule growth. Coupled to pulling from internal sources, the resulting astral microtubule length asymmetry generates a centering force (blue arrow) for the forming spindle apparatus within the forming blastomere. This same asymmetric influence, coupled to spindle elongation and a hypothesized stress force within the spindle, results in spindle alignment in an orientation parallel to the furrow (and perpendicular to the spindle) for the previous cell cycle (green arrows)

be described as follows (Fig. 4.5). During anaphase, at a time when nuclear envelope membranes are being reformed, a microtubule interaction zone forms at the plane along which the furrow will cleave. Because of the rapid cycling of early blastomeres, this time period also coincides with the separation of centrosomes and start of formation of the spindle for the following cell cycle. The nascent spindle is located in relatively close proximity to the microtubule interaction zone at the furrow corresponding to the previous cell cycle. The close proximity of astral microtubules to this aster interaction zone limits the length of astral microtubules directed toward the furrow. Under these circumstances, pulling forces on these shorter astral microtubules (extending between the interaction zone and the aster center) are weaker than forces on the opposite side of the aster center (extending toward the central and larger portion of the blastomere). This pulling force differential results in a net force on the spindle that moves it away from the furrow plane toward the center of the blastomere, resulting in spindle centering. Asymmetric pulling forces on the radially structured metaphase astral microtubules can also

explain the alignment of the spindle in the same direction as the furrow from the previous cycle, if the sister centrosomes are connected and exert a stress force on each other. Because aster microtubules oriented in the same orientation as the aster interaction zone (for the previous cell cycle) do not experience a microtubule length limit, internal pulling along the same in the direction of the previous furrow would not be restricted. Together, these forces result in alignment of the spindle for the new cell cycle along the same axis as the furrow plane for the previous cell cycle. This in turn results in the orientation of the new furrow, for any given cell cycle, in a plane perpendicular to that of the previous cell cycle. In zebrafish, where this sequence of events has been studied in more detail, the process is repeated to generate the embryonic cleavage pattern at least until about the sixth cell cycle, when the blastomeres become small enough to be contacted by the metaphase asters. As described in the following sections, this mechanism has been proposed to mediate the stereotypical cleavage patterns in zebrafish and *Xenopus* early embryos.

4.3.3.3 Cleavage Pattern Determination in Zebrafish

In the zebrafish, embryonic cleavage is known to be not only highly synchronous, a characteristic of pre-MBT cleavage patterns (see Chap. 9), but also stereotypical in terms of the orientation of the furrow plane during each cell cycle (Kimmel et al. 1995). As mentioned above, zebrafish embryos have meroblastic cleavage. This cleavage pattern is presaged by the structure of the oocyte, which has two primary regions, a wedge-shaped region at the animal pole of the egg where the oocyte nucleus is arrested in metaphase II of meiosis and the remaining region that contains a mixture of ooplasm and yolk granules (Selman et al. 1993). In zebrafish, the sperm site of entry is found at a specific location within the oocyte animal pole region which corresponds to the approximate center of the blastodisc that forms after egg activation (Hart and Donovan 1983; Hart et al. 1992). This likely facilitates sperm-aster centering with respect to a plane parallel to that of the blastodisc itself (which we refer to as the *x*–*y* plane). Although not yet shown, centering in a perpendicular dimension (in the *z*-direction, along the height of the forming blastodisc and the animal–vegetal axis of the embryo) may still occur. Similar to sperm-aster centering in *Xenopus* (Wühr et al. 2010), sperm-aster centering along the *z*-axis in zebrafish could occur through asymmetric forces on the sperm-aster due to microtubule length restriction on the side of the cortex (see below).

The earliest embryonic cleavage cycles exhibit a largely stereotypic pattern (Kimmel et al. 1995; Fig. 4.6a). During the first five cycles, blastomeres divide along the *x*–*y* plane (with *x* being the dimension along the first cell division and *y* along the second cell division) and in an orientation that alternates 90° every cell cycle. This generates, in subsequent cell cycles, a pattern of one-tiered blastomere arrays of 2 × 1 (two-cell embryo), 2 × 2 (four-cell), 4 × 2 (eight-cell), 4 × 4 (16-cell), and 8 × 4 (32-cell). Furrow positioning for the sixth cell cycle bisects the blastomeres along a *z*-plane, generating a two-tiered 8 × 4 blastomere array (64-cell embryo). This cleavage pattern is remarkably constant, although detailed live imag-

ing has found variations in the cell cleavage pattern during the earliest cell cycles (Olivier et al. 2010). Later cell cycles continue to be temporally synchronized but exhibit a pattern of furrow placement of increased variability. Because furrow induction and positioning are determined by the spindle midzone and its orientation, the observed pattern is generated by changes in spindle alignment in each successive cleavage.

The mechanistic model described above is consistent with spindle orientation changes in the early zebrafish and therefore its largely invariant cleavage pattern. According to observations and the model, metaphase asters in the early cell cycles are too small to sense the shape of the cell via microtubule–cortex interactions. However, aster alignment depends on internal pulling forces that are asymmetric due to the microtubule interaction zone at the cleavage plane for the previous cell cycle. As described above, these asymmetric forces result in alignment of the spindle in an orientation parallel to the furrow (and perpendicular to the spindle from the previous cell cycle). During each cell cycle, this asymmetric force results in both spindle orientation and spindle centering and, importantly for the overall cleavage pattern, cell furrows forming at alternating perpendicular angles.

This regular cleavage pattern explains the alternating furrow orientation pattern for the first cell divisions, but why do blastomeres stay in a single plane, forming a one-tiered structure, and why does the pattern change during the sixth cell cycle to generate a two-tiered structure? In terms of the spindles, why do spindles lie in the *x–y* plane during the first five cell cycles, whereas during the sixth cell cycle, spindles reorient vertically along the z-axis? The first question, of why spindles remain along a single x–y plane, may be related to a cell shape-sensing mechanism. The cell cortex gradually becomes close enough to the spindle to allow astral microtubules to increasingly contact the cortex and respond according to shape-sensing forces (Wühr et al. 2010; Xiong et al. 2014). Blastomeres are initially relatively elongated along the x–y plane compared to the z-dimension, i.e., they are longer and wider rather than taller, which would tend to align spindles along the x–y plane. The answer to the second question, of why spindles tend to reorient along the z-axis during the sixth cell cycle, may be related to the same mechanism if, as blastomeres divide and acquire a smaller size, their dimensions along the x and y axes become smaller, relative to the z-axis, which has remained relatively unchanged (e.g., blastomeres become taller, in the z-axis, than wider, along the x–y dimensions). Thus, a shape-sensing mechanism may cause spindles to realign from the x–y plane to the z-axis when new blastomere dimensions promote this realignment. An effect of changing cell dimensions on cleavage plane orientation has also been implicated in the regulation of the thickness of epithelial layers at later stages of development (Da Silva-Buttkus et al. 2008; Luxenburg et al. 2011; Lázaro-Diéguez et al. 2015). The stereotypic pattern of division orientation in zebrafish embryos ceases to be apparent at the seventh cycle and beyond (Kimmel et al. 1995; Hoh et al. 2013).

The emerging picture is that blastomeres of the early embryo may utilize a mechanism in which cell shape is sensed by the combined action of the mitotic spindle, oriented by an asymmetry defined by the zone of microtubule exclusion, and interphase asters allowing cell shape sensing. Together with changes in blastomere

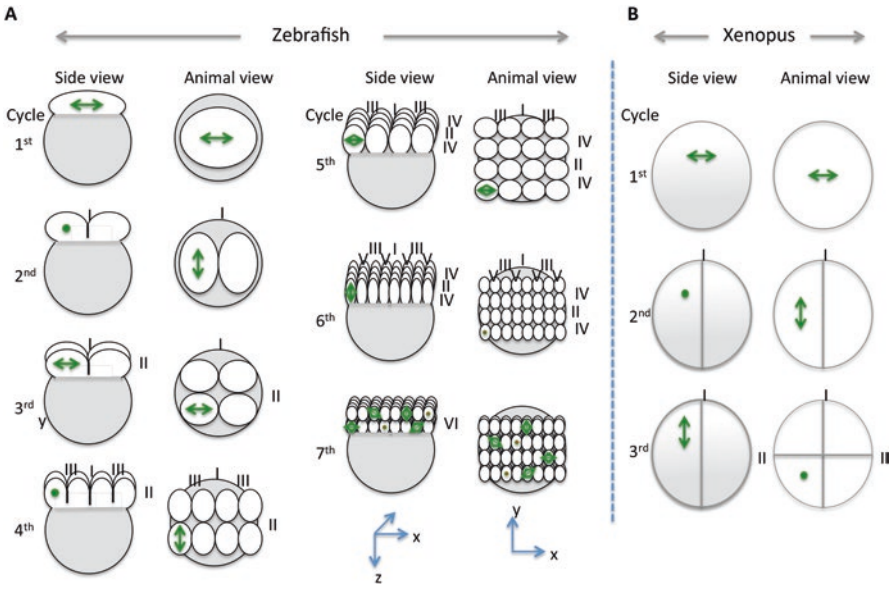

Fig. 4.6 Cleavage pattern orientation in early zebrafish and *Xenopus* embryos. (**a**) Stereotypic cleavage pattern in zebrafish. During the first five cell cycles, spindles (double arrows) remain aligned to a single, horizontal (x–y) plane parallel to the blastodisc plane, alternating 90° every cell cycle. During the sixth cell cycle, spindles tend to align in the vertical (z-axis) orientation, generating two tiers of blastula cells. Spindle orientation becomes random during the seventh cell cycle. (**b**) Canonical cleavage pattern in *Xenopus*. The first two cell cycles have spindles aligned to the x–y plane, also exhibiting an alternating 90° pattern, resulting in meridional cleavages. Spindle tends to reorient along the z-axis during the third cell cycle to generate a cleavage plane parallel to the equator. During the following cell cycles, the spindle aligns with the longest axis of the cell (Strauss et al. 2006; Wühr et al. 2010). Graded shading represents asymmetric yolk distribution, which is enriched in vegetal regions. Asymmetrically distributed yolk is hypothesized to interfere with microtubule length and/or pulling forces to generate a force that places the spindle in an eccentric position, biased toward the animal pole. In both (**a**) and (**b**), spindle orientation is indicated with green double arrows (which appear as a dot when entering the plane of the page). The order of furrow formation (corresponding to the cell cycle in which those furrows form) is indicated by Roman numerals

dimensions, this combined mechanism results in changes in spindle orientation that begin to transform a two-dimensional single-cell layer into a three-dimensional blastula.

4.3.3.4 Cleavage Pattern Determination in *Xenopus*

The mechanistic model described above, which explains the early zebrafish cleavage pattern, is also consistent with the generation of cell cleavage pattern in *Xenopus laevis*, another embryo with large blastomeres (Wühr et al. 2010; Fig. 4.6b). As in zebrafish, blastomere cleavage planes exhibit an alternating 90° pattern (Nieuwkoop

and Faber 1967; Sawai and Yomota 1990). Also similar to zebrafish, spindles acquire their final orientation early in the mitotic cycle, before astral microtubules are long enough to reach the cortex (Wühr et al. 2010).

In contrast to zebrafish, fertilization of anuran amphibian eggs, such as *Xenopus*, does not occur through a spatially defined sperm entry point, but instead occurs at random locations in the animal hemisphere (Elinson 1975; Schatten 2012). This generates in the *Xenopus* embryo an immediate need for sperm-aster centering along the x–y plane. This centering likely occurs by internal pulling forces acting on the sperm-aster, formed immediately after fertilization and generated by reconstitution of a centrosome around the sperm-derived centrioles, which act as an MTOC for the sperm-aster. The location of the sperm centrosome immediately below the surface will automatically result in centering: the membrane generates a restriction on the astral microtubule lengths, and pulling forces from the opposite (internal) side generate an overall asymmetric force that centers the sperm-aster (Wühr et al. 2010). The aster's closeness to the cortex induces an asymmetry generating a long sperm-aster axis that is roughly parallel to the tangent of the cortex at the sperm entry point. The aster seems to sense this long axis and transfer it to the nascent first mitotic spindle (see below). This might explain the old observation that the first cleavage plane typically cuts through the sperm entry point (Roux 1903).

During the first division cycles, *Xenopus* MTOCs tend to move toward the animal-most third of the embryo (Wühr et al. 2008). This movement in the animal direction may also be explained if, as in zebrafish, yolk granules present in more vegetal regions limit astral microtubule attachments. More yolk could inhibit microtubule growth or result in fewer cytoplasmic structures for dynein to pull on, thus again providing a force differential that pulls the spindles animally until reaching an equilibrium point at the observed location. However, the proposed mechanism by which asters and yolk interact is not understood. Induction of furrows in *Xenopus* initiates at the animal region and only gradually moves vegetally (Danilchik et al. 1998), consistent with asymmetric location of the spindles, which can act to induce a furrow first on the more closely located animal cortex. The first two divisions occur with spindles aligned along an x–y plane parallel to the equator, alternating 90 °C to generate four cells whose furrows span meridians along the animal–vegetal axis. This arrangement is similar to the cleavage pattern in zebrafish and, even with the obvious contrast that cleavage in *Xenopus* is holoblastic, is easily explained by the microtubule exclusion model described above. As in zebrafish, maintenance of the spindles in the x–y plane may rely on a differential force that precludes z-axis tilting during these cycles. The frog egg is not perfectly spherical but slightly oblate, and vegetal yolk likely weakens pulling forces on asters in the z-direction, two conditions that would promote spindle orientation along the x–y plane.

Xenopus embryos, also like zebrafish, exhibit a transition of spindle alignment from the x–y plane to a z-axis orientation (as in zebrafish, the latter also corresponds to the animal–vegetal axis of the embryo). However, this transition normally occurs in the third cycle in *Xenopus*, compared to the sixth cycle in zebrafish. This earlier transition in *Xenopus* may reflect a differing balance between microtubule pulling lengths along the x–y axis (limited by membranes laid out between blastomeres)

and vegetally located yolk. The result of this z-axis shift in spindle orientation is cell furrowing along a plane parallel to the equator (above the equator due to the spindles being located closer to the animal pole), generating four smaller animal blastomeres and four larger vegetal blastomeres. Subsequent cleavage planes generally follow a rule for alternating shifts in spindle orientation albeit showing increasing variation: cleavage in cycle 4 again tends to generate longitudinal furrows, reflecting a realignment of the spindle with the x–y plane, whereas cleavage in cycle 5 tends to generate furrows parallel to the equator, reflecting a second z-axis reorientation. The alternating alignment of the spindle along the x–y plane and the z-axis may reflect, as in zebrafish, a cell shape-sensing mechanism influenced by changing dimensions of the blastomeres as they undergo cell division (Strauss et al. 2006; Wühr et al. 2010).

Thus, and in spite of exhibiting an entirely different type of cleavage pattern (meroblastic compared to holoblastic), the global pattern of cleavage orientation in zebrafish and *Xenopus* embryos can be explained spatially and temporally through the cleavage stages using the same simple mechanistic model (Wühr et al. 2010). This model initially relies on microtubule length-dependent forces and the influence of the furrow from the previous cell cycle, together with additional intracellular modulation, such as the distribution of yolk. Over time cells acquire a smaller size and the patterning system may transition to the spindle being able to directly sense the cortex (Strauss et al. 2006; Wühr et al. 2010; Xiong et al. 2014). Together, these influences generate a three-dimensional blastula.

The fact that species as phylogenetically distant as teleosts and amphibians appear to obey a conserved set of cell-biological mechanisms, which generate manifestly different cleavage patterns from different initial starting conditions, suggests that a common set of rules may provide the basis for the various cell arrangements observed in many early vertebrate embryos. During evolution, such unifying rules may be all that is necessary to accommodate limited changes in starting blastodisc dimensions and/or the influence of modifying factors (i.e., embryo size, affinity of internal anchors, amount or nature of yolk particles) to generate cleavage pattern variation. It will be interesting to test this hypothesis through further studies in additional species.

4.3.4 Cell Cleavage Orientation in Other Vertebrate and Proto-vertebrate Systems

Studies in amphibians and teleosts have, until now, contributed the most to our mechanistic understanding of cleavage plane determination in vertebrate embryos. However, a full comparative picture will involve patterns in other vertebrate systems such as Aves, reptiles, and mammals. The chordates include invertebrates such as tunicates in addition to vertebrates, and mechanisms involved in cell cleavage pattern have been well described in ascidian (sea squirt) tunicate embryos. We summarize our current knowledge in these systems below.

4.3.4.1 Meroblastic Cleavage in Aves and Reptiles

Most knowledge concerning the cleavage stages of avian embryos comes from studies in the domestic chicken (*Gallus gallus*). However, understanding of cellular and molecular processes during cleavage stages is limited even in this well-known developmental model organism (Lee et al. 2013; Sheng 2014; Nagai et al. 2015). This is because, by the time the egg is laid, the embryo is long past the blastula stage. Thus, studying cleavage-stage embryos in the chick requires the use of methods for obtaining eggs still developing in utero (Lee et al. 2013).

The chick embryo undergoes meroblastic cleavage, with blastomeres dividing atop a large yolky mass (reviewed in Sheng 2014; Fig. 4.7). Fertilization is notable because, while only a single female and single male pronucleus will give rise to the zygotic genome, polyspermy is not uncommon (Lee et al. 2013). These supernumerary sperm, which can be few or many, are found in both yolk and blastoderm. Their function, if any, is unclear, but they are capable of producing pseudo-furrows that do not fully ingress.

Another notable feature of these embryos is that the point at which the first two cleavage furrows meet is not centered at the middle of the embryo (Sheng 2014; Nagai et al. 2015). There is a known correlation between asymmetric inheritance of maternally deposited factors and establishment of PGCs, but whether this is associated with off-center early cleavages is unclear. It has also been proposed that the off-center cleavages cause blastomeres to inherit heterogeneously deposited maternal factors asymmetrically as they cellularize, leading to symmetry breaking and axis induction. However, the ability to experimentally change the dorsal–ventral axis after egg laying challenges this early-establishment hypothesis (reviewed in Sheng 2014).

The first two cleavages in the chick are stereotypical, with the second forming perpendicular to the first in the plane of the blastoderm. The third furrow follows this pattern, though evidence suggests that divisions at this point become asymmetric, resulting in smaller cells in the interior and larger cells at the periphery (Lee et al. 2013). Cell division becomes asynchronous at the fourth cleavage, and presumably some asynchronous division continues (Sheng 2014).

It is also roughly around this time that the embryo begins to form two layers of blastomeres. As cleavage progresses, the embryo will reach 5–6 layers of cells in thickness before thinning again during and after gastrulation (Sheng 2014). A study found that approximately 75 % of surface cells (i.e., those in the uppermost layer) divided in a direction parallel to the blastoderm plane, yielding two daughters in the same layer, while deeper cells show more variation, with approximately 56 % dividing in a direction 30–90° from that of the blastoderm plane (Nagai et al. 2015; see below). These data suggest that cleavage orientation only partly explains increasing layer number, but other mechanisms, such as rearrangement of already cellularized blastomeres, may also be involved. The increase in layers also roughly corresponds to the onset of formation of the subgerminal cavity, a space that separates part of the blastoderm from the yolk. This structure may also influence blastoderm thickness.

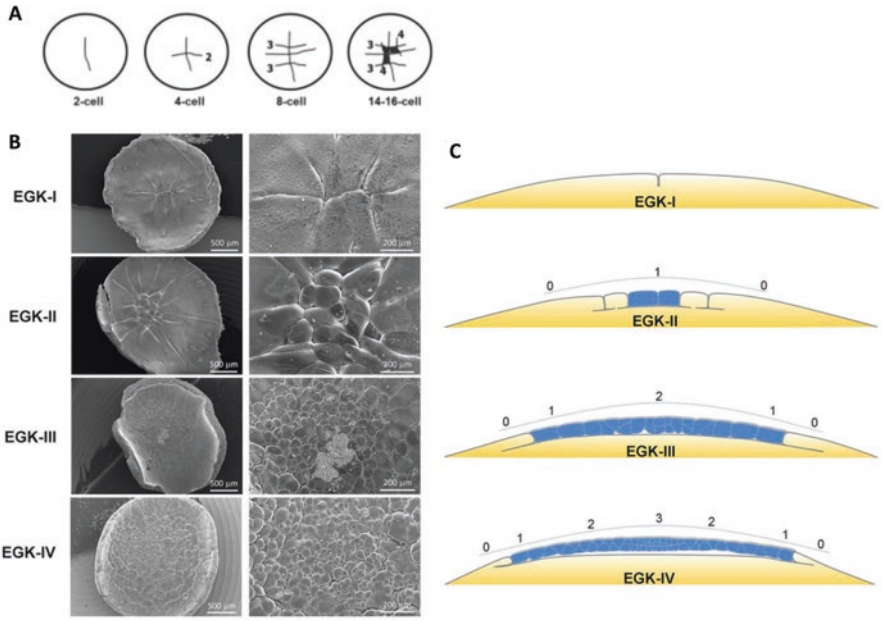

Fig. 4.7 Cleavage pattern in the early chick embryo. Early cleavage in chick embryos during the early to mid-cleavage stages (EGKI–EGKIV) tends to exhibit a perpendicularly alternating pattern, diagrammed in (**a**) and visualized through scanning electron micrographs in (**b**). (**c**) Diagram of a side view of the animal pole of the embryo, depicting meroblastic cleavage, with cellularized cells in blue and the number of layers indicated by numbers. Reproduced from Lee et al. (2013) (a) and Nagai et al. (2015) (**b**, **c**), with permission

Study of chick cleavage stages reveals some striking similarities to groups outside amniotes. For example, evidence suggests that zygotic gene activation in the chick occurs around the seventh or eighth cell division (precision is difficult due to cell cycle asynchrony). This places the midblastula transition somewhere between cycles 7 and 9, quite similar to the timing observed in zebrafish and *Xenopus* (Nagai et al. 2015). These researchers also find evidence of a yolk syncytial layer in the chick, though it is unclear if it plays a regulatory role similar to the YSL in zebrafish (see Chap. 7). The division of a blastomere with the cleavage plane parallel to the blastoderm, resulting in an inner cell and an outer cell that will go on to different fates, is reminiscent of a similar process in *Xenopus* (Sheng 2014). This evidence, combined with overall morphological similarity of cleavage-stage embryos in birds, reptiles, and teleost fish, suggests deep ancestry or convergent evolution of many characteristics of cleavage-stage embryonic development in vertebrates (Nagai et al. 2015).

Reptiles warrant a mention here. They are an important group to study to understand evolution of embryonic cleavage patterning in vertebrates and are known to undergo meroblastic cleavage. Unfortunately, data on their earliest cleavage stages

are scarce (Wise et al. 2009; Matsubara et al. 2014). Reasons for this can include difficulty of establishing breeding colonies in the lab, small clutch sizes, fertilization time uncertainty for wild-caught pregnant females, difficulty in culturing embryos due to extreme temperature and humidity sensitivity, and difficulty isolating embryos because of embryo adhesion to the egg's inner surface. However, recent work has begun to overcome these obstacles (reviewed in Wise et al. 2009; Matsubara et al. 2014). A method for culturing embryos of the Japanese striped snake (*Elaphe quadrivirgata*) (Matsubara et al. 2014) is a major step forward. While the earliest stage depicted in the study was a gastrula-stage embryo, it appeared quite similar to a chick embryo at the same stage. The data focused on somitogenesis, but this method has promise for examining early cleavage stages in snakes.

Other studies have suggested various lizards, such as the leopard gecko (*Eublepharis macularius*), as a model for development in that group (reviewed in Wise et al. 2009) and presented staging series. So far though, the majority of these focus on embryos in eggs that have already been laid, which is too late for characterization of cleavage stages. However, easy husbandry of these animals, combined perhaps with methods similar to those mentioned above for snakes and chicks, has potential to further the study of cleavage patterning in this phylogenetically important group of reptiles.

4.3.4.2 Early Cleavage Divisions in Mammals

Analogous to fish, amphibians, birds, and reptiles, mammalian zygotes initially undergo a series of cleavage divisions following fertilization to produce an increasing number of progressively smaller cells without changing the overall size of the embryo. However, the introduction and optimization of in vitro fertilization (IVF) and embryo culture techniques have revealed several notable differences in how these early divisions occur between mammals and other vertebrate animals. First, mammalian species exhibit rotational cleavage, whereby meridional division is observed along the animal–vegetal axis in the first cleavage, but during the second cleavage, the daughter cells can divide either meridionally or equatorially by dividing perpendicular to the animal–vegetal axis (Gulyas 1975; Fig. 4.8). As a consequence, each blastomere inherits equivalent cytoplasmic material from both the animal and vegetal region at the two-cell stage and potentially differentially allocated animal and vegetal portions when the embryo divides from two cells to four cells (Gardner 2002). The type of second division each daughter cell undergoes determines which of the four distinct classes (meridional–meridional, meridional–equatorial, equatorial–meridional, or equatorial–equatorial) a four-cell embryo will become, and this may impact both cell fate and developmental potential as previously suggested (Piotrowska-Nitsche and Zernicka-Goetz 2005). More specifically, it has been shown that four-cell mouse embryos containing at least two blastomeres with both animal and vegetal material are much more likely to develop to term than embryos where all blastomeres have either only animal or only vegetal cytoplasmic inheritance (i.e., the equatorial–equatorial class; Piotrowska-Nitsche et al. 2005).

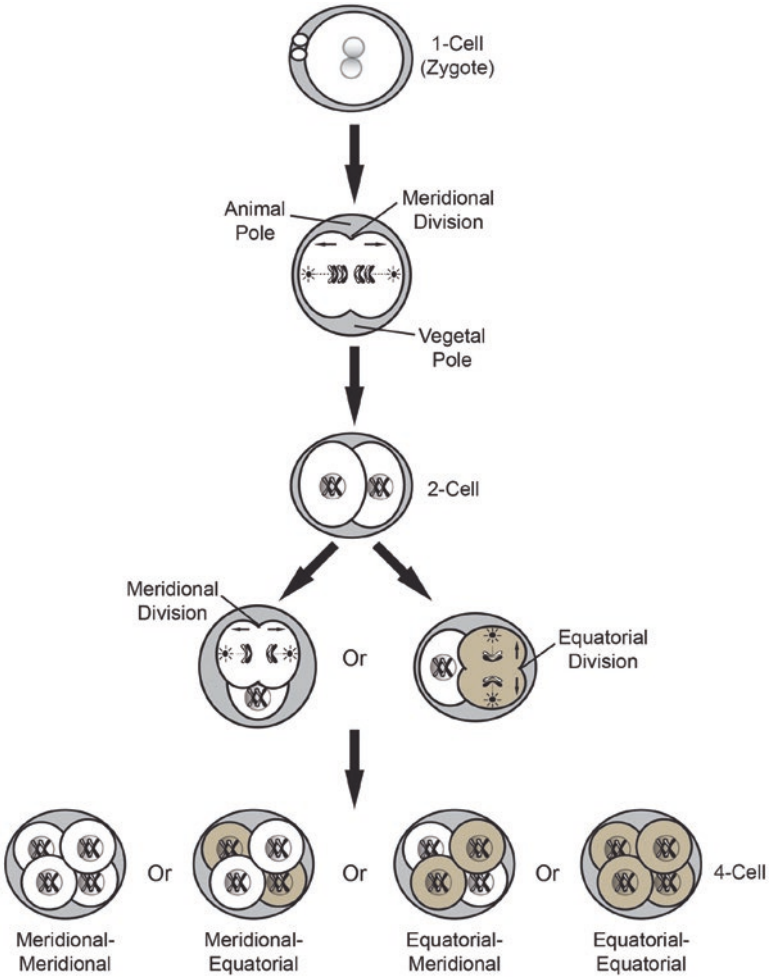

Fig. 4.8 Cleavage pattern in the early mammalian embryo. The first division is meridional, along the animal and vegetal poles, and the second division can be either meridional or equatorial. This generates four possible cellular arrangements at the four-cell stage with meridional–equatorial and equatorial–meridional divisions distinguishable by virtue of one of the blastomeres in the two-cell embryo dividing prior to the other. In embryos that exhibit the equatorial–equatorial pattern, putative factors localized to the animal and vegetal poles segregate to different daughter cells. See text for details

Based on their differential distribution between the animal and vegetal poles in the zygote, several candidates have been proposed to mediate this cleavage-related asymmetry (reviewed in Ajduk and Zernicka-Goetz 2015). Apart from findings of asymmetric localization, however, none of these candidate proteins have been shown to function in the determination of whether a meridional or equatorial division occurs at the two-cell stage. Thus, the exact molecular mechanism(s)

mediating the developmental fate of each blastomere and distinction between the four classes of four-cell mammalian embryos remains to be determined (Ajduk and Zernicka-Goetz 2015).

In addition to unique cell orientation, the time between cleavage divisions in mammalian embryos is more prolonged, typically between 8 and 24 h apart depending on the species, compared to that in many nonmammalian vertebrates. For instance, the time between the first and second mitosis in mice is approximately 20 h and for the majority of other mammals, including humans, 8–12 h (O'Farrell et al. 2004; Wong et al. 2010; Weinerman et al. 2016). Besides longer time intervals, blastomeres in early cleavage-stage mammalian embryos also undergo asynchronous cell division rather than simultaneously dividing like in other vertebrates. Therefore, mammalian preimplantation embryos do not increase exponentially in cell number from the two- to four- or four- to eight-cell stage but can contain an odd number of blastomeres at certain times in development (Gilbert 2000). Lastly, in contrast to other vertebrate animals, the mammalian genome becomes activated much earlier, and many of the mRNA transcripts and protein products produced from embryonic genome activation (EGA) are required for subsequent cell divisions. Because of this requirement, the stage of embryo development that coincides with EGA is most susceptible to cleavage arrest or "block" in several mammalian species (Ko et al. 2000). The mouse embryo exhibits EGA earliest, at the two-cell stage; however, minor transcription of certain mRNAs also occurs in mouse embryos at the one-cell stage and is often referred to as zygotic gene activation (ZGA) (Flach et al. 1982; Ko et al. 2000; Hamatani et al. 2004; Wang et al. 2004; Zeng et al. 2004). Similar to the mouse, human embryos have also been shown to undergo minor transcriptional activity of preferential mRNAs prior to the major wave of EGA, and some of the transcripts include cell cycle regulators (Dobson et al. 2004; Zhang et al. 2009; Galán et al. 2010; Vassena et al. 2011). Thus, it is likely that preimplantation embryos from other mammalian species also exhibit "waves" of gene expression during the transition from maternal to embryonic transcriptional control, which may impact embryo behavior and cell division timing if these cell cycle-related genes are aberrantly expressed. Regardless of which wave it occurs under, the production of embryo-derived transcripts is clearly established by day 3 of human preimplantation development even in embryos that arrested prior to the eight-cell stage (Dobson et al. 2004; Zhang et al. 2009; Galán et al. 2010; Vassena et al. 2011), suggesting that EGA is a function of time rather than cell number per se.

Several studies have shown that male mammalian preimplantation embryos may actually cleave faster than female embryos cultured in vitro (Xu et al. 1992; Pergament et al. 1994; Peippo and Bredbacka 1995), although other studies detected no difference in the sex ratios between early- or late-cleaving human embryos (Ng et al. 1995; Lundin et al. 2001). This suggests that potential sex-related differences in the cleavage rate of male versus female human embryos do not occur until later during post-implantation development or that, alternatively, this phenomenon is restricted to only certain mammalian species. Nevertheless, early cleavage in general is a strong indicator of embryo viability since human embryos that undergo the first mitotic division sooner appear to have greater potential for successful implanta-

tion and positive pregnancy outcome (Lundin et al. 2001). More recent findings, however, suggest that it is the duration of the first cleavage division rather than its onset, together with the time intervals between the first three mitotic divisions, that is highly predictive of which human embryos will reach the blastocyst stage (Wong et al. 2010). Since this initial report, other studies have confirmed the importance of early cleavage divisions and identified additional cell cycle or morphological parameters predictive of developmental success (Cruz et al. 2011, 2012; Meseguer et al. 2011, 2012; Azzarello et al. 2012; Dal Canto et al. 2012; Hashimoto et al. 2012; Hlinka et al. 2012; Rubio et al. 2012; Liu et al. 2014, 2015; Stensen et al. 2015) as well as underlying chromosomal composition (Chavez et al. 2012, 2014; Campbell et al. 2013; Basile et al. 2014; Yang et al. 2014). Whether the first three mitotic divisions are similarly predictive of embryo viability and/or chromosomal status for other mammalian species is still under investigation, but an examination of early mitotic timing in murine, bovine, and rhesus monkeys has suggested that this is likely the case (Pribenszky et al. 2010; Sugimura et al. 2012; Burruel et al. 2014). As mentioned above, however, the precise timing between the first cell divisions can vary between different mammalian species (O'Farrell et al. 2004; Wong et al. 2010; Weinerman et al. 2016), and the underlying cause(s) remains largely unknown. Besides a later EGA onset in comparison to the mouse, human embryos have also been shown to express diminished levels of cell cycle checkpoints and robust expression of cell cycle drivers at the cleavage stage (Harrison et al. 2000; Los et al. 2004). This can impact not only embryo chromosomal stability, as shown by the high incidence of whole chromosomal abnormalities (aneuploidy) in cleavage-stage human embryos (Vanneste et al. 2009; Johnson et al. 2010; Chavez et al. 2012; Chow et al. 2014), but may also produce preimplantation embryos that cleave at a faster rate over other mammals. Moreover, time-lapse monitoring of early embryonic development has demonstrated that human embryos also frequently undergo multipolar divisions, whereby zygotes or blastomeres divide into three or more daughter cells rather than the typical two. Indeed, it has been estimated that approximately 12 % of human zygotes cultured in vitro are characterized by multipolar divisions (Chamayou et al. 2013), and this phenomenon could further explain differences in mitotic timing between mammalian species. While the potential impact of higher-order divisions at the two-cell stage and beyond may not be as detrimental and is still being investigated, embryos that exhibit multipolar divisions at the zygote stage are much less likely to form blastocysts and implant than zygotes that undergo a bipolar division (Hlinka et al. 2012).

4.3.4.3 Proto-vertebrates

Studies in ascidians have provided important insights into patterns of cell cleavage in a lineage basal to vertebrates, which may reflect ancestral developmental mechanisms. Cleavage pattern in ascidian species is holoblastic, invariant, and characterized by bilateral symmetry (Conklin 1905; Nishida and Satoh 1983; Nishida 1987). The pattern of cell cleavage orientation in ascidians provides insights into our

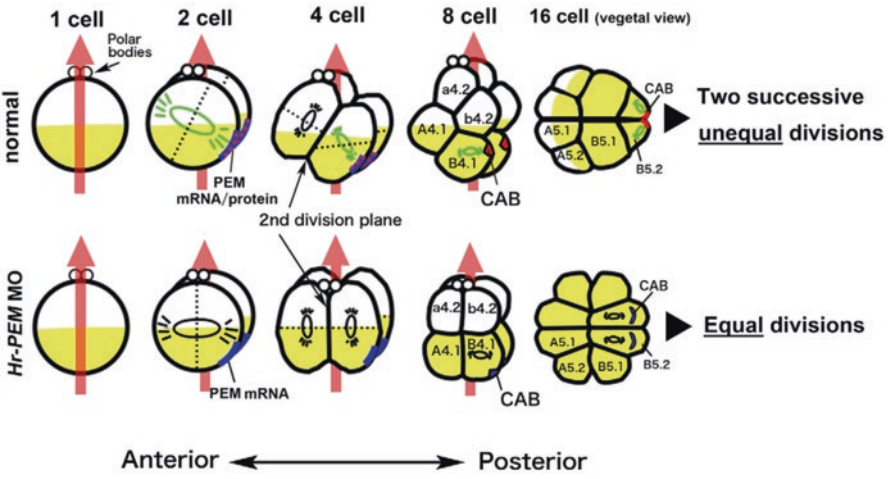

Fig. 4.9 Early embryonic cleavage pattern in ascidians. Early cleavages in general follow an alternating perpendicular pattern, consistent with a dependence on cell shape. Spindle orientation in the posterior-vegetal cell is influenced by the attraction of one of the spindle poles to the CAB (blue), which becomes condensed and attached to the posterior-vegetal cortex in the eight-cell and 16-cell embryo. This causes the eccentric placement of the spindle at the posterior end of the posterior-vegetal blastomere during these stages, resulting in consecutive asymmetric cell divisions that generate a larger blastomere anteriorly and a smaller blastomere posteriorly. Spindle orientation is also influenced by the condensing CAB during formation of the two-cell embryo, resulting in a shift of the cleavage plane axis with respect to the polar bodies at the embryo animal pole and animal–vegetal axis (orange arrow) of the embryo, as well as during formation of the four-cell embryo, resulting in the observed protrusion of posterior-vegetal blastomeres (B4.1) at this stage. Spindle centering in anterior- and posterior-animal blastomeres, which do not contain the CAB, occurs normally resulting in symmetric cell division. Attraction of the spindle pole by the CAB is dependent on the function of PEM, a maternal product localized to the CAB. Reproduced, with permission, from Negishi et al. (2007)

general understanding of cell cleavage patterning in early embryos. The mechanism appears to involve symmetric cleavages in alternating orientations, consistent with shape-sensing spindle orientation mechanisms acting in large embryonic cells. This underlying cell division pattern is further modified in a subset of cells by a specialized structure capable of influencing the orientation and position of the spindle through multiple cell cycles (at least cycles 3–6), adding asymmetric details to the blastula, which begin to sculpt the embryo. This modified structure, capable of influencing spindle placement and cell pattern arrangement, also contains factors that function in cell shape specification. Thus, basal mechanisms of cell division interact with a specialized structure to coordinate cell division cues and cell fate signals to generate the basic body plan (Fig. 4.9).

The first two cleavage planes in ascidian embryos are meridional, passing through the animal and vegetal poles, with the second cleavage oriented perpendicular to the first to generate four equal-sized blastomeres. The third cleavage plane is also perpendicular to the first and second cleavage but equatorial, divid-

ing each blastomere to generate an eight-cell blastula with four cells in each of the animal and vegetal halves of the embryo. These first three cleavages are nearly symmetric, with the exception of an outward tilt of the division axis for the two posterior blastomeres during the third cell division, resulting in a slight outward protrusion of the resulting posterior-vegetal blastomeres. The next three cell cycles continue a cell division pattern that is symmetric in animal and anterior-vegetal blastomeres but is asymmetric in posterior-vegetal blastomeres. These posterior-vegetal blastomeres instead divide asymmetrically, each cell cycle generating a small cell posteriorly and a larger cell anteriorly. The resulting cells in the blastula exhibit unique lineages and cell fates (Conklin 1905; Nishida 1987).

The ascidian cleavage pattern thus exhibits some similarities to those observed in vertebrate systems, especially vertebrates such as teleosts and amphibians. In particular, the holoblastic, mutually orthogonal divisions of ascidians during the first three cycles are aligned in a pattern that is at least superficially identical to that in the canonical cleavage pattern in *Xenopus*: two divisions with spindles oriented along the x–y plane (to generate blastomeres along a single plane) and then a third along the z-axis (to generate two tiers of blastomeres). Although not yet directly tested, this symmetric, alternating pattern may result from the same mechanisms described above that govern spindle orientation in other vertebrates, namely, a combination of asymmetric forces generated by the furrow for the previous cell cycle and cortex sensing mediated by astral microtubules (see Sect. 4.3.3 and below).

With regard to cell cleavage pattern, two differences stand out in ascidians compared to amphibians. The first difference is the bilateral symmetry of the embryo. Subcellular mechanisms maintaining bilateral symmetry in cell arrangement have not yet been studied. It is possible that this feature involves no more than the absence of cell mixing between the two embryonic halves prior to differentiation, itself possibly reflecting strong intercellular adhesive forces along the earliest cleavage planes, with cell division tightly coordinated with cell-autonomous fate commitment.

A second unique characteristic of ascidian cleavage pattern is the asymmetric cell division of posterior-vegetal cells. A posteriorly localized cytoplasmic structure, termed the centrosome-attracting body (CAB), has been shown to be involved in both the posterior tilting of the cleavage axis during the third cell cycle, generating the posterior-vegetal protrusion of the blastula, and the asymmetric cell division of the posterior-vegetal cells during the next three cell cycles (Hibino et al. 1998; Nishikata et al. 1999). The CAB is derived from cytoplasm associated with the posterior-vegetal cortical region that is enriched in cortical ER (cER) and associated factors. The cER becomes enriched at the vegetal pole through cytoplasmic reorganization during the first cell cycle (Roegiers et al. 1995, 1999; Sardet et al. 2003; Prodon et al. 2005) and subsequently undergoes a posterior displacement to coalesce by the third cell cycle into a tight mass at the posterior cortex, constituting the CAB (Hibino et al. 1998; Iseto and Nishida 1999; Nishikata et al. 1999). Interestingly, observed patterns of cER reorganization are similar in three evolutionarily distant ascidian species, *Halocynthia roretzi*, *Ciona*

intestinalis, and *Phallusia mammillata*, indicating a high degree of conservation for cytoplasmic mechanisms involved in cell cleavage in ascidian lineages (Sardet et al. 2003; Prodon et al. 2005). Ablation of the posterior-vegetal cytoplasm from which the CAB forms results in the absence of spindle tilting and asymmetric cell division at subsequent cell cycles (Nishida 1994, 1996). The CAB has an electron-dense appearance at the ultrastructural level (Iseto and Nishida 1999) and is known to contain a number of localized mRNAs, the so-called type I postplasmic/PEM RNAs, some of which are known to have cell-determining functions (Nishida 2002; Sardet et al. 2006).

The CAB is also able to associate with the cytoskeleton. Indeed, the posteriorly located CAB appears to direct the rearrangement of microtubules between the CAB and the interphase nucleus, forming a bundled array of microtubules which undergo shortening. This results in the posterior movement of the nucleus, which is followed by the attachment of one of the centrosomes of the metaphase spindle to the CAB. The combined process results in a posteriorly displaced spindle apparatus, which is attached through one centrosome to the posterior cortex. This eccentric placement of the spindle results in the asymmetric cell division that occurs in these cells. Segregation of the CAB to the posterior-most membrane insures that the posterior-most blastomere inherits this structure, which continues to promote the eccentric spindle location in the following cell cycle. The electron-dense nature of the CAB as well as its ability to localize mRNAs is similar to that of nuage or germplasm, the specialized cytoplasm that specifies primordial germ cells (Wylie 2000; see Chap. 8), suggesting that these may be related structures. Consistent with this interpretation, the smallest, most posterior blastomeres in the 64-cell blastula are fated to become primordial germ cells (Shirae-Kurabayashi et al. 2006). Although a short microtubule bundle is not observed at the third cell cycle stage, the posterior tilting of the spindle that occurs during this earlier stage in posterior-vegetal blastomeres is thought to have the same underlying cause as spindle eccentric movement, namely, the attraction of one centrosome toward the CAB (Negishi et al. 2007). The influence of CAB precursor components at the posterior-vegetal cortex has been proposed to influence spindle orientation as early as the second cell cycle, resulting in an observed shift of the second division cleavage plane with respect to polar bodies at the animal pole of the embryo (Negishi et al. 2007).

One of these CAB-localized mRNAs that code for the novel protein posterior end mark (PEM) has been shown to be directly involved in CAB-induced microtubule reorganization. In embryos with PEM functional knockdown, the CAB appears to form normally, but the microtubule bundle linking the centrosome to the posterior cortex does not form (Negishi et al. 2007). Embryos with PEM functional knockdown also lack the tilting of the spindle characteristic of the third cell cycle as well as the cleavage plane shift at the second cell cycle (Negishi et al. 2007). Thus, PEM mRNA is localized to the CAP, and its protein product has a function essential for the association of the CAP at the posterior cortex with the spindle centrosome, involved in both the cell division tilting and the eccentric placement of the spindle leading to asymmetric cell division.

4.4 Cell Division Machinery During the Early Cleavage Stage

Cell division in the early embryo is influenced by features characteristic of the egg-to-embryo transition, such as the shift from meiotic to mitotic cycles, the inheritance of a limited supply of cellular building blocks, and specializations for large cellular size and unique embryo architecture. We address these topics in this section.

4.4.1 Maternal Loads and Scaling of Spindle Size During Early Cell Divisions

The early embryo develops with unique restrictions since, prior to zygotic gene activation at the midblastula transition (see Chap. 9), all embryonic processes by necessity are driven solely by maternal products. Thus, while cells at later stages of the embryo and the adult produce on their own new products essential for cell growth and division, cells in the early embryo are limited to the supply of cellular building blocks originally stored in the mature egg. Species have evolved specialized systems for the storage in the egg and controlled use of maternal products during early embryonic development. In addition to energy and essential building blocks, the embryo must generate subcellular structures as it becomes multicellular. It has been shown that the overall protein composition from the fertilized egg to the midblastula transition only changes minimally (Lee et al. 1984; Peshkin et al. 2015). Hence, the embryo must generate vital subcellular structures that can fulfill their tasks under drastically different dimensional scales, while the cell size changes by orders of magnitude from the fertilized egg to the midblastula transition. In particular, the cellular machinery must adapt to use a finite initial supply of building blocks for vital subcellular structures as they are being used by the embryo and in the context of a several-fold change in blastomere size. Interestingly, the limited supply of histones and replication factors and their utilization by the exponentially increasing DNA amount have been shown to trigger the onset of the midblastula transition (Newport and Kirschner 1982a, b; Collart et al. 2013; Amodeo et al. 2015). In this section, we use the scaling of spindle-associated structures as an example of maternal product inheritance and adaptation to different length scales in the early cleaving vertebrate embryo.

An important advance in the analysis of intracellular processes involved in early embryonic cell division was the ability to reconstitute asters and bipolar spindles in *Xenopus* extracts, first from oocytes to generate structures analogous to meiotic spindles (Lohka and Maller 1985; Sawin and Mitchison 1991; Mitchison et al. 2013) and later from early embryos to generate sperm-asters and mitotic spindles (Wühr et al. 2008). In both situations, remarkably normal spindles formed, but, in spite of the absence of any cell boundaries in this in vitro system, the spindles exhib-

ited similar sizes to the in vivo equivalent. Furthermore, an upper limit to spindle size was also shown to occur in *Xenopus* embryos: during the first four cell cycles, the spindle size is relatively constant at an upper limit similar to that observed in spindles formed in vitro using an early mitotic or meiotic extract (Wühr et al. 2008; Good et al. 2013; Hazel et al. 2013). The small size of the mitotic spindle compared to the cell size requires some special adaptation for proper DNA segregation. While the mitotic spindle still is responsible for the initial separation of sister chromatids, the majority of the DNA movement into the center of the future daughter cell is executed by cell-spanning anaphase/telophase asters (see Sect. 4.3.2.3).

Starting at the fifth cell cycle, spindle length begins to scale with blastomere length, exhibiting an approximately linear relationship. These observations showed a transition between spindle length control mechanism, with very early and late blastomeres exhibiting different control mechanisms. The observations that the upper spindle length limit is observed in in vitro-reconstituted spindles (Wühr et al. 2008) as well as in embryos where cells are too large to be contacted by metaphase spindle asters (Wühr et al. 2010; see above) suggest the presence of a length-determining mechanism intrinsic to the spindle. The precise nature of this mechanism remains unknown, but it is thought to depend on a balance between microtubule nucleation dynamics and the function of microtubule-associated motors, as proposed for the meiotic spindle (Burbank et al. 2007; Cai et al. 2009; Dumont and Mitchinson 2009; Reber et al. 2013). Subsequently in smaller cells, spindle length does become reduced coordinately with blastomere cell size.

As stated above, spindle size does scale with blastomere size during the later blastomere cycles (Wühr et al. 2008). A simple model by which subcellular structures may scale to the decreasing size of embryonic blastomeres invokes a limiting-component mechanism, developed through studies in the nematode *C. elegans* embryo (Decker et al. 2011). Under this mechanistic model, the size of subcellular structures, in this case centrosomes, scales according to cell volume due to the inheritance during cell division of a limiting amount of structure precursor material that necessarily decrements with each cell division.

The limiting-component mechanism appears to also apply to spindle formation in *Xenopus laevis*, as shown by the analysis of spindles forming in cytoplasmic compartments produced by microfluidic technology (Good et al. 2013; Hazel et al. 2013). In these compartments, spindle size correlates with cell volume. By deforming the compartments to maintain cell volume while changing droplet diameter, the authors showed that spindle length depends on a volume-sensing mechanism as opposed to a boundary-sensing mechanism. Direct measurements shows a decrease in free cytoplasmic tubulin in smaller blastomeres, consistent with spindle scaling being dependent on the concentration of a limiting spindle factor precursor (Good et al. 2013). Interestingly, encapsulated cytoplasm from early stages shows an upper limit in spindle length in vivo as well as in within large droplets (Good et al. 2013; Hazel et al. 2013). Together, these findings indicate that spindle size is regulated by two separate yet interacting systems, one dependent on cytoplasmic composition which imparts an upper limit to spindle length in large cells and a second which

depends on cytoplasmic volume and a limiting subunit such as free tubulin and results in spindle scaling in smaller cells.

A limiting-component mechanism may be a simple and widely used property of animal embryos, including those of vertebrates. Spindle size in early mammalian early embryos is also consistent with a limiting cytoplasmic factor (Courtois et al. 2012), and centrosome size in the early zebrafish embryo is significantly larger in early blastomeres than in later ones (Lindeman and Pelegri 2012). Various components may also interact. For example, centrosome size is known to influence spindle length (Greenan et al. 2010), and it will be interesting in the future to assess the role of centrosome apportioning to spindle scaling. Mechanisms similar to those proposed to regulate spindle and centrosome size, dependent on limiting components inherited in the egg, likely act in the regulated generation of other subcellular organelles in the early embryo.

4.4.2 Specialization of the Cytoskeleton in the Early Embryo

The morphogenetic forces that produce each species' unique embryonic cleavage pattern must integrate with several other cellular activities during cell division to construct the pregastrular embryo. These activities include the establishment of a specialized basolateral membrane domain in each cleavage plane, the zippering together of apical–basolateral margins along advancing furrows to produce a tight-junctional osmotic barrier, and, ultimately, the osmotically driven inflation of interstitial spaces such as the blastocoel. Where the egg begins with only a single outer (apical) surface, the blastula must develop a functional, polarized epithelium to physiologically isolate the interstitial space and/or blastocoel from the outside world. During cleavage, blastomeres become adherent and distinct apical and basolateral membrane domains develop, separated by apical tight junctions (Muller and Hausen 1995; Merzdorf et al. 1998; Fesenko et al. 2000). This process, referred to as compaction, occurs at different developmental times in different organisms. For example, as discussed below (Sect. 4.4.4), in mammalian embryos, compaction is a distinct phase beginning at about the eight-cell stage or later (Ducibella and Anderson 1975; Fleming et al. 2000), while in holoblastically cleaving eggs of amphibians and sturgeon (Zotin 1964; Bluemink 1970; Kalt 1971a, b; Bluemink and deLaat 1973), it occurs contemporaneously with the earliest cleavages.

4.4.2.1 Basolateral Membrane Formation in Xenopus Cleavage

Amphibian early embryos are distinctive among dividing cells for the comparatively large amount of membrane added continuously during the cleavage process (Bluemink and deLaat 1973). Cleavage furrowing is said to be unipolar because it begins at one pole of the egg, and the contractile band then assembles and travels as an arc extending progressively around the egg surface, eventually forming a complete ring which then constricts inward while large amounts of new surface area are

added near the base of the advancing furrow. The new plasma membrane therefore has a composition different from that of the original egg surface (Kalt 1971a, b; Sanders and Singal 1975; Byers and Armstrong 1986; Aimar 1997). The main source of the new basolateral membrane appears to be a pool of post-Golgi vesicles produced during oogenesis which contributes membrane lipids, glycoproteins, and extracellular matrix components to the growing surface (Kalt 1971a, b; Servetnick et al. 1990). Roberts et al. (1992) provided direct evidence that Golgi-derived vesicles generated during late oogenesis can fuse specifically with the new membrane.

4.4.2.2 Microtubule-Dependent Exocytosis of Basolateral Membrane in the Cleavage Plane

The massive localized delivery of new basolateral membrane to the advancing cleavage furrow is known to be microtubule dependent in both Xenopus and zebrafish. A distinctive array of antiparallel microtubule bundles, referred to as a furrow microtubule array, develops along the base of advancing furrows in both *Xenopus* (Danilchik et al. 1998) and zebrafish (Jesuthasan 1998; Fig. 4.10). Their appearance and general geometry distinguish furrow microtubule arrays from other

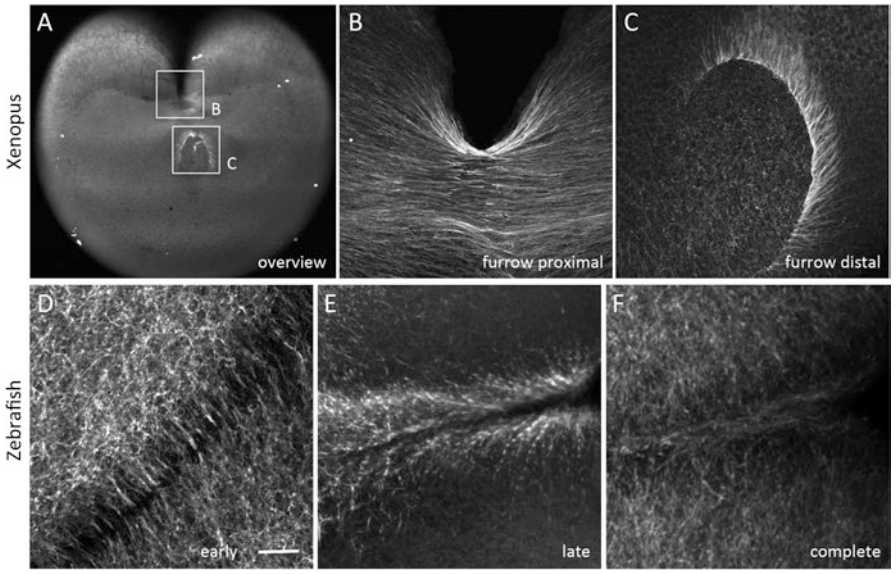

Fig. 4.10 FMA reorganization in *Xenopus* and zebrafish. (**a**–**c**) FMA in *Xenopus*: overview (**a**) and magnified views at the base of the furrow (**b**) and in more vegetal regions of the advancing furrow (**c**). (**d**–**f**) FMA in zebrafish furrows: early furrow shows a parallel arrangement of FMA tubules, oriented perpendicular to the plane of the furrow (**d**), maturing furrow shows distally accumulating FMA tubules in a tilted, V-shaped arrangement (**e**), and complete furrows show FMA disassembly (**f**). Scale bar in (**d**) corresponds to 10 μm for panels (**d**–**f**). Panels (**d**)–(**f**) courtesy of Celeste Eno

microtubule-containing structures in the cleavage plane, such as interzonal spindle microtubules. The rapid expansion of new membrane sustained by the furrow microtubule array is sometimes regarded as an embryonic amplification of the mid-body-dependent localized exocytosis required for abscission of dividing cells of organisms ranging from yeasts to plants and animals (Straight and Field 2000; Glotzer 2001).

4.4.2.3 Protrusive Activity During Cleavage Furrow Closure

Various kinds of membrane protrusions develop along cleavage furrow margins (Danilchik and Brown 2008; Danilchik et al. 2013). Long microvilli are seen associated with stress folds at the cortex, and lamellipodia and filopodia extend near the furrow base itself. These protrusions are actively motile and can make relatively robust contacts across the extracellular space separating the furrow margins. Abrogating normal actin assembly by microinjection of constitutively active rho and cdc42 disrupts normal furrow margin protrusive activity and cleavage furrowing, suggesting that the protrusions play a role in normal furrow closure and blastomere adhesion (Danilchik and Brown 2008).

4.4.2.4 Blastocoel Formation

In amphibian embryos, the initiation of blastocoel formation is evident well before the first contractile ring has finished closing (Kalt 1971a, b). During advance of the cleavage furrow, a new domain of plasma membrane becomes inserted in the plane of the plasma membrane on either side of the contractile ring, resulting in two expanding basolateral surfaces facing each other between separating blastomeres (Bluemink 1970; Kalt 1971a, b). A similar phenomenon likely occurs in the zebrafish (Feng et al. 2002; Urven et al. 2006; Eno et al. 2016). As discussed above, this rapid expansion of new membrane depends on localized exocytosis of maternally derived vesicles immediately behind the advancing contractile ring. The new cleavage planes express maternally encoded C- and EP-cadherins which facilitate adhesion between sister blastomeres (Heasman et al. 1994a, b; Kühl and Wedlich 1996) and integrins. Each successive cleavage event inserts more basolateral surface between dividing daughter cells; the blastocoel, resting at the intersection of all the early cleavage planes, thus is entirely lined by basolateral surface and, with the development of apical–basolateral tight junctions, becomes osmotically isolated from the outside world. Although definitive extracellular matrix fibers, including fibronectin, do not develop until mid- to late-blastula stage (Boucaut et al. 1984; Davidson et al. 2004, 2008), the volume of the blastocoel is nevertheless entirely filled with extracellular matrix at all stages (Keller 1986, Danilchik, unpublished). The blastocoel undergoes continuous expansion and change in shape throughout the cleavage stage, via osmotic uptake of water (Slack and Warner 1973; Han et al. 1991; Uochi et al. 1997), as well as the progressive epibolic thinning of the

blastocoel wall (Longo et al. 2004). Other forces involved with blastular morphogenesis remain obscure but certainly include localized regulation of cell–cell interactions, as illustrated by the effective obliteration of the blastocoel following interference with cadherin-dependent adhesion or Eph–ephrin-mediated cell–cell repulsion (Heasman et al. 1994a, b; Winning et al. 1996).

4.4.3 *Transition of Cell Division Factors from Oocytes to Embryos*

Egg activation, typically coincident with fertilization, is regarded as a natural boundary between egg and zygote. Indeed, egg activation involves many processes that turn a quiescent cell into an actively dividing one. A dramatic example of this sharp transition is the cortical calcium wave associated with egg activation and/or sperm entry, which triggers the exocytosis of cortical granules and remodeling of surrounding egg membranes to act as a block against polyspermy (see Chap. 1). Another dramatic example is the initiation of maternal transcript polyadenylation upon egg activation, which results in the translation of their cognate protein products for use during embryonic development (see Chap. 2).

Besides the abrupt physiological transition brought about by egg activation and the associated process of fertilization, several other profound alterations in cellular processes are known to take place between the end of oogenesis and early embryonic development. This is exemplified by the transition between two different mechanisms for spindle formation in mouse embryos (Courtois et al. 2012). Vertebrates exhibit degeneration of centrioles during oogenesis; centrioles are provided solely by the sperm, an arrangement that ensures a constant centriole number through generations and is an obstacle to parthenogenetic development (Symerly et al. 1995; Delattre and Gönczy 2004). Thus, in vertebrates, spindle formation during meiosis utilizes a centriole-independent pathway in which microtubules self-organize into a pair of microtubule foci which, although wider than those organized by centriole pairs, can nevertheless direct the formation of a barrel-shaped bipolar spindle (Heald et al. 1996, 1997; Gaglio et al. 1997; Walczak et al. 1998; Schuh and Ellenberg 2007). During cleavage stages, embryonic cells typically use bipolar spindles whose formation relies on centrioles inherited through the sperm. However, in rodent lineages, not only oocyte centrioles but also sperm centrioles degenerate (Woolley and Fawcett 1973; Schatten 1994; Manandhar et al. 1998), and the early rodent embryo has to rely on a centriole-independent mechanism to generate MTOCs and spindles. Courtois et al. (2012) found that, unlike most organisms, the mechanism for MTOC formation in the early mouse embryo fails to undergo a sharp transition at fertilization. Instead, early mouse embryos form MTOCs and spindles that have morphological properties similar to those in oocytes during meiosis. During the first eight embryonic cleavages, MTOC and spindle morphology change gradually from the meiotic pattern to one that is typical of later embryonic stages,

e.g., a centriole-dependent MTOC nucleation and the presence of conventional centriole markers. These studies further revealed a similar requirement for the centriole-independent formation of meiotic and early mitotic spindles on microtubule-dependent motors, such as dynein and kinesin-5 (Schuh and Ellenberg 2007; Courtois et al. 2012), showing not only a continuing reliance on meiotic factors during the early mitotic divisions but also the deployment of these factors in similar cellular programs.

Another example of gradual transitioning from oocytes to embryos is the finding in zebrafish of copies of housekeeping genes, which are specialized for maternal expression and which function during both meiosis and early mitosis. This phenomenon has been observed in a maternally expressed form of the protein survivin, which, as mentioned above, is a component of the CPC complex involved in furrow induction and maturation. A mutation in the gene motley was found to affect one of two *survivin* (a.k.a. *birc*) genes in the zebrafish genome, *birc5b* (Nair et al. 2013). This gene exhibits predominantly maternal expression, whereas the related gene *birc5a* is expressed both maternally and throughout zygotic development. Mutations in *motley/birc5b* as well as Birc5b protein localization indicate a specialized role for this gene copy in cytokinesis during both meiotic divisions and early zygotic mitotic divisions. A similar dual function in meiotic and early embryonic mitotic divisions is observed in the case of another maternal zebrafish gene, *tmi* (Nair and Pelegri, unpublished). These examples highlight the continuation of cellular programs across the generational boundary occurring at fertilization, possibly because, in the absence of ongoing transcription, reutilization of programs involved in oocyte formation is an effective way to implement processes subsequently required for early embryonic development. Further studies will be required to understand the prevalence of such cellular program reutilization across the fertilization boundary as well as the role of gene duplication in the generation of genes acting in such processes.

4.4.4 Mammalian Embryo Compaction

In mammals, the cleavage-stage embryo undergoes several mitotic divisions until compaction, or intracellular adhesion, occurs to form a morula. Initially described by Mulnard and Huygens (1978) in mouse embryos and further characterized by others (Ziomek and Johnson 1980; Batten et al. 1987; Natale and Watson 2002), the formation of a morula represents the first morphological disruption in embryo radial symmetry (Fig. 4.11). It is thought that compaction is required for subsequent morphogenetic events such as lineage specification, but how this process is regulated is generally not well understood (Kidder and McLachlin 1985; Levy et al. 1986). In the mouse, compaction is mediated by the formation of adherens junctions, or protein complexes between cells, of which epithelial cadherin (E-cadherin) is a major component (Vestweber et al. 1987). As a type-1 transmembrane protein, E-cadherin relies on calcium ions (Ca^{2+}) and its intracellular binding partners, alpha-catenin (α-catenin) and beta-catenin (β-catenin), to function. However, since E-cadherin

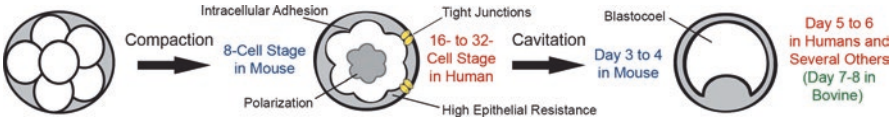

Fig. 4.11 Mammalian embryo compaction and blastulation. The processes of compaction, intracellular adhesion, and polarization result in the formation of a morula at the eight-cell and 16- to 32-cell stage in mouse and human embryos, respectively. In mammals, compaction is mediated by the development of adherens junctions and results in the development of the first developmental asymmetry in the embryo. Once compaction is complete, the assembly of tight junctions in the embryo initiates cavitation and the formation of a fluid-filled cavity called the blastocoel. Most mammalian species, including humans, form blastocysts between days 5 and 6, whereas mouse embryos begin blastulation earlier between days 3 and 4 and bovine embryos later between days 7 and 8

and α-/β-catenin are expressed in mouse embryos as early as the zygote stage, it is unclear how the process of compaction is initiated (Vestweber et al. 1987; Ohsugi et al. 1996). Of note, compaction still occurs even if transcription is inhibited beginning at the four-cell stage (Kidder and McLachlin 1985) and in fact can be prematurely induced by incubating four-cell embryos with protein synthesis inhibitors (Levy et al. 1986). This suggests that all the components required for embryo compaction have been synthesized by the four-cell stage, and, given that premature compaction is observed in the presence of protein kinase activation as well, it also indicates that compaction is under the control of posttranslational regulation via phosphorylation (reviewed in Cockburn and Rossant 2010). Indeed, both E-cadherin and β-catenin become phosphorylated at the time of compaction (Pauken and Capco 1999), and a recent report demonstrated that E-cadherin-dependent filopodia are responsible for the cell shape changes necessary for compaction in mouse embryos (Fierro-González et al. 2013). Using live-cell imaging and laser ablation, this study determined that filopodia extension is tightly coordinated with blastomere elongation and that the inhibition of filopodia components, E-cadherin, α-/β-catenin, F-actin, and myosin-X, prevented cellular elongation and mouse embryo compaction. Whether other mammalian embryos establish and/or maintain cell elongation by similarly extending filopodia is not known, but an earlier time for the initiation of compaction correlates with implantation success in human IVF embryos (Landry et al. 2006; Skiadas et al. 2006).

Along with cell elongation, intracellular polarization also occurs during compaction, whereby the outward-facing, or apical, region of each blastomere becomes distinct from the inward-facing (basolateral) regions at least in mouse embryos. In particular, the blastomere nuclei move basolaterally, whereas both actin and microtubules concentrate apically concomitant with differential localization of membrane and polarity protein complexes (Reeve and Kelly 1983; Johnson and Maro 1984; Houliston and Maro 1989). Cell–cell contact appears to be essential for establishing the orientation of polarity, since blastomeres polarize along the axis perpendicular to cell contact and apical poles form farthest from the contact point. However, additional mechanisms are also likely involved (Ziomek and Johnson 1980; Johnson

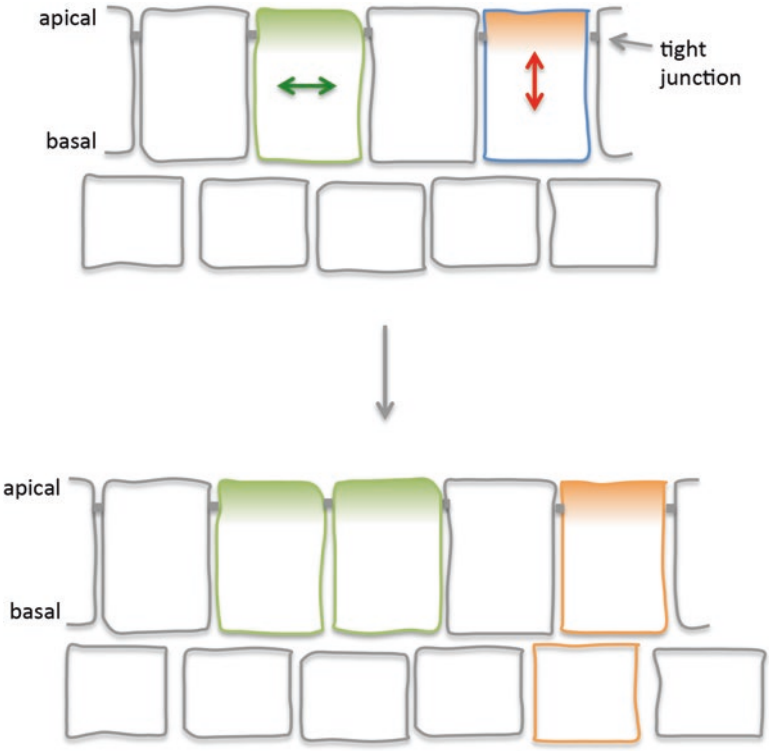

Fig. 4.12 Cell division in a polarized epithelium results in different patterns depending on spindle orientation. Spindle orientation perpendicular to the direction of cell apical–basal polarization (green) results in a cleavage plane that maintains polarization in both daughter cells. Spindle orientation parallel to the direction of cell polarization (red) results in one daughter cell that is polarized and another that is not and which exits the epithelium. Spindle orientation is indicated by a double arrow

and Ziomek 1981a, b). Once the embryo has compacted and polarized, subsequent cell divisions are influenced by the orientation of the cleavage plane so that the established polarity is inherited in the daughter cells (Fig. 4.12). If a blastomere divides at an angle parallel to its axis of polarity, both daughter cells will be polar and remain on the outside of the embryo. However, if a blastomere undergoes mitosis perpendicular to its axis of polarity, one daughter cell will be polarized and contribute to the outside of the embryo, whereas the other daughter cell will be apical and become a part of the inside of the embryo (Johnson and Ziomek 1981a, b; Sutherland et al. 1990). These symmetric versus asymmetric cleavage divisions eventually result in the generation of two distinct cell populations; the cells on the inside will become a part of the inner cell mass (ICM), and the cells on the outside will contribute to the trophectoderm (TE) layer of the blastocyst.

As expected, much of what we know about embryo compaction and blastocyst formation has been derived from studies in mice, and therefore relatively little is

known about these processes in other mammalian species, including humans. Notably, compaction occurs much earlier in mouse embryos, at the eight-cell stage, than in human embryos, where it begins at the 16-cell stage and, even later, at the 32-cell stage, in bovine embryos (Steptoe et al. 1971; Edwards et al. 1981; Nikas et al. 1996; Van Soom et al. 1997). Almost immediately following compaction, the formation of a fluid-filled cavity called the blastocoel is initiated by the assembly of tight junctions, which includes occludin, cingulin, as well as other components, and the establishment of high epithelial resistance in TE cells until the 32-cell stage (Fleming et al. 1993; Sheth et al. 1997, 2000). Once the blastocoel is formed, human embryos are likely to undergo at least one additional round of cell division to form a ~256-cell blastocyst, whereas mouse blastocysts typically comprise ~164 cells (Niakan and Eggan 2013). Until recently, it was unknown how these differences in the timing of compaction or number of cells may affect polarization and asymmetric cell divisions in the human embryo. In contrast to the mouse, wherein TE and ICM fates are established in a positional and cell polarization-dependent manner at the morula stage as described above, human embryos appear to establish the TE as well as the epiblast and primitive endoderm lineages concurrently at the blastocyst stage (Petropoulos et al. 2016). This study also noted that human embryo compaction is not as prominent as in the mouse, with only partial compaction occurring in a certain number of blastomeres on embryonic day 4. Consequently, it is not until embryonic day 5 and upon blastocyst formation that distinct inner and outer compartments are observed in human embryos. Whether other mammals undergo compaction via a cell polarization-dependent mechanism at the morula stage for lineage specification or establish the first lineages concomitant with blastocyst formation is unclear, but partial compaction has been observed in bovine, porcine, and rabbit embryos (Reima et al. 1993; Koyama et al. 1994). Taken together, this suggests that although mammalian embryos morphologically resemble each other during preimplantation development, there are several notable species-specific differences that may limit extrapolation between mammals.

4.5 Evolutionary Relationship Between Holoblastic and Meroblastic Cleavage Types

The phylogenetic distribution of holoblastic and meroblastic cleavage indicates that the latter has evolved independently five times in craniates (a phylogenetic group containing the vertebrates and hagfish (Myxini)) (Collazo et al. 1994; Collazo 1996; Fig. 4.13). Several closely related groups outside of craniates, such as ascidians, tunicates, and echinoderms, exhibit holoblastic cleavage, suggesting that this type of cleavage is the ancestral mode. Within craniates, meroblastic cleavage appears to have evolved independently in Myxini (hagfish), Chondrichthyes (sharks, skates, and rays), Teleostei (largest infraclass of ray-finned fishes), Actinistia (coelacanths), and Amniota (certain non-eutherian mammals (e.g., egg-laying monotremes), birds,

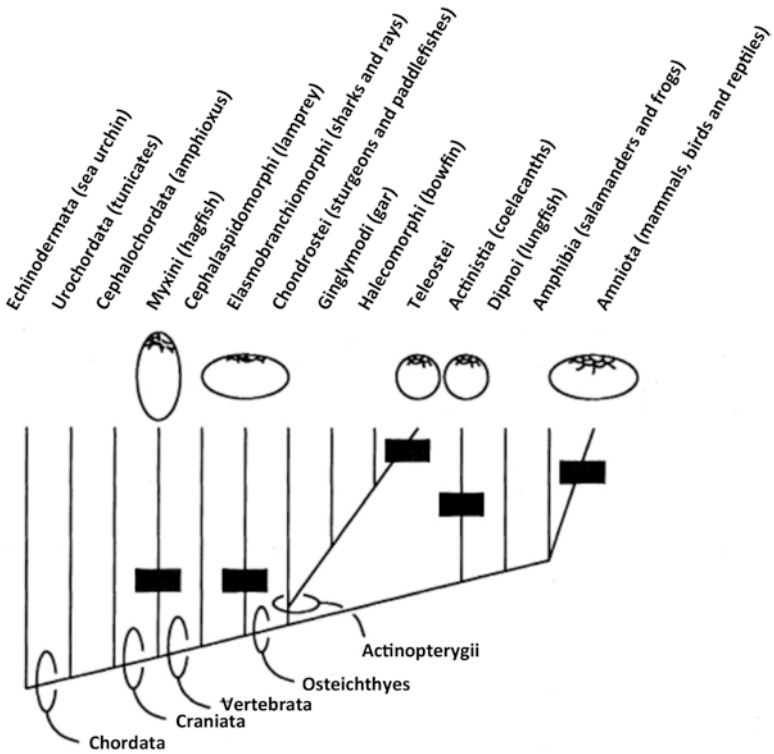

Fig. 4.13 Independent appearance of meroblastic cleavage in various vertebrate phylogenetic lineages. Phylogenetic tree of lineages from sea urchin to vertebrates, showing that meroblastic cleavage (black rectangles) arose multiple times and independently within these lineages. Diagram reproduced from Collazo et al. (1994), with permission

and reptiles). Convergent evolution of meroblastic cleavage is further supported by differences in early development between the various lineages that undergo meroblastic cleavage (Collazo 1996). Independent evolution of meroblastic cleavage appears to reflect a selective advantage. Once arisen within a lineage, meroblastic cleavage is typically not lost, again consistent with an evolutionary advantage. A notable exception to this pattern is the inferred reversion of cleavage pattern within Amniota, from meroblastic to holoblastic as found in eutherian mammals and marsupials.

In amphibian holoblastic cleavage, like that observed in the model vertebrate *Xenopus*, all blastomeres eventually contribute to one of the three germ layers. The ancestral nature of holoblastic cleavage is largely responsible for the widely held assumption that amphibian-like cleavage represents the ancestral form of holoblastic cleavage in vertebrates. However, evidence from bichir (*Polypterus*), a basal actinopterygian (ray-finned fish), and lamprey (*Lampetra japonica*), a basal vertebrate, challenges this view (Takeuchi et al. 2009). Vegetal cells in embryos of these organisms do not express mesodermal or endodermal markers as in the case in amphibi-

ans. Analyses of such embryos suggest that some vegetal cells do not contribute to any of the three germ layers and are instead nutritive yolk cells only. This fact, combined with the evolutionary position of basal fish like bichirs and lampreys, points to the conclusion that, while holoblastic cleavage is ancestral in vertebrates, the particular form observed in amphibians, with all blastomeres contributing to embryonic tissues, is derived. This conclusion is further bolstered by the observation that a maternally expressed homologue of vegT, which is crucial for early amphibian endoderm development, appears to only be found in amphibians and not other vertebrate species, such as mice, bichirs, lampreys, and teleosts (Takeuchi et al. 2009).

The ancestral trait of having explicitly nutritive yolk cells in vertebrates may have provided early embryos an evolutionarily advantageous ability to implement cell division in the absence of yolk granules while nevertheless maintaining embryonic nutritive stores, an advantage that may have been maintained in meroblastic cleaving embryos. Developing embryos are known to rely on exquisitely precise cellular processes, such as cytoskeletal reorganization and the recycling of membrane particles during cell division, and it is easy to imagine that the presence of yolk particles may interfere with, or add variability to, this process. Selection against such interference could be one cause of a transition to a meroblastic cleavage system in an animal's lineage.

The inference of explicitly nutritive yolk cells in ancestral vertebrates may also make it easier to understand precisely how meroblastic cleavage might evolve from holoblastic cleavage. For example, fusion of yolky blastomeres into a single nondividing mass is proposed to be the second of two changes that occurred leading to evolution of the teleost embryo, the first being loss of bottle cells that are still present near the beginning of gastrulation in more basal taxa (Collazo et al. 1994). Given this, groups such as bowfins (*Amia*) and gars (*Lepisosteus*) (Ballard 1986a, b; Long and Ballard 2001), which exhibit cleavage patterns that appear to be partially meroblastic (Fig. 4.14a), may be representative of ancestral transitional states along a continuum from holoblastic to meroblastic cleavage.

An important correlation that has long been observed in the study of cleavage pattern evolution is that meroblastic cleavage often correlates with large egg size (Collazo 1996). Egg size correlation can be most clearly seen in amniotes where large eggs and meroblastic cleavage are predominant, with the small embryos of eutherian mammals and marsupials having returned to essentially holoblastic cleavage. Furthermore, as discussed above, the generally small eggs of amphibians exhibit a likely derived form of holoblastic cleavage. In such cases, selected traits such as differing degrees of reliance on egg nutritive stores may underlie the correlation between cleavage type and egg size, although other explanations have been proposed (Collazo 1996).

Teleosts constitute a major exception to this correlation, as there appears to be a sharp decrease in egg size in their stem phylogenetic lineage (Collazo 1996). Conversely, the Puerto Rican tree frog, *Eleutherodactylus coqui*, has an egg approximately 20 times the size of *Xenopus laevis* but in spite of this enormous size maintains a holoblastic cleavage pattern. How these particular species may escape the

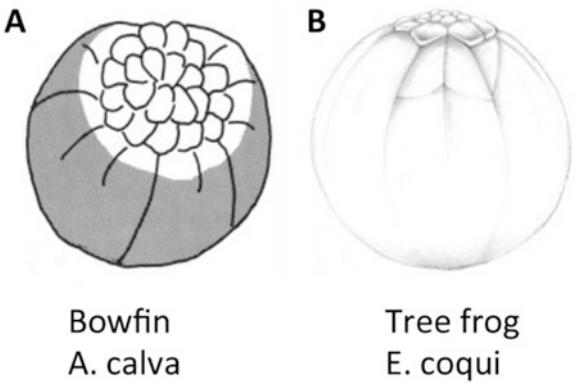

Fig. 4.14 Intermediates between holoblastic and meroblastic cleavage patterns. (**a**) Partially meroblastic cleavage in bowfin fish (*A. calva*). The yolk-rich vegetal region ceases to cleave after the formation of 16 cleavage furrows. (**b**) Formation of nutritional endoderm in the Costa Rican tree frog (*E. coqui*). Yolk-rich vegetal cells divide although do not eventually become part of the embryo proper (Buchholz et al. 2007). Diagrams reproduced from Ballard (1986a, b), in panel (**a**) and Buchholz et al. (2007) in panel (**b**), with permission

general rule of egg size and type of cleavage is unknown. However, blastula embryos for *E. coqui* exhibit a distinct population of nutritional vegetal cells that are not destined to become endodermal tissue (or any other embryonic cell type) (Buchholz et al. 2007; Fig. 4.14b), and, as mentioned above, the presence of such nutritional cells may represent an important step in the evolutionary transition from holoblastic to meroblastic cleavage (Buchholz et al. 2007; Elinson 2009).

Thus, it appears that holoblastic cleavage is the ancestral pattern of cleavage in craniates. Meroblastic cleavage, in which only a portion of the embryo is made up of dividing blastomeres, has evolved independently at least five times within this group. This evolution is sometimes, though not always, correlated with increased egg size. Meroblastic cleavage evolution in teleosts is a significant exception and has been shown to involve two evolutionary changes, loss of bottle cells and fusion of yolk into a single mass. The presence of a population of nutritive cells, whether ancestral or derived in a lineage, may be a key innovation on the way toward meroblastic cleavage. These innovations likely conferred a selective advantage, possibly an increased ability to implement subcellular programs required for early embryogenesis.

4.6 Conclusions

In this chapter, we have described mechanisms underlying patterns of cell cleavage arrangement in early vertebrate embryos. Much of our knowledge on this topic stems from studies in tractable developmental systems such as amphibians and teleosts.

These studies highlight major challenges that the cleavage-stage embryo, in these and likely other vertebrate species, has to overcome. These include structures such as spindle asters that are too small relative to the large early blastomeres, a limited supply of cellular building blocks within a changing landscape of cell size and organization, and the requirement for cytoskeletal specializations adapted to very large cells. We discuss how the vertebrate embryo appears to use simple rules to drive development even under these limitations, such as the use of interphase astral microtubules from a given cycle to orient the spindle for the following cell cycle or the use of limiting inherited reagents, such as tubulin, to scale spindles according to cell size in later-stage embryos. Such simple rules provide elegant solutions to overcome constraints associated with the transition from an egg into a three-dimensional embryonic blastula. Notably, we examine how a combination of cell shape-sensing cues, including those from a microtubule exclusion zone at the furrow for the previous cell cycle to orient the spindle, explain the sequence of blastomeric divisions leading to the basic cell arrangement in both zebrafish and *Xenopus* embryos, and possibly other vertebrates as well, though this remains to be determined. Interestingly, dynamic changes in the embryonic developmental landscape contribute to developmental decisions as they occur. For example, changes in cell dimensions in teleost blastomeres likely result in the eventual shift of the spindle plane from a horizontal (x–y) axis to a vertical (z) axis, generating a two-tiered blastula.

Although not as well studied, similar rules may exist across the range of vertebrate species, for example, mechanisms for spindle scaling in mammals and mechanisms of cleavage plane positioning in proto-vertebrates such as ascidians, suggesting that cellular mechanisms involved are highly conserved. This hypothesis is bolstered by examination of the phylogenetic distribution of major cleavage patterns across vertebrates and their close relatives.

It is possible that a wide variety of cleavage patterns can be explained with the same simple rules but with different initial parameters or conditions. For example, the presence of yolk enriched in the vegetal pole in Xenopus likely creates a pulling force bias on the spindle, resulting in the animal movement of the spindle and eventually an asymmetric cell division leading to a smaller animal pole cell and a larger vegetal pole cell. As another example, a change in shape in the initial blastodisc may lead to changes in the relative proportions of blastomere allocated to different dimensions in the resulting blastula. Basic cell shape-sensing mechanisms also interact with specialized cytoplasmic structures, such as the CAB in ascidians, resulting in the generation of added cell cleavage pattern variation. The observed embryonic patterns thus appear to be the outcome of a temporal sequence based on initial embryonic conditions (starting shape, amount, and type of relevant factors) as they are modified by ongoing cycles of cell division by the factors inherited by the egg itself. Future studies will continue to address detailed mechanistic aspects that drive these early embryonic processes and will also allow us to understand how changes in various conditions and parameters lead to diversity in blastomere arrangements encountered in different species. Cell cleavage pattern is a backdrop on which cell fate decisions are overlain, and it will also be important to better understand the interconnection between these two types of processes. Future studies

will surely continue to provide us with a view of the elegant mechanisms embryos use to solve the unique problem of transforming an egg into a multicellular blastula.

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Chapter 5
Localization in Oogenesis of Maternal Regulators of Embryonic Development

Matias Escobar-Aguirre, Yaniv M. Elkouby, and Mary C. Mullins

Abstract Cell polarity generates intracellular asymmetries and functional regionalization in tissues and morphogenetic processes. Cell polarity in development often relies on mechanisms of RNA localization to specific subcellular domains to define the identity of future developing tissues. The totipotent egg of most animals illustrates in a grand way the importance of cell polarity and RNA localization in regulating multiple crucial developmental events. The polarization of the egg arises during its development in oogenesis. RNAs localize asymmetrically in the early oocyte defining its animal-vegetal (AV) axis, which upon further elaboration in mid- and late-oogenesis stages produces a mature egg with specific localized factors along its AV axis. These localized factors will define the future anterior-posterior (AP) and dorsal-ventral (DV) axes of the embryo. Furthermore, AV polarity confines germ cell determinants to the vegetal pole, from where they redistribute to the cleavage furrows of the 2- and 4-cell stage embryo, ultimately specifying the primordial germ cells (PGCs). The sperm entry region during fertilization is also defined by the AV axis. In frogs and fish, sperm enters through the animal pole, similar to the mouse where it enters predominantly in the animal half. Thus, AV polarity establishment and RNA localization are involved in all the major events of early embryonic development. In this chapter, we will review the RNA localization mechanisms in vertebrate oocytes that are key to embryonic patterning, referring to some of the groundbreaking studies in frog oocytes and incorporating the current genetic evidence from the zebrafish.

Keywords Balbiani body • Bucky ball • Oocyte polarity • Germplasm • RNA localization • Oogenesis • Fertilization • Cytoskeleton • Axis formation • Animal-vegetal axis

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5.1 Introduction

Studies in *Xenopus* have identified many animally and vegetally localized factors in the egg that distribute either to the germ cells, the germ layers, or specify the axes in the early embryo (Bubunenko et al. 2002; Claussen and Pieler 2004; Claussen et al. 2011; Cuykendall and Houston 2010; Houston et al. 1998; Kaneshiro et al. 2007; Ku and Melton 1993; Kwon et al. 2002; Rebagliati et al. 1985; Weeks and Melton 1987). Genetic screens in the zebrafish or knockdown approaches in *Xenopus* have revealed the function for some of these factors and discovered new ones. In the zebrafish, the *bucky ball* (*buc*) and *macf1* genes were identified in a maternal-effect mutant screen and provide the only known genetic entry points for studying the early events of AV polarity establishment during oogenesis (Bontems et al. 2009; Dosch et al. 2004; Gupta et al. 2010; Marlow and Mullins 2008; Wagner et al. 2004). Vegetally-localized RNAs acting in DV axis formation include *wnt8* in zebrafish (Erter et al. 2001; Lekven et al. 2001; Varga et al. 2007) and *wnt11* in *Xenopus* (Cha et al. 2008, 2009; Ku and Melton 1993; Tao et al. 2005), while vegetally localized VegT in *Xenopus* functions in germ layer formation (Clements et al. 1999; Xanthos et al. 2001). Genetic screens in zebrafish also identified *glutamate receptor interacting protein 2* (*grip2a* or *hecate*) (Ge et al. 2014) and *syntabulin* (*tokkaebi*) (Nojima et al. 2010) as key vegetally-localized regulators of DV axis formation in the egg. The repertoire of functionally important localized factors in the egg will certainly continue to expand in the future.

In this chapter, we will review the RNA localization mechanisms in vertebrate oocytes that are key to embryonic patterning, referring to some of the groundbreaking studies in frog oocytes and incorporating the current genetic evidence from the zebrafish. The mechanisms of RNA localization will be discussed in relation to how they act in AV polarity establishment and its consequences in embryonic patterning, germ cell specification, and fertilization.

5.2 Function of Localized Products

5.2.1 Determinants of the Embryonic Body Axes Originate in the Polarized Oocyte

In vertebrates the main embryonic body axes are first formed and patterned during blastula and gastrula stages. From a seemingly symmetrical sphere of totipotent cells, gastrulation transforms the embryo into the bilateral vertebrate body plan, comprised of dorsal-ventral (DV), anterior-posterior (AP), and left-right axes. With the more dramatic changes that follow, the patterning of these axes is fine-tuned with cells continuously being specified and differentiating, with further morphogenesis governing organogenesis. The first cues that guide this magnificent process of development are, however, already seeded in the developing oocyte, long before the formation of the zygote.

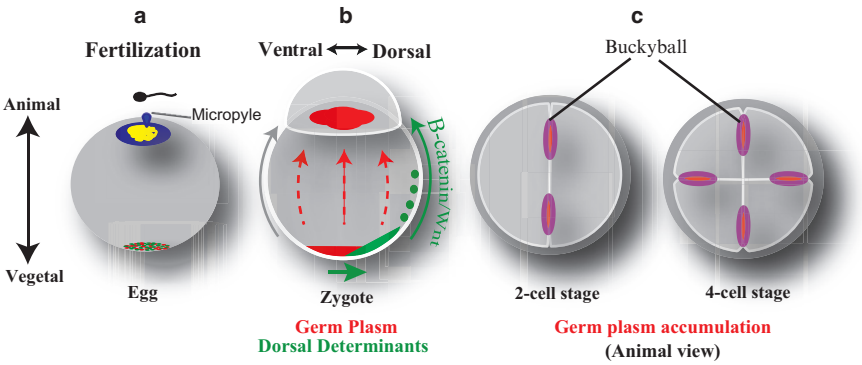

Fig. 5.1 Function of localized maternal products. Establishment of the AV axis in the egg defines the (**a**) sperm entry region, (**b**) dorsal-ventral (DV) axis, and (**c**) the location of the germ plasm, which specifies the PGCs. (**a**) In frog, fish, and mouse, the oocyte nucleus migrates to the animal pole during oocyte maturation and extrudes the first polar body. Frog and fish eggs are fertilized in the animal half, and in zebrafish the sperm enters through the micropyle that forms at the animal pole. Following fertilization, the second polar body is also extruded at the animal pole. At the egg vegetal pole, the germ plasm (*red*) and dorsal determinants (*green*) are localized. (**b**) Upon fertilization, dorsal determinants are transported from the vegetal pole to the future dorsal side of the embryo. Here, activation of the Wnt pathway specifies the dorsal fate. (**c**) The germ plasm, including Bucky ball, accumulates at the cleavage furrows of the 2- and 4-cell stage embryo, illustrated for the zebrafish embryo here

Establishment of the DV axis of the embryo is governed by maternal factors localized to the vegetal pole of the egg (Fig. 5.1b) (see Chap. 6) (De Robertis and Kuroda 2004; Langdon and Mullins 2011). Among these, the factors that determine the site of the dorsal organizer of the embryo are called dorsal determinants, and they are specifically localized in the egg during oogenesis (Langdon and Mullins 2011). In both *Xenopus* and zebrafish, the activity of the dorsal determinants results in the nuclear localization of the Wnt pathway effector, β-catenin, specifically in a group of blastomeres on the future dorsal side of the blastula embryo (De Robertis and Kuroda 2004; Langdon and Mullins 2011). This local activity of β-catenin cooperates with broader Nodal and FGF signals to induce dorsal gene expression (De Robertis and Kuroda 2004; Langdon and Mullins 2011).

Evidence indicates that the dorsal determinants are Wnt ligands. Albeit implied from the role of β-catenin in dorsal organizer specification, their entity had been illusive for some time. In *Xenopus* the dorsal determinant is a unique combination of Wnt11 and Wnt5a ligands, each traditionally considered a non-canonical Wnt ligand (Cha et al. 2008, 2009; Tao et al. 2005). Surprisingly, Wnt11 and Wnt5a are connected via O-sulfate bonds and function as a dimerized ligand to activate the canonical Wnt pathway, inducing β-catenin nuclear localization on the future dorsal side of the embryo (Cha et al. 2008, 2009; Tao et al. 2005). In zebrafish Wnt8 is the implicated dorsal determinant (Lu et al. 2011).

Despite using different Wnt ligands, dorsal determinant activity is regulated by similar principles in *Xenopus* and zebrafish. The *wnt* ligand mRNAs are initially

localized to the vegetal pole of the egg and zygote (Fig. 5.1a) (Ku and Melton 1993; Lu et al. 2011; Nojima et al. 2004, 2010). Upon fertilization in *Xenopus* and egg activation in zebrafish, microtubules reorganize to pave the way for these mRNAs to translocate asymmetrically in an animal direction, resulting in their new localization specifically on the future dorsal side of the embryo (Fig. 5.1b) (Heasman 2006; Ku and Melton 1993; Lu et al. 2011; Tao et al. 2005). Prior to this cortical rotation, these mRNAs are translationally silent. While *wnt8* translation regulation has yet to be dissected in zebrafish, *wnt11* mRNA in *Xenopus* was shown to load onto polysomes only after detaching from the microtubules at its dorsal destination during cleavage stages (Schroeder et al. 1999). A similar mechanism is expected for zebrafish Wnt8. The coordinated localization and translation of these mRNAs ensures their dorsally localized protein product and results in a dorsal to ventral Wnt gradient (Fig. 5.1b).

Two factors identified in maternal-effect screens in zebrafish, *syntabulin* (*tokkaebi*) and *grip2a* (*hecate*), are also localized to the vegetal pole of the egg and play key roles in dorsal determinant translocation following egg activation (Ge et al. 2014; Nojima et al. 2010). Consistent with this role, females mutant for these genes produce embryos that are ventralized. Syntabulin is a microtubule-motor linker protein that can bind the Kinesin I motor heavy chain, Kif5b (Nojima et al. 2010). Syntabulin protein localizes to the vegetal pole of the egg and translocates asymmetrically in a microtubule-dependent manner, consistent with it playing a role in translocating dorsal determinants on the microtubule array. The *grip2a* mRNA also localizes to the vegetal pole and itself is translocated asymmetrically like the *wnt* transcripts following egg activation (Fig. 5.1b) (Ge et al. 2014). In eggs of *grip2a* mutant females, the vegetal pole microtubule network is compromised and the asymmetric translocation of *wnt8* and *grip2a* itself fails (Ge et al. 2014). The vegetal localization of *grip2a* and *syntabulin* are conserved in *Xenopus*, as well as the function of Syntabulin (Colozza and De Robertis 2014; Nojima et al. 2010). However, *grip2a* is, in addition, later localized to PGCs in *Xenopus* (Ge et al. 2014; Kaneshiro et al. 2007; Kirilenko et al. 2008; Tarbashevich et al. 2007).

The *wnt8, wnt11, syntabulin,* and *grip2a* mRNAs are all localized to the vegetal pole of the egg (Ge et al. 2014; Kirilenko et al. 2008; Ku and Melton 1993; Lu et al. 2011). Moreover, with the exception of *wnt11* (Ku and Melton 1993), their localization to the vegetal pole occurs early in oogenesis and is executed by the Balbiani body (Bb) (Kirilenko et al. 2008; Lu et al. 2011; Tarbashevich et al. 2007). These mRNAs localize to the Bb and the future vegetal pole of the early oocyte, where they remain anchored throughout oogenesis and in the egg. Thus, the AV axis of the oocyte is key to forming the dorsal organizer and subsequently establishing the basic vertebrate embryonic body plan (Fig. 5.1).

5.2.2 Germ Layer Formation and Its Regulation by Oocyte Polarity

All the organs and cell types in an animal body originate from the three embryonic germ layers, the ectoderm, mesoderm, and endoderm during early development. The specification of the germ layers is intimately related to the AV axis and layered upon the DV

axis of the early embryo. In this setting, the ectoderm forms from cells in the animal region, the endoderm from the most vegetal region, and the mesoderm from the margin (see Chap. 7) (De Robertis et al. 2000; Kimelman 2006). All animal embryos generate their germ layers during the process of gastrulation (see Solnica-Krezel and Sepich 2012). The outcome of this massive reorganization is the formation of the endoderm as the innermost tissue, the ectoderm as the outermost, and the mesoderm between them.

The major inducer of mesoderm is the Nodal signaling pathway (reviewed in Kimelman 2006; Schier 2003). Nodal ligands are secreted from vegetal cells and activate the pathway vegetally as well as in marginal cells of the embryo (De Robertis et al. 2000; Kimelman 2006). This marginal zone is specified as mesoderm in *Xenopus* and mesendoderm in zebrafish (De Robertis et al. 2000; Kimelman 2006; Schier and Talbot 2005). In addition to the uniform secretion of Nodal along the DV axis, on the dorsal side of the embryo, *nodal* ligands are expressed slightly earlier, resulting in a brief transient higher accumulation of Nodal signal in this region (Kimelman 2006). In addition, the nodal ligand *squint* (*sqt*) mRNA was described in zebrafish to exhibit localization dynamics that enrich it dorsally at the 4-cell stage (Gore et al. 2005), which could further reinforce a Nodal gradient. In *Xenopus* the endoderm forms in the vegetal hemisphere, while in zebrafish, the mesendoderm comprises the marginal cells, with the endoderm specified in the most vegetal cell tier (Ober et al. 2003; Schier and Talbot 2005). This setting puts the endoderm precursor cells in both animals in greater proximity to the vegetal Nodal signal source, and this vegetal-to-animal Nodal signaling gradient is thought to play a role in distinguishing between mesodermal and endodermal cell fates (De Robertis et al. 2000; Kimelman 2006; Schier and Talbot 2005; van Boxtel et al. 2015).

The expression of *nodal* in *Xenopus* is regulated by the vegetal pole localized factor VegT, a T-box transcription factor (Clements et al. 1999; Kimelman 2006; Xanthos et al. 2001). *VegT* mRNA is released from the vegetal cortex upon fertilization and slowly diffuses in the vegetal cytoplasm. During the third cell division in *Xenopus*, a cleavage furrow forms along the embryo equator, perpendicular to the AV axis, and this cell division confines *VegT* transcripts to the vegetal hemisphere. VegT protein is then only produced in the vegetal hemisphere, which induces expression of *nodal* ligands specifically in this region (Clements et al. 1999; Kimelman 2006; Xanthos et al. 2001). In zebrafish, Nodal signaling from the extraembryonic yolk syncytial layer (YSL), which underlies the blastoderm, is required to induce *nodal* ligand expression in the marginal blastoderm cells (Fan et al. 2007; Hong et al. 2011). Within the YSL, another maternal T-box gene, Eomesodermin, together with the Mix homeodomain transcription factor Mxtx2, induces *nodal* ligand expression (Du et al. 2012; Xu et al. 2014). The *eomesodermin* mRNA localizes to the zebrafish oocyte cortex, similarly to *vasa*, although it is not known if it localizes there via the Bb like *vasa* (Bruce et al. 2003; Kosaka et al. 2007).

The *vegT* mRNA is localized to the oocyte vegetal pole not via the Bb but by a later pathway in oogenesis (see Sect. 5.4). In this pathway, the Kinesin-1 and Kinesin-2 motors bind and transport mRNPs, including *vegT* mRNA, to the vegetal cortex, utilizing specialized microtubules that radiate their plus-ends vegetally (King et al. 2005; Messitt et al. 2008). *vegT* mRNA remains anchored to the vegetal pole cortex throughout oogenesis and in the egg, before being released in the early

embryo (King et al. 2005). Therefore, the initial positional information that sets up the embryonic germ layers and body axis is stored in and provided by the vegetal pole. Oocyte polarization during early oogenesis is therefore critical for these fundamental processes of embryogenesis.

Interestingly, the final destinations of vegetally localized transcripts in the *Xenopus* egg differ following fertilization. While *vegT* and *wnt11* are released from the cortex and diffuse in the cytoplasm, *syntabulin* and *grip2a* transcripts localize to the cleavage furrows with the germplasm and are inherited specifically by the primordial germ cells (PGCs) (Claussen et al. 2011; Colozza and De Robertis 2014; Ge et al. 2014; Kaneshiro et al. 2007). Despite its mRNA localizing to the PGCs, Syntabulin protein is expected to localize more broadly in *Xenopus*, as it does in zebrafish, to fulfill its role in dorsal axis determination (Colozza and De Robertis 2014; Nojima et al. 2010). The distinct post fertilization localization of *vegT* and *wnt11* versus *syntabulin* and *grip2a* may reflect their distinct localization mechanisms during oogenesis via the late localization pathway versus the early Bb pathway, respectively. It is possible that distinct vegetal cortical docking mechanisms cause the distinct postfertilization cortical release or cleavage furrow localization, or alternatively different regulatory information in their respective mRNAs deliver them to distinct locales following fertilization.

5.2.3 *Germ Cell Determinants Originate in the Polarized Oocyte*

Animals have developed two primary strategies to specify the germ line (see Chap. 8 for more details). In mammals, the germ cell lineage is induced in the epiblast by the action of BMP4 produced in the extraembryonic ectoderm (Lawson et al. 1999). In other vertebrates, and in most insects, germ cells are specified by the inheritance of the germplasm (GP), a cytoplasmic aggregate containing RNA and proteins sufficient to determine the primordial germ cell (PGC) fate (Kobayashi et al. 1994; reviewed in Extavour and Akam 2003). In zebrafish and *Xenopus*, GP accumulates at the 2- and 4-cell stages at the ends of the cleavage furrows (Fig. 5.1c) (Hashimoto et al. 2004; Kloc et al. 2001; Yoon et al. 1997). Removal of GP diminishes or ablates PGC formation in fish and frogs (Ikenishi et al. 1974; Smith 1966; Whitington and Dixon 1975; Yoon et al. 1997; Zust and Dixon 1975). Conversely, GP transplantation induces PGC formation (Kobayashi et al. 1994), thus providing compelling evidence for GP function in germ cell specification.

In *Xenopus* the GP becomes localized during oogenesis to the oocyte vegetal cortex, where it remains localized in the egg (Figs. 5.1a and 5.2b) (Heasman et al. 1984;

Fig. 5.2 (continued) molecular motors (late pathway). Thus, at this stage early and late localized RNAs reside at the vegetal cortex (*orange*). During this stage, additional mRNAs (e.g., Vg1 in zebrafish) are localized animally (*light blue*), and the micropyle becomes evident at the animal pole. In stage IV, the oocyte matures, the nucleus migrates to the animal pole, and the nuclear envelope disassembles in frogs, fish, and the mouse. At this stage, the oocyte transitions from diplotene of meiosis I to metaphase of meiosis II, extruding the first polar body at the animal pole in all three species

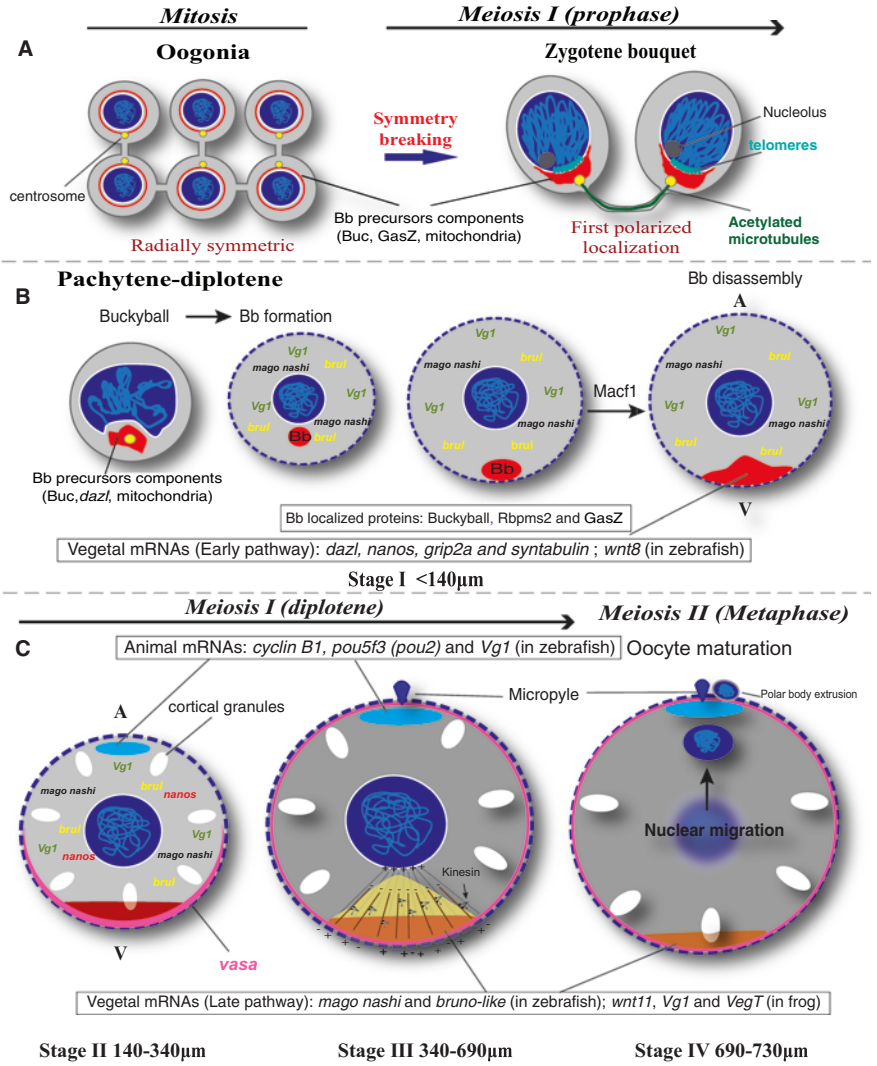

Fig. 5.2 RNA localization and AV axis establishment during oogenesis. (**a**) In premeiotic oogonia, Bb precursors are distributed radially in the cytoplasm, telomeres are scattered intranuclearly, and the centrosome (*yellow*) is at a perinuclear position, possibly positioned facing the cytoplasmic bridge of the last mitotic division. When meiosis initiates, specifically during zygotene stages, Bb precursors (*red*) localize around the centrosome and adjacent to the telomere cluster (*cyan*) of the zygotene bouquet configuration. Thus, the nuclear axis of the bouquet configuration predicts the AV axis of the oocyte. At this stage, acetylated tubulin cables (*dark green*) form and may connect sister oocyte pairs through the cytoplasmic bridges of the last mitotic oogonial division (a pair is shown). (**b**) At the subsequent pachytene stage, a nuclear cleft forms at the centrosome position where Bb precursors aggregate. The nuclear cleft remains throughout pachytene and early diplotene. In mid-diplotene, the nuclear envelope rounds out and the mature spherical Bb forms marking the position of the prospective vegetal pole of the oocyte. The formation of the Bb and its disassembly requires Bucky ball and Macf1 function, respectively. Hence, Bucky ball and Macf1 regulate RNA localization to the vegetal pole through the early pathway and are also required for AV polarity establishment. (**c**) At stage II, Bb RNAs are localized to the vegetal pole (*red*). Distinct RNAs localize to the animal pole (*light blue*) and cortical granules (*white ovoid*) are formed. *vasa* RNA is broadly localized at the vegetal pole (*pink*) and expands radially cortically at subsequent stages. At stage III, previously unlocalized RNAs (*yellow*) are transported to the vegetal pole on microtubules tracks (*brown*) by the action of

Houston and King 2000; Kloc et al. 2002). In the *Xenopus* embryo, the vegetal cells that inherit the GP then adopt the PGC fate (Houston 2013; Whitington and Dixon 1975). In zebrafish it is more complex: some GP components localize to the oocyte and egg vegetal pole (e.g., *dazl* RNA) (Kosaka et al. 2007), whereas *vasa* RNA, for instance, is initially vegetally localized in the oocyte and then becomes radially localized to the oocyte cortex, where it remains in the egg (Kosaka et al. 2007). After egg activation in zebrafish, the GP components from distinct locations accumulate at the cleavage furrows of the 2- and 4-cell stage embryo (Fig. 5.1c) (Braat et al. 1999; Knaut et al. 2000; Riemer et al. 2015; Yoon et al. 1997). The difference between frog and fish is likely due to the distinct architectures of these embryos. In zebrafish the yolk lies at the vegetal pole of the egg separate from the blastomeres that form in the animal half, whereas in the frog, the yolk and cytoplasm are not segregated from each other, and blastomeres will comprise the entire AV extent of the egg. So in the zebrafish, the GP must reaggregate at the cleavage furrows at the yolk-cytoplasm interface at an animal-vegetal mid-region (Fig. 5.1b and c), whereas in the frog, the vegetal-most blastomeres inherit directly the vegetal-oocyte localized GP to become the PGCs.

In both frogs and fish, the germplasm components are first localized in stage I oocytes to a structure called the Balbiani body (Bb) (Fig. 5.2b) (Chang et al. 2004; Heasman et al. 1984; Kloc and Etkin 1995; Kloc et al. 1996, 1998; Kosaka et al. 2007; Schnapp et al. 1997; Wilk et al. 2005). Here we will generally use the zebrafish oocyte staging convention (Selman et al. 1993), which is slightly different to that in *Xenopus*. The Bb, also called the mitochondrial cloud in *Xenopus*, is a highly conserved structure present from insects to humans where specific RNAs, proteins, and organelles become localized (reviewed in Kloc et al. 2004b). These RNAs and proteins, including GP components, translocate via the Bb to the oocyte vegetal cortex (Fig. 5.2b) (Kloc and Etkin 1995; Melton 1987; Wilk et al. 2005; Yisraeli et al. 1989, 1990; Zhou and King 1996b). In Sect. 5.4, we will discuss what is known about the RNA localization mechanism via the Bb.

5.2.4 *Fertilization: Oocyte Polarity Defines the Sperm Entry Region*

The AV axis defines the region of sperm entry in most vertebrates. Frog, zebrafish, and mouse eggs are all fertilized predominantly in the animal half, although each differs somewhat from the others (Grey et al. 1974; Hart et al. 1992; Piotrowska and Zernicka-Goetz 2001). In the mouse, the animal 11 % of the egg lacks villi, reducing the membrane surface area for sperm binding, and fertilization is excluded from this region (Hiiragi and Solter 2004). However, fertilization occurs predominantly in the remainder of the animal half of the mouse egg, largely due to the greater

perivitelline space found animally (Motosugi et al. 2006). The animal pole exclusion zone for sperm entry represents inherent polarity of the mouse egg derived from the oocyte. During oocyte maturation, which occurs in late stages of oogenesis, the oocyte nucleus (also called the germinal vesicle) migrates to the oocyte periphery and completes meiosis I, extruding the first polar body. This defines the animal pole in the mouse oocyte and future egg and confers it with distinct properties, such as the fertilization exclusion zone (Motosugi et al. 2006). The animalward migration of the GV and polar body extrusion processes in oocyte maturation are conserved, except that AV polarity in frogs and fish has already been defined much earlier in oogenesis.

In zebrafish, the egg is fertilized through a micropyle (Fig. 5.1a), which is generated during oogenesis by a single specialized follicle cell evident in stage III oocytes at the animal pole (Fig. 5.2c). The micropyle is an opening in the vitelline membrane that allows only a single sperm to fertilize the egg (monospermy) in zebrafish (Amanze and Iyengar 1990). In the absence of AV polarity establishment in zebrafish *bucky ball* (*buc*) mutant eggs, multiple ectopic micropyles form, resulting in polyspermy (Marlow and Mullins 2008). Interestingly, Buc functions in the oocyte and thus non-cell autonomously acts in micropyle formation, suggesting cross talk between the oocyte and surrounding follicle cells in micropyle biogenesis (Heim et al. 2014).

Not all fish use a single micropyle as a mechanism to block polyspermy. Sturgeons, for example, can have several micropyles at the animal pole (Cherr and Yanagimachi 2014). To prevent polyspermy, sturgeons rely on the cortical granule reaction, typical of most animals. Cortical granules are specialized vesicles stored beneath the oocyte cortex that are exocytosed after fertilization (Fig. 5.2c). They modify the vitelline membrane and extracellular space in a way that precludes the entry of additional sperm at this stage. Interestingly, zebrafish sperm lack an acrosome, whereas sturgeon sperm possess one (Cherr and Clark 1985; Cherr and Yanagimachi 2014). The acrosome is a large vesicle in the sperm head of many animals that is secreted upon binding to the egg. The acrosome contains enzymes that modify the vitelline envelope allowing the sperm to penetrate it and fertilize the egg. The zebrafish sperm need not have an acrosome, since the single micropyle in the zebrafish egg generates a hole in the vitelline envelope. In the sturgeon, the micropyle may not allow the sperm to directly contact the egg membrane, and therefore sturgeon sperm cells necessitate an acrosome reaction for fertilization (Cherr and Yanagimachi 2014; Morisawa 1999; Morisawa and Cherr 2002). Overall, these differences in gamete morphology and strategies for blocking polyspermy in fish are relevant to AV polarity establishment. Zebrafish *buc* mutant females produce eggs with multiple micropyles that are no longer restricted to a single pole. The relationship between Buc function (see sections below) and the multiple micropyles found in other fish is an exciting evo-devo question that awaits future studies.

5.3 Animal-Vegetal Oocyte Polarity

5.3.1 Key Events in Oogenesis

After their birth from a germ line stem cell, oocytes become polarized through the differentiation process of oogenesis. The process of oogenesis begins when a germ line stem cell divides to form an oogonial cell. Oogonia are larger than germ cell stem cells and undergo additional mitotic divisions (Fig. 5.2a). The transition of germ line stem cells into oogonia is uncharacterized in vertebrates, and its regulation is unknown. During their mitotic divisions, oogonia undergo incomplete cytokinesis, whereby individual oogonial cells remain interconnected by cytoplasmic bridges to their sister cells, thus forming a cyst (Fig. 5.2a). The early development of oocytes in a cyst is a conserved theme in animals (Pepling et al. 1999). Since cells in a cyst can share their cytoplasm or specific factors within it, it is thought that the cyst allows for their synchronized development. Oogonia divide several times, comprising the cyst, where it has been defined and is engulfed by somatic follicle cells (Leu and Draper 2010).

After their final division, oogonia initiate meiosis I. Ample evidence in the mouse establishes the retinoic acid (RA) signaling pathway as the major regulator of meiotic initiation (Anderson et al. 2008; Bowles and Koopman 2007; Koubova et al. 2014). While RA players are expressed in follicle cells of the zebrafish ovary (Rodriguez-Mari et al. 2013), such data in zebrafish and frogs are scarce. Upon initiation of meiosis, oocytes undergo a specialized and unusually long prophase. Major developmental events occur during the elongated prophase I (Fig. 5.2). The most prominent ones are the genetic process of recombination and the vast cellular growth of the oocyte. The first meiotic division is completed in late stages of oogenesis, during oocyte maturation (Fig. 5.2c). The second meiotic division is only completed after fertilization.

The cyst in which oogonia reside eventually breaks down, and individual oocytes begin folliculogenesis. Folliculogenesis involves the surrounding of individual oocytes by somatic follicle cells. Follicle cells may play an active role in the completion of cytokinesis and breakdown of the cytoplasmic bridges in the cyst, as found recently in germ line stem cells in the Drosophila testis (Lenhart and DiNardo 2015). Ultimately, several layers of follicle cells surround each oocyte, with granulosa cells in the inner layers and theca cells in the outer ones. Folliculogenesis progresses in parallel to oocyte growth.

5.3.2 Symmetry Breaking and Early Polarization During Oocyte Differentiation

Until recently, the first indication of polarity in the oocyte was the detection of the Balbiani body (Bb) at a mid-diplotene stage of prophase I or, an earlier more dispersed asymmetric localization of Bucky ball protein in zebrafish (Heim et al. 2014;

Riemer et al. 2015). It has now been shown in zebrafish that the first stages of Bb formation and oocyte polarization occur much earlier, at the onset of meiosis (Elkouby et al. 2016). In the premeiotic oogonial stages, Bb precursor components, such as Bucky ball, GasZ, and mitochondria, are distributed symmetrically in the perinuclear cytoplasm (Fig. 5.2a). However, this symmetry is broken when these precursors initiate their specific and asymmetric localization at the early meiotic zygotene bouquet stage. At these stages, oocyte polarity aligns to a nuclear polarity that is a characteristic feature of the zygotene stage (Fig. 5.2a). During zygotene, the telomeres of the chromosomes become tethered to the nuclear envelope and move and rotate actively in the nucleus, finally orienting their telomeres so they are clustered to one pole of the nuclear envelope, a configuration called the chromosomal bouquet (Scherthan 2001; Shibuya et al. 2014b). This dynamic process is mediated by microtubules and the centrosome and facilitates chromosomal pairing and meiotic recombination (Ding et al. 2007; Link et al. 2014; Sato et al. 2009; Scherthan 2001; Shibuya et al. 2014a, b).

Remarkably, Bb precursors initially localize to the cytoplasmic region that apposes the bouquet telomere cluster and contains the centrosome (Elkouby et al. 2016). Significantly, disruption of microtubules causes a loss of telomere clustering of the bouquet and a parallel loss of mitochondrial enrichment to form the Bb precursor aggregate, indicating that these processes are coordinately regulated (Elkouby et al. 2016). Furthermore, epistasis experiments showed that initial oocyte polarization was normal in *buc*$^{-/-}$ oocytes, indicating that these early symmetry-breaking events lie functionally upstream to the Bucky ball protein.

Subsequent to the bouquet stage, the Bb precursor components localize as multiple aggregates within a nuclear indentation positioned at the location of the centrosome and the presumptive former telomere cluster of the bouquet (Fig. 5.2b). We have termed this structure a nuclear cleft. The nuclear cleft then gradually rounds out, and the Bb aggregate becomes more condensed and spherical, giving rise to the typical mature Bb of mid-diplotene stages. Therefore, the centrosome and telomere cluster association at zygotene stages marks the future vegetal pole of the oocyte and is proposed to form a meiotic-vegetal center that couples oocyte patterning to meiotic genetic events (Elkouby et al. 2016).

5.3.3 Potential Upstream Regulation of Oocyte Symmetry Breaking

The question arises if further regulation upstream of the bouquet may be important in setting up the axis of polarity. The tissue organization in cysts could be important for polarization. It has been suggested that the cytoplasmic bridges that form due to incomplete cytokinesis of oogonial cell divisions in the cyst may be the sites of Bb precursor aggregation. Clusters of mitochondria have been observed in frog oogonia close to the cytoplasmic bridges (Kloc et al. 2004a). However, whether this aggregation of mitochondria is linked to the mitochondria of the mature Bb is not known.

Evidence in zebrafish suggests that the polarity axis is prepositioned by the plane of the last oogonial division (Elkouby et al. 2016). Similar to oogonia, zygotene oocytes remain organized in nests and pairs of oocytes appear interconnected by a cytoplasmic bridge that contains acetylated tubulin extending through it typical of a mitotic midbody (Fig. 5.2a). As cytokinesis completes during abscission, the midbody microtubules are normally deacetylated, which was also observed in post-zygotene stage oocytes. Interestingly the location of the midbody/cytoplasmic bridge coincides with the location of the centrosome cytoplasm of both interconnected oocytes, where the early Bb is forming (Fig. 5.2a). This suggests that polarity is predisposed to be orthogonal to the last oogonial division plane, which positions the centrosome and aligns the future AV axis. Polarization is then in effect executed with the recruitment of Bb precursors to the centrosome by the bouquet organization at zygotene stages.

Many questions remain open. It is unknown how the meiotic-vegetal center is activated at zygotene to initiate telomere clustering and recruit Bb precursors. The localization of Bb precursors required intact microtubules at the bouquet stage (Elkouby et al. 2016); however, further elaboration of the localization mechanism remains unknown. How Bb precursors continue to aggregate in the nuclear cleft and what is the role of the nuclear cleft in this process are unknown. Lastly it is unclear if follicle cells, either in the early cyst or during later folliculogenesis, contribute to polarization. Theoretically, follicle cells could affect oocytes in the cyst by regulating their division plane or express localized queues that could form signaling gradients.

5.3.4 *Bucky Ball and Macf1: Genetic Entry Points to Oocyte Polarity*

Two maternal-effect mutants identified in zebrafish, *bucky ball* (*buc*) and *macf1* (*microtubule cross-linking factor 1*), have provided the only known genes required for AV polarity establishment in vertebrates (Fig. 5.2b) (Bontems et al. 2009; Gupta et al. 2010; Marlow and Mullins 2008). In contrast to a normal egg with cytoplasm in the animal pole blastodisc and yolk at the vegetal pole, *buc* and *macf1* mutant eggs show radially distributed cytoplasm around a centrally localized yolk. Moreover, the primary defect in *buc* and *macf1* mutants is evident much earlier, specifically during the Bb localization pathway in stage I oocytes (discussed further in the next section). *buc* mutant oocytes fail to form the Bb and RNAs that are normally vegetally localized are instead unlocalized and remain dispersed in the cytoplasm. In contrast, in *macf1* mutants the Bb forms and RNAs localize to it; however, they fail to anchor to the prospective vegetal cortex, and instead they remain in an apparent persisting Bb (Gupta et al. 2010). Thus, Buc and Macf1 provide a molecular link to the early processes that establish AV polarity in the oocyte. The function of these proteins is discussed further in Sect. 5.5 below.

5.4 Mechanisms of RNA Localization in the Oocyte

Once AV polarity is established, it will ultimately define the sperm entry site, the embryonic axes, and underlie the mechanism of germ cell specification. Throughout oogenesis, dorsal determinants and germplasm components distribute within distinct compartments of the oocyte reflecting its AV polarity. Early studies in *Xenopus* identified several vegetally localized RNAs and determined their localization dynamics during oogenesis (Cuykendall and Houston 2010; Houston et al. 1998). Among them, *Xcat/nanos* (Forristall et al. 1995; Mosquera et al. 1993) and *Vg1/gdf1* (Yisraeli and Melton 1988) became models for unraveling RNA localization mechanisms. *nanos* RNA localizes to the Bb in stage I oocytes and then translocates via the Bb to the vegetal cortex, where it becomes docked during Bb disassembly at the end of stage I in zebrafish (stage II in frog) of oogenesis. On the other hand, *Vg1* localization to the vegetal pole begins during stage III and culminates in anchoring to the vegetal cortex by stage VI in frog (Mowry and Cote 1999). The localization patterns of *nanos* and *Vg1* became signatures for defining two pathways of RNA localization, an early pathway for RNAs that are transported via the Bb (also called the METRO pathway) (Kloc and Etkin 1995; Wilk et al. 2005) and a later pathway for RNAs, like *Vg1*, that localize after the Bb disassembles (Mowry and Cote 1999). These two pathways are further distinct mechanistically in that the early pathway is not known to require the cytoskeleton, whereas the late pathway requires microtubules for successful localization of RNAs to the vegetal pole.

Although zebrafish and frog oocytes use similar pathways of RNA localization, they also show differences in the localization patterns for some RNAs. For example, *nanos* RNA localizes to the Bb initially, but in zebrafish it becomes unlocalized in stage II oocytes after Bb disassembly (Kosaka et al. 2007). Another example is *Vg1* RNA, which localizes in stage III oocytes to the vegetal pole in *Xenopus*, while in zebrafish it is docked to the animal pole (Bally-Cuif et al. 1998; Marlow and Mullins 2008). Further, the RNA of the *bucky ball* homolog in frog *Xvelo* does not localize to the Bb in stage I but is vegetally localized later in oogenesis (Claussen and Pieler 2004; Nijjar and Woodland 2013). In zebrafish, *bucky ball* localizes in stage I oocytes to the Bb and vegetal cortex after Bb disassembly and then reappears at the animal pole in later stages (Bontems et al. 2009). These differences open new questions regarding the evolutionary modifications to the RNA localization processes in these organisms, which likely reflect evolutionary changes in the location of the embryo blastomeres relative to the vegetal pole and yolk (yolk is separated in zebrafish vegetally from, or in frog is integral to, the blastomeres).

In frogs and zebrafish, the early pathway, also known as the METRO pathway, functions during stage I of oogenesis and relies on the Bb to translocate RNAs to the oocyte vegetal cortex (Kloc and Etkin 1995; Kloc et al. 1996; Kosaka et al. 2007; Marlow and Mullins 2008). Transcripts that are localized via the Bb include *nanos*, *dazl*, *vasa*, *syntabulin*, *grip2*, *xlsirt*, among others (Cuykendall and Houston 2010; Ge et al. 2014; Houston 2013; Kosaka et al. 2007; Marlow and Mullins 2008; Nojima et al. 2010). These RNAs localize to the Bb, translocate

with it to the oocyte cortex, where the Bb disassembles and the transcripts become anchored to the cortex. Many of the RNAs localized through the early pathway will constitute the germplasm during early development but also include developmental patterning factors (Forristall et al. 1995; Kloc and Etkin 1995; Yisraeli et al. 1990). In the late pathway, RNAs like *Vg1* in frog (Cote et al. 1999), and *bruno-like* and *mago nashi* in zebrafish (Kosaka et al. 2007), localize to the vegetal pole in stage III of oogenesis.

5.4.1 The Early Pathway: Rue Balbiani

The delivery of the early pathway mRNAs to a restricted region of the oocyte cortex specifies this region as the vegetal pole, and their localization is executed by the Balbiani body (Bb). The mature Bb is first evident at a perinuclear position during mid-diplotene stages of oogenesis. At this stage the Bb is loaded with its specific mRNAs and aggregated mitochondria. The Bb ultimately docks at the oocyte plasma membrane, where it disassembles and its content is unloaded (Fig. 5.2b). The unloaded RNPs are then anchored to this region of the cell membrane, defining it as the oocyte vegetal pole.

The Bb is a universally conserved structure found in oocytes from insects (Cox and Spradling 2003; Jaglarz et al. 2003), arthropods (Jedrzejowska and Kubrakiewicz 2007), fish (Marlow and Mullins 2008), frogs (Dumont 1978), birds (Carlson et al. 1996; Rodler and Sinowatz 2013; Ukeshima and Fujimoto 1991), rodents (Pepling et al. 2007; Weakley 1967), and primates (Barton and Hertig 1972) to humans (Albamonte et al. 2013; Hertig 1968). In all these species, two common themes of the Bb are apparent: (1) the aggregation of mitochondria and electron-dense material, presumably corresponding to mRNA, and (2) the localization of the aggregate at a perinuclear position. In fact, in the original discovery of the Bb in 1845 (Hertig 1968), it was described as "a yolk nucleus." In mammals, the Bb is present in early oocytes, though it is not known if it polarizes the early mammalian oocyte or if it is related to the asymmetric formation of the antral cavity or to the asymmetries that are established during oocyte maturation (discussed above).

Among vertebrates, the Bb has been most studied in *Xenopus* and zebrafish. Only a few components of the Bb have been identified. These include germline-specific mRNAs (like *dazl, vasa,* and *nanos* (Kosaka et al. 2007; Mosquera et al. 1993; Zhou and King 1996a)) and mRNAs encoding dorsal determinants and its localizing machinery (like *wnt8*, *grip2a*, and *syntabulin*) (Colozza and De Robertis 2014; Ge et al. 2014; Kirilenko et al. 2008; Lu et al. 2011; Nojima et al. 2010; Tarbashevich et al. 2007), as well as mRNA-binding proteins (like Rbpms2 (Hermes) (Kosaka et al. 2007; Song et al. 2007)) and RNP scaffold proteins (like GasZ (Marlow and Mullins 2008; Yan et al. 2004; Yano et al. 2004)). Zebrafish females mutant for the Bb-localized transcripts *grip2a* (*hecate*) (Ge et al. 2014) or *syntabulin* (*tokkaebi*) (Colozza and De Robertis 2014; Nojima et al. 2010) show normal Bb formation and overall AV polarity, although they display a maternal-effect mutant ventralization defect in their embryos.

Vegetal pole specification, as executed by the Bb, is essential for embryonic development, as indicated by the *bucky ball* (*buc*) mutant in zebrafish (Fig. 5.2b) (Bontems et al. 2009; Heim et al. 2014; Marlow and Mullins 2008). *Buc* oocytes fail to form the Bb; Bb mRNAs are not localized to the oocyte cortex and consequently the vegetal cortex is not specified. Instead, animal mRNAs are expanded radially, and these oocytes develop forming a radial animal pole. Instead of formation of a single micropyle, the animal specialized structure that enables sperm entry, *buc* mutant eggs form multiple micropyles. Another consequence of the radial animal fate is that embryonic cell divisions occur radially around the yolk, instead of restricted to a defined animal pole. This phenotype is catastrophic to embryogenesis, and embryos die at cleavage stages (Dosch et al. 2004; Marlow and Mullins 2008).

5.4.2 Early Balbiani Body-Dependent RNA Localization

Few proteins have been implicated in localizing mRNAs to the Bb. Indeed Rbpms2 (Hermes) is the only one known to play such a role during oogenesis. In frogs and fish, Rbpms2 was shown to localize to the vegetal cortex through the early Bb pathway and interact with vegetally localized RNAs (Kosaka et al. 2007; Marlow and Mullins 2008; Zearfoss et al. 2004). In frogs, Rbpms2 binds to *nanos* (formerly called *Xcat-2*) and *Xvelo* (*buc* homolog) RNAs among others (Nijjar and Woodland 2013; Song et al. 2007; Zearfoss et al. 2004), whereas in zebrafish it has only been shown to bind with *buc* RNA (Heim et al. 2014). Similarly, in both species Rbpms2 was shown to interact with Buc/Xvelo protein (Heim et al. 2014; Nijjar and Woodland 2013).

Much more is known about the cis-acting Bb localizing elements (LE) in various mRNAs. The first LE that was identified was the mitochondrial cloud (Bb) localizing element (MCLE) in the 3′UTR of the *Xenopus nanos* mRNA (Zhou and King 1996a). Deletion analysis showed that 250 nucleotides (nts) in the proximal end of the 3′UTR is required for *nanos* localization to the Bb. Specifically, six repeats of the motif UGCAC within this region are required for its Bb localization (Zhou and King 1996b). Within the UGCAC, the CAC sequence was proposed as a core motif, required generally for localizing mRNAs. It was proposed that variations in the surrounding sequences facilitate specific protein binding and/or activities that would drive specific localization (Betley et al. 2002). Indeed, CAC motifs were also required for Xpat and Xnif mRNA localization to the Bb (Betley et al. 2002; Claussen and Pieler 2004).

Interestingly, the *nanos* MCLE motif can drive a reporter to localize to the Bb in zebrafish oocytes (Kosaka et al. 2007). However, endogenous zebrafish Bb mRNAs, like *dazl*, *vasa*, and *nanos,* contain no or a few sparse MCLE sequence motifs, and even these are found outside the region required for localization of these transcripts to the Bb (Kosaka et al. 2007). A similar endogenous MCLE sequence therefore is unlikely to localize these zebrafish transcripts to the Bb. Deletion analysis of the zebrafish *dazl* mRNA revealed a 375nt fragment in the

proximal end of its 3′UTR that is required for Bb localization (Kosaka et al. 2007). Interestingly, another fragment was required not for *dazl* Bb localization but for its later vegetal localization in the egg, probably necessary for its anchoring to the cortex. It was therefore revealed that distinct 3′UTR sequences mediate *dazl* localization at specific stages (Kosaka et al. 2007).

Further studies in *Xenopus* addressed the dynamics of recruitment of injected mRNAs into the mature Bb. In these experiments, fluorescently labeled *nanos* and *Xdazl* mRNA were injected into oocytes and their distribution tracked by live imaging (Chang et al. 2004). The injected *nanos* and *Xdazl* accumulated significantly within the Bb after 9–17 h. Another study reported consistent accumulation in 6–18 h of an injected fluorescently labeled *nanos MCLE* 3′UTR fragment to the Bb (Heinrich and Deshler 2009), which depended on the MCLE UGCAC sequences (Chang et al. 2004). Interestingly, FRAP analysis of the injected *nanos* and *Xdazl* RNAs localized to the Bb revealed no to very low recovery of signal after bleaching a region of the Bb (Chang et al. 2004). While photobleaching a non-Bb cytoplasmic region recovered completely in 3 min, recovery from photobleaching within the Bb only reached the low cytoplasmic levels after almost 6 h. Additionally, when Bb regions were bleached, no movement of localized mRNAs from non-bleached Bb areas to the bleached area was detected. Importantly, these results suggested that mRNAs localize to the Bb by a "diffusion and entrapment" mechanism (Chang et al. 2004). According to this model, Bb mRNAs freely diffuse in the cytoplasm until they randomly encounter the Bb. When they reach the Bb, the mRNAs are trapped in its dense meshwork of organelles. This RNA localization mechanism was shown to be microtubule independent (Chang et al. 2004).

These results suggest that the Bb is almost a quiescent structure. Both recovery of bleached mRNAs within the Bb, and their initial accumulation in the Bb took hours to a day. Therefore, the dynamics of mRNA components within the Bb, as well as the turnover of these components between it and the cytoplasm, are extremely slow. These experiments were performed on oocytes with a well-developed Bb. In earlier stages of Bb formation, however, when Bb components are first brought together, delivered, and packed, the dynamics of the Bb may be very different.

5.4.3 The Late Pathway: Bb-Independent RNA Localization

The late vegetal mRNA localization pathway during stages III–V in *Xenopus* depends on microtubules (MTs) that radiate from a dense perinuclear array to the cortex in continuous filaments, and by stage VI oocytes display a perinuclear cap enriched in tubulin at the vegetal side of the nucleus (Gard 1991). The molecular mechanisms that drive RNA localization are better understood for the late pathway, in particular for the *Xenopus Vg1* RNA (Fig. 5.2c). Interestingly, in zebrafish *Vg1* (*gdf3*) RNA localizes to the animal pole, instead of the vegetal pole of stage III oocytes. Studies in frog oocytes show that a 340-nt localizing element (LE) in the 3′UTR of *Vg1* contains repetitive sequence motifs necessary for the vegetal

localization of injected *Vg1* RNA. These motifs are the sequences UUUCU (VM1) and UUCAC (E2) that are necessary also for localization of RNAs through the early pathway. The clustering of E2 and/or VM1 motifs might be important for LE recognition (Betley et al. 2002; Choo et al. 2005; King et al. 2005).

RNA localization ultimately relies on RNA-binding proteins that bind to the LE and target it to the vegetal oocyte cortex. Studies in frog have identified a number of proteins that bind the *Vg1* RNA. Among these Igf2bp3 (also called Vg1RBP and Vera) and Ptbp1 (also called VgRBP60 and hnRNPI) were shown to bind the *Vg1* LE and function in *Vg1* localization. Whereas Ptbp1 binds to Vg1 through the VM1 motifs of the LE, Igf2bp3 does so through the E2 sites (Lewis et al. 2004). Mutations in the *Vg1* LE affect Igf2bp3 and Ptbp1 binding and disrupt *Vg1* localization (Deshler et al. 1997, 1998; Havin et al. 1998).

The proteins that bind and localize RNAs first associate with the RNA either in the nucleus or the cytoplasm to form the RNP transport complex. Igf2bp3 and Ptbp1 associate with Vg1 and VegT RNAs in the nucleus, and the RNP complex is then exported to the cytoplasm (Kress et al. 2004). The RNA-binding protein Staufen, first identified in flies to play a role in RNA localization (Brendza et al. 2000, 2002), was shown in frogs to bind *Vg1* and Kinesin I in the cytoplasm (Yoon and Mowry 2004). Moreover, expression of mutated forms of Staufen, lacking two out of five RNA-binding domains and the tubulin-binding domain, disrupts Vg1 localization and suggests that Staufen may be a functional link between the RNA and the MT transport machinery required for localizing RNA (Micklem et al. 2000; Yoon and Mowry 2004).

Little is known in zebrafish about the mechanisms and RNA-binding proteins (RBPs) acting in localizing RNAs through the late pathway. As in frogs, Staufen and Ptbp1are expressed during oogenesis (Bateman et al. 2004; Mei et al. 2009). Staufen depletion in embryos causes defects in PGC migration and survival (Ramasamy et al. 2006; Yoon and Mowry 2004); however, its function in RNA localization in oogenesis has not been tested. Ptbp1a/Brom bones functions in egg activation, where it is required for IP3-mediated Ca^{2+} release in egg activation. A second copy of the *ptbp1* gene, *ptbp1b,* was found in zebrafish that is also expressed during oogenesis, suggesting the possibility that these genes act redundantly in RNA localization (Mei et al. 2009). Further studies are required to address specifically the function of these RBPs in RNA localization during zebrafish oogenesis.

The current understanding of the RNA localization process in *Xenopus* oocytes suggests that early and late pathways may not represent two independent events, but rather a continuum mechanism of RNA localization. Interestingly, when RNAs that localize through the early pathway are injected into stage III oocytes in *Xenopus*, they can still localize to the vegetal cortex (Choo et al. 2005; Claussen et al. 2004; Zhou and King 1996a). Moreover, the late pathway *Vg1-LE* when injected into stage I oocytes can localize to the Bb (Choo et al. 2005). Importantly, these RNAs require the same motifs in their 3′UTRs for early and late pathway localization, suggesting that the RNA-protein machinery functioning in the late pathway may be similar to that used in the early pathway (Choo et al. 2005; Claussen et al. 2004).

Technologies like the MS2 RNA-labeling system are helping to elucidate the real-time dynamics of RNA localization in different contexts. The MS2 system

was originally developed in yeast for live imaging of endogenous *ash1* mRNA asymmetric localization to the bud tip (Bertrand et al. 1998). The MS2 system is composed of two elements: the MS2 coat protein from bacteriophage (MCP) fused to GFP and multiple MCP-binding sites composed of stem-loops usually inserted within the 3′UTR of the RNA target. Moreover, the technology was adapted to study live dynamics of endogenous RNAs in oocytes. In flies, live tracking of an engineered *nanos-ms2* RNA revealed that *nanos* localizes to the oocyte posterior through a diffusion and entrapment mechanism (Forrest and Gavis 2003). In stage III frog oocytes, by injecting RNAs for the MS2 protein and *Vg1*-LE-ms2, the transport dynamics of *Vg1*-LE to the vegetal pole and the role of molecular motors in this process were characterized (Gagnon and Mowry 2010). Following a similar strategy, the MS2 system was recently adapted in zebrafish showing the localization of injected *nanos-ms2* RNA to PGCs in the embryo, as revealed by MS2-GFP provided by a transgene (Campbell et al. 2015a). Similar strategies will be possible to use to visualize the dynamics of endogenous RNAs in the early Bb localization pathway in vertebrate oogenesis, which has so far been inaccessible to this type of study.

5.5 RNA Localization and AV Polarity: An Intimate Connection

The identification of *bucky ball* and *macf1* (*magellan*) genes demonstrated that Bb formation and disassembly, respectively, is essential to defining the AV axis in vertebrates. In *buc* mutants, the Bb is absent, and RNAs that normally localize to the Bb and vegetal cortex appear diffuse in the cytoplasm. In *macf1* mutants the Bb forms and RNAs localize to it; however, disassembly of the Bb is defective, and the Bb-localized RNAs never become anchored to the oocyte cortex. In both mutants, the oocytes develop into eggs lacking polarity, showing a radial distribution of the cytoplasm with no recognizable blastodisc. *buc* and *macf1* are the only known genetic entry points to studying oocyte polarity in vertebrates; hence, understanding their functions is fundamental to deciphering the molecular mechanisms of AV polarity establishment.

5.5.1 Bucky Ball: The Balbiani Body Gene

The *buc* transcript and protein localize to the Bb in zebrafish and, after Bb disassembly, to the oocyte vegetal cortex (Fig. 5.2a and b) (Heim et al. 2014; Riemer et al. 2015). Furthermore, Buc protein localizes to the germplasm in the early embryo (Fig. 5.1c) (Riemer et al. 2015), where it can increase PGC number in overexpression experiments (Bontems et al. 2009). So far, two Buc-interacting proteins

have been reported: Rbpms2 (Hermes) (Heim et al. 2014) and Kinesin-1 (Campbell et al. 2015a). Rbpms2 is postulated to function with Buc in Bb formation, whereas Kinesin-1 plays a distinct role in germ cell formation. Hence, Buc function connects the early stages of AV polarity and RNA localization with early developmental events of germ line specification.

Studies of the *buc* gene structure show the importance of the introns and 3′UTR to its function in AV polarity establishment. Several transgenes were generated containing either all or none of the *buc* gene introns (*gbuc* or *intronless-buc*, respectively), with a full-length or truncated 3′UTR (Heim et al. 2014). Analysis of eggs from *buc* mutant females carrying a single copy of a transgene showed that the egg AV polarity defect is rescued in a higher ratio using *gbuc* containing the full-length 3′UTR than the truncated version. These results suggest that the *buc*-3′UTR plays a regulatory role, possibly in its localization or translation; however, it is not essential, since some embryos were rescued to wild type. In *buc* mutants, the *buc-intronless* transgenes either with the full-length or truncated 3′UTR could not rescue egg or oocyte polarity. In fact, these *buc-intronless* transgenes induced a partially penetrant dominant-negative (DN) effect, producing oocytes or eggs with no AV polarity. This DN effect is observed in a *buc* heterozygous or wild-type background, with either *buc-intronless* carrying the full-length or truncated 3′UTR.

In another transgene construct where *buc-intronless* carries the original buc^{p106} mutation (*bucp106-intronless*) (Marlow and Mullins 2008; Bontems et al. 2009), it fails to rescue *buc* mutants (Heim et al. 2014), as expected. However, unexpectedly this transgene, when homozygous, induces a DN effect in females. Furthermore, a *buc-intronless* transgene in a wild-type background can cause oocyte polarity defects and formation of what appears to be supernumerary Bbs (Heim et al. 2014). The protein and RNA constituents of these Bbs have not yet been determined, but they look like typical Bbs by histological analysis. Additional studies will be needed to determine the nature of these ectopic Bbs: for example, if they arise due to incomplete coalescence of Bb precursors, to formation of new Bbs at distinct time points, or to fragmentation of a Bb. It will also be interesting to determine if these supernumerary Bbs disassemble at the cortex as in wild-type oocytes or if some or all do not, or do so abnormally leading to the AV egg defects observed in these mutants. If these supernumerary Bbs can disassemble and dock their contents to the cortex, one would expect it to lead to an expansion of vegetal pole identity, possibly at the expense of animal identity, the opposite phenotype to a *buc* loss-of-function mutant oocyte.

The molecular cause of the DN effect of the *buc-intronless* transgenes remains to be determined. It is possible that the *buc* transcript lacking introns fails to recruit the exon junction complex (EJC) or other factors within the nucleus that could regulate *buc* translation or RNA localization. In a wild-type background, *buc-intronless* may lead to an excess of Buc protein, with this imbalance causing the formation of ectopic Bbs and ultimately affecting oocyte polarity. As for the DN effects observed in *bucp106-intronless* homozygous transgene, one could think of a scenario where the imbalance is produced by titration of potential translational regulatory machinery, hence lowering the levels of Buc protein to suboptimal for Bb formation and as a consequence leading to

the same *buc* mutant polarity defect. Overall, however, the cause of the defects induced by the different *buc* transgenes on AV polarity is still unclear.

A new buc-GFP transgenic line containing the entire *buc* locus was recently reported that faithfully recapitulates Buc localization and function (Riemer et al. 2015). Like the endogenous Buc protein, Buc-GFP localizes to the Bb in early stage I oocytes and after Bb disassembly, it spreads at the vegetal cortex. After fertilization, Buc-GFP accumulates along with the germplasm at the ends of the cleavage furrows of 2- and 4-cell stage embryos. Surprisingly, Buc-GFP and endogenous Buc can be detected up to 48 hpf in germ cells, colocalizing with Vasa in perinuclear granules. More importantly, *buc* mutant females carrying the *buc*-GFP transgene lay eggs that develop into wild-type embryos, suggesting that Bb formation and RNA localization during oogenesis is rescued. The transgenic line reported in this study (Riemer et al. 2015) holds promise to address several aspects of Buc function in the oocyte and early embryo, offering the possibility to perform live imaging experiments and track Buc behavior during germplasm segregation in the Bb and accumulation at the cleavage furrows in the early embryo and to explore its role in germ cell granules in the embryo.

It is tempting to speculate that Buc may function and be regulated by mechanisms similar to those for the GP and oocyte posterior pole regulator Oskar (Osk) in Drosophila. In flies, *osk* mutant females produce embryos that lack PGCs and fail to form abdominal segments (Ephrussi et al. 1991). Like many other localized mRNAs, *osk* RNA relies on 3′UTR motifs for its proper localization, as well as a unique stem-loop sequence within the coding region, and splicing of the first intron. The first intron splicing assembles the EJC next to the stem-loop, which together are required for *osk* mRNA transport and posterior pole localization (Ghosh et al. 2012). The *buc-intronless* transgenes suggest the importance of the introns in *buc* regulation or function. However, additional studies will be required to determine the nature of the intron requirement, whether it be for *buc* RNA localization via EJC assembly as with *osk* or instead due to transcriptional regulatory elements localized within an intron, or to facilitate mRNA transport out of the nucleus, or another mechanism.

Osk and Buc may have some homologous functions in GP assembly. In Drosophila at the oocyte posterior pole, *osk* RNA is translated, where accumulation of Osk protein enhances the recruitment of GP components that specify the germ line (Ephrussi and Lehmann 1992; Glotzer et al. 1997; Jenny et al. 2006; Markussen et al. 1995; Staudt et al. 2005). Heim et al. (2014) proposed a similar mechanism in zebrafish for Buc, whereby asymmetric/localized translation of *buc* leads to the recruitment of Bb-localized RNAs, including *buc* RNA, which further produces Bb-localized Buc protein in a positive feedback mechanism of Bb component entrapment and hence Bb formation. One major difference between Osk and Buc is that Osk can bind GP RNA components directly, whereas Buc is not known to do so and may rely on other interacting proteins to assemble RNA into the GP, like Rbpms2. Alternatively or in addition, a critical step in oocyte polarity may be Buc protein aggregation. In early stage I oocytes, Buc protein is asymmetrically localized but not yet in a single aggregate (Elkouby et al. 2016; Heim et al. 2014), suggesting that a factor may mediate Buc aggregation or that Buc must accumulate to a particular level before it aggregates into a single bolus as the Bb.

Kinesin-1 has been identified to interact with Buc protein and shown to function in the germ line of the embryo. Kinesin-1 (Kif5Ba) acts in the accumulation of GP to the cleavage furrows in the 4-cell stage embryo (Campbell et al. 2015b). Kif5Ba is required for the recruitment of Buc to the cleavage furrows along with several RNA components of the GP. Consistent with this important role in GP assembly, *kif5ba* mutants do not form PGCs and develop into sterile adults. Interestingly, *kif5ba* RNA does not localize to the Bb in stage I oocytes and the *kif5ba* mutant does not cause defects in oocyte polarity (Campbell et al. 2015b). Thus, Buc remains as the only known factor to have GP assembly functions in the oocyte and early embryo.

5.5.2 *Macf1: Cytoskeleton Integration Underlies AV Polarity Establishment*

Microtubules (MT), actin, and intermediate-filament (IF) cytoskeletal components are all detected in different compartments during oocyte progression. Cytokeratins, for instance, are detected first in the Bb (Gard et al. 1997) and in the cytoplasm as oogenesis progresses in *Xenopus*. On the other hand, polymerized MTs are not detected in the Bb and show intricate cytoplasmic networks in late-stage *Xenopus* oocytes (Gard 1991, 1992, 1999; Gard et al. 1995). Actin distributes cortically and is present also intranuclearly (Gard 1999; Gupta et al. 2010; Marlow and Mullins 2008). Microtubule actin cross-linking factor 1 (Macf1), a member of the spectraplakin family, functionally integrates the cytoskeleton and is required for maintaining the Bb structure and for guiding the Bb and associated RNAs to the cortex for vegetal anchoring (Fig. 5.2b) (Gupta et al. 2010). Paradoxically, disruption of MT and actin filaments in early stage I oocytes does not affect Bb structure nor the localization of RNAs to the Bb through the early localization pathway. Nevertheless, Macf1 function suggests that the cytoskeleton is relevant for the early pathway of RNA localization. Thus, elucidating Macf1 function and the cytoskeleton-dependent mechanism of RNA localization will shed light into the unknown processes of cytoskeleton integration that are required for AV polarity establishment (Gard et al. 1997).

The functions of spectraplakins in cell polarity have been reported in a variety of cell types (Alves-Silva et al. 2012; Applewhite et al. 2010; Bottenberg et al. 2009; Karakesisoglou et al. 2000; Kodama et al. 2003; Lin et al. 2005; Sanchez-Soriano et al. 2009; Sonnenberg and Liem 2007; Wu et al. 2008, 2011). Shot, the Macf1 homolog in flies, is required for the organization of the fusome and for oocyte progression. The fusome is a membranous structure that extends between the cytoplasmic bridges of the 16 cells of the oogonial cyst and which functions in oocyte specification (Roper and Brown 2004). Shot localizes to the fusome and functions to recruit MT for the transport of factors, including centrosomes, toward the oocyte. Shot in Drosophila probably acts much earlier during oogenesis than Macf1 in zebrafish, since the *macf1* mutant phenotype is visible only around mid-stage I of oogenesis, long after the oogonial cyst stage (Gupta et al. 2010).

Spectraplakins are difficult to address functionally, since they are large proteins encoded by long transcripts with a variety of isoforms that challenge traditional cloning and transgenic techniques. Moreover, their function relies on the presence of specific functional domains that are cell type and context dependent. Fortunately, genome-editing approaches offer new possibilities to study them, including in zebrafish, where *macf1* loss of function does not cause embryonic lethality like in mammals (Kodama et al. 2003). Thus, zebrafish oocytes offer a unique possibility to interrogate the requirement of Macf1 single modules in cell polarity. Such studies will help to understand the relationship between the cytoskeleton, the Bb and the RNA localization machinery that together drive AV polarity establishment during early oogenesis.

An interesting question is how the translocation of the Bb to the future vegetal pole is coordinated with the simultaneous growth of the oocyte. Between the point when the mature Bb is first detected and the time the Bb reaches the future vegetal cortex, docks, and disassembles, the oocyte is at least twofold larger (Fig. 5.2). This growth involves increasing the cytoplasmic volume and expanding the cytoplasmic membrane. How these changes affect the organization of cytoskeletal or other cellular features that lead to Bb vegetal localization and disassembly is unknown. Understanding the developmental context of oocyte differentiation will be important to dissecting its polarization mechanisms, including tracing early stages of Bb formation as well as analyzing its translocation, docking, and disassembly at the future vegetal pole.

5.6 The Animal Pole Localization of RNAs

At the animal pole, the egg is fertilized in frogs and fish and the blastodisc forms in zebrafish. Like at the vegetal pole, RNAs localize to the animal pole during oogenesis, though they localize after the Bb disassembles, suggesting that vegetal pole identity is acquired first. In zebrafish, RNAs like *cyclin B1* (*cycB*) and *pou5f3* (previously called *pou2*) localize to the animal pole in stage II and early stage III, respectively, remaining there in a cortical and tight distribution throughout oocyte maturation (Fig. 5.2c) (Howley and Ho 2000). Interestingly, *Vg1* RNA in zebrafish localizes to the animal pole right below the micropyle (Marlow and Mullins 2008), unlike in frogs where Vg1 localizes to the vegetal pole (Mowry and Melton 1992). As stated earlier, the lack of vegetal pole identity in zebrafish *buc* mutants causes the formation of multiple micropyles and a radial animal identity with no recognizable blastodisc in the egg (Dosch et al. 2004; Marlow and Mullins 2008). Similarly, *pou5f3* and *cycB* RNAs show a radial localization in *buc* mutant stage III oocytes, indicating that Buc defines the egg AV axis by also restricting the localization of animal RNAs in the oocyte.

In stage IV, zebrafish oocytes undergo maturation. Through this process, oocytes progress from prophase of meiosis I (MI) to metaphase of meiosis II (MII), extruding the first polar body at the animal pole. Several changes occur during this pro-

cess, including GV migration to the animal pole and translation of RNAs promoting meiotic progression (Fig. 5.2c). Before the oocyte matures, animally localized RNAs like *cycB* are translationally repressed. CycB protein is required during oocyte maturation, along with Cdc2 kinase, as part of the maturation-promoting factor (MPF) that drives meiotic progression (Kondo et al. 1997; Masui 1972; Nagahama and Yamashita 2008). Thus, *cycB* mRNA localization to the animal pole is important for CycB localized translation and activity, which allows, in conjunction with other factors, oocyte meiotic progression and competence.

Interestingly, *cycB* localization is regulated both by its 3′UTR as well as sequences within its coding region (Yasuda et al. 2010). In a transgenic reporter assay, the cycB 3′UTR is sufficient for localization to the animal pole in stage III zebrafish oocytes. However, localization is transient, and by stage IV of oogenesis, the reporter RNA is no longer localized. It was determined that a 9 bp sequence within the ORF is required for its stable cortical localization. Importantly this sequence is conserved in vertebrate *cycB* genes suggesting its conserved function (Yasuda et al. 2013). This 9 bp sequence also functions in mediating the precise timing of *cycB* translation, since when it is mutated, the reporter is translated prematurely following oocyte maturation induction (Yasuda et al. 2013).

In frogs, the timing of *cycB* translation is regulated by two RNA-binding proteins, the cytoplasmic polyadenylation element-binding protein (CPEB) and Pumilio 1 (Pum1) (Mendez and Richter 2001; Nakahata et al. 2001; Pique et al. 2008). While CPEB-dependent polyadenylation leads to recruitment of *cycB* RNA into polysomes, Pum1 regulates the timing of its translation. Thus, RNAs containing CPEB are translated at different times during maturation. Pum1 functions in the assembly of *cycB* RNA granules that are translationally silent before maturation. In zebrafish and mouse oocytes, *cycB* RNA granules disassemble after oocyte maturation induction, which coincides with the onset of CycB protein synthesis. Failure of Pum1 binding to the *cycB* 3′UTR causes precocious translation of CycB protein in maturing oocytes. In addition to Pum1 binding, actin filaments are also required for *cycB* translational timing during maturation. Actin filaments regulate RNA granule assembly and disassembly dynamics and in doing so affect the timing of *cycB* RNA translation (Kotani et al. 2013; Yasuda et al. 2010).

5.7 The Hydrogel and the Intrinsically Disordered Proteins Theory

The Bb is a specialized massive mRNP granule. Many different types of mRNP granules exist across cell types and species. These include P granules in *C. elegans* oocytes and embryos, germ granules in zebrafish and mice germ cells, the chromatoid body in mouse spermatids, polar granules in Drosophila oocytes, neuronal granules, stress granules, and P bodies. A common theme to all mRNP granule types is the tight clustering of mRNA and proteins, which is distinct from other soluble

material in the cytoplasm, and the lack of a membrane around the granule, which together provide partial isolation but also allows continued interaction with the cytoplasm.

How are mRNP granule components maintained separated from the cytoplasm? One emerged strategy is the utilization of a hydrogel granule scaffold. A hydrogel can form by polymerization of substrate molecules that generate a meshwork with a viscosity similar to that of glycerol (Brangwynne et al. 2009a; Han et al. 2012; Toretsky and Wright 2014). The hydrogel is thus less soluble than the generally more liquid cytoplasm (Brangwynne et al. 2009a; Han et al. 2012; Toretsky and Wright 2014). The hydrogel's viscous meshwork can provide a scaffold to hold the mRNP granule content together (Brangwynne et al. 2009a; Han et al. 2012; Toretsky and Wright 2014). Such a condensation of the granule material would keep it physically separated from the remaining liquid cytoplasm. This separation, however, needs to be partial, since mRNP granules have the capacity to both assemble and disassemble under certain conditions. Therefore, the physical separation of granules that enables their function must be balanced with the ability of cytoplasmic factors to interact with the granules for both their trafficking and disassembly at the right time and place. Indeed, the phosphorylation state of the hydrogel-forming molecules can regulate their condensation-dissolution state (Wang et al. 2014).

One type of molecule that can form hydrogels is the nucleoporin family of proteins that build the nuclear pore complexes (NPC). Nucleoporins contain multiple FG repeat domains. The FG domains of nucleoporins in the NPC are found as looping extensions from the NPC into the vicinity of the pore, forming a complex meshwork. It has been demonstrated that this meshwork of nucleoporins actually forms a hydrogel that functions as a barrier (Hulsmann et al. 2012). Nuclear export/import factors are thus required to interfere with the hydrogel bonds to allow for passage of their cargo. Indeed, specific nucleoporins were able to form hydrogels in vitro, which depended on the FG repeat domains (Frey et al. 2006).

For *C. elegans* embryonic germplasm P granules, important studies have demonstrated the mechanism for the formation and dissolution of these mRNP granules (Brangwynne et al. 2009a; Wang et al. 2014). Embryonic P granules segregate along the anteroposterior axis in early cleavage embryos to specify the germ line cell. The segregation of P granules to the posterior of the embryo was shown to be a result of a condensation-dissolution gradient (Brangwynne et al. 2009b). In the posterior, granule components are allowed to condense and phase separate into a liquid droplet glycerol-like consistency, forming granules. In the anterior, however, conditions favor the dissolution of granule components into the cytoplasm and therefore granule disassembly. The conditions regulating the condensation/dissolution states turned out to be protein phosphorylation (Wang et al. 2014).

The substrate forming the hydrogel-like structure in P granules was found to be the MEG family of proteins (Wang et al. 2014). The MEG proteins are intrinsically disordered proteins (IDPs), i.e., proteins that contain long stretches of residues that do not autonomously fold into any specific structure but remain random in orientation. In high concentration, IDPs have been suggested to promote the phase separation of mRNP granules (Toretsky and Wright 2014), similar to the FG repeats. Direct phosphorylation

of MEG-1 and MEG-3 by the MBK-2/DYRK kinase led to dissolution of granules, whereas their dephosphorylation by the phosphatase PP2A induces their condensation (Wang et al. 2014). Indeed, MBK-2/DYRK is localized to the anterior half of the embryo, while PP2A is ubiquitously distributed. This results in the condensation/dissolution gradient of P granules along the anteroposterior axis (Wang et al. 2014).

Intriguingly, the Bucky ball protein, which is required for Bb formation, is an IDP (Jamieson-Lucy and Mullins, unpublished data) (Bontems et al. 2009; Toretsky and Wright 2014). Since the Bb is a large aggregate of mRNP granules, and given the conservation of a hydrogel-like structure of mRNP granules, it is tempting to speculate that Bucky ball functions as a polymerizing substrate, providing the scaffold upon which different Bb components amass. Buc and other Bb precursors are initially enriched in a region around the centrosome at zygotene stages, and aggregation of the Bb precursors into the mature Bb occurs in the nuclear cleft at subsequent stages (Elkouby et al. 2016). Interestingly, Bb mitochondria initially localize normally in the nuclear cleft in *buc*$^{-/-}$ oocytes and only disperse later, suggesting that oocyte polarity is initiated normally in the absence of Buc (Elkouby et al. 2016). It is possible that Buc accumulation over a specific threshold in the nuclear cleft may be required for Bb precursor condensation into a single aggregate granule. This would argue for a scaffolding role of Buc that is similar to the C. elegans MEG proteins. It will be important in the future to identify potential regulators of Buc condensation-dissolution, possibly by controlling its phosphorylation state. Such a condensed hydrogel structure would also explain the extremely slow turnover and diffusion of mRNAs within the mature Bb and between it and the cytoplasm in *Xenopus* (Chang et al. 2004), as discussed above.

5.8 Potential Contribution of Germ Granules/Nuage to the Balbiani Body

Nuage is another universal feature of germ cells that has some relationship to the Bb. The nuage consists of mRNPs seen in TEM as electron-dense material that when associated with mitochondria has also been termed “mitochondrial cement.” The nuage is now established to function in the piRNA pathway and across different species harbors several piRNA pathway proteins such as the Argonaute family, Tudor domain proteins, Maelstrom, Vasa, and GasZ (Findley et al. 2003; Juliano et al. 2011; Ku and Lin 2014; Ma et al. 2009).

Nuage granules are usually distributed in the perinuclear cytoplasm. This is the case in mouse developing sperm and oocytes, as well as in primordial germ cells and young oocytes (≤20 μm in diameter) in zebrafish (Houwing et al. 2007, 2008; Huang et al. 2011; Juliano et al. 2011; Kamminga et al. 2010; Ku and Lin 2014; Ma et al. 2009). In zebrafish, nuage containing granules have been termed “germ granules” because of their germ cell specificity and because they contain the germplasm protein Vasa. These granules dissociate in zebrafish oocytes at early diplotene stages (Houwing et al. 2007, 2008; Huang et al. 2011; Kamminga et al. 2010) and are next detected at early embryonic cleavage stages (Strasser et al. 2008). They contain

piRNA enzymes and function in piRNA biogenesis, as has also been shown for the Vasa protein (Xiol et al. 2014).

Nuage and perinuclear granules process piRNAs and contain both piRNA core enzymes like Miwi/Ziwi and Mili/Zili, as well as RNA-binding proteins like Vasa. Importantly, they also contain scaffold proteins that bind and connect the RNA-binding proteins with other RNA-binding proteins and with the piRNA enzymes (Findley et al. 2003; Huang et al. 2011; Ma et al. 2009). In the absence of these scaffolds, the nuage granules fall apart and the piRNA pathway is impaired (Findley et al. 2003; Huang et al. 2011; Ma et al. 2009). One such scaffold protein in the mouse nuage is the GasZ protein (Lim et al. 2013; Ma et al. 2009). GasZ is intriguingly detected in the Bb of zebrafish and *Xenopus* oocytes (Marlow and Mullins 2008; Yan et al. 2004). It was recently shown that the perinuclear piRNA pathway granules also polarize and transition from being radially distributed in oogonia to being localized to the cytoplasm apposing the bouquet telomere cluster, along with the Bb precursor components (Elkouby et al. 2016). This included localization of Vasa, Zili, Ziwi, and GasZ. It is possible that perinuclear granule components contribute to the Bb, such as GasZ, or alternatively that GasZ has multiple distinct functions in piRNA biogenesis and in the Bb, such as structurally maintaining these granules as a scaffolding protein.

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Chapter 6
Vertebrate Axial Patterning: From Egg to Asymmetry

Douglas W. Houston

Abstract The emergence of the bilateral embryonic body axis from a symmetrical egg has been a long-standing question in developmental biology. Historical and modern experiments point to an initial symmetry-breaking event leading to localized Wnt and Nodal growth factor signaling and subsequent induction and formation of a self-regulating dorsal "organizer." This organizer forms at the site of notochord cell internalization and expresses primarily Bone Morphogenetic Protein (BMP) growth factor antagonists that establish a spatiotemporal gradient of BMP signaling across the embryo, directing initial cell differentiation and morphogenesis. Although the basics of this model have been known for some time, many of the molecular and cellular details have only recently been elucidated and the extent that these events remain conserved throughout vertebrate evolution remains unclear. This chapter summarizes historical perspectives as well as recent molecular and genetic advances regarding: (1) the mechanisms that regulate symmetry-breaking in the vertebrate egg and early embryo, (2) the pathways that are activated by these events, in particular the Wnt pathway, and the role of these pathways in the formation and function of the organizer, and (3) how these pathways also mediate anteroposterior patterning and axial morphogenesis. Emphasis is placed on comparative aspects of the egg-to-embryo transition across vertebrates and their evolution. The future prospects for work regarding self-organization and gene regulatory networks in the context of early axis formation are also discussed.

Keywords Vertebrate embryology • Axis formation • Cortical rotation • Spemann organizer • Dorsoventral patterning • Anteroposterior patterning • Embryonic induction • Nieuwkoop center • Anterior visceral endoderm • Gastrulation

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Abbreviations

AP	Anteroposterior, Anterior-to-posterior
AVE	Anterior visceral endoderm
BMP	Bone morphogenetic protein
CRD	Cysteine-rich domain
DEP domain	Dishevelled, Egl10, Pleckstrin
DFC	Dorsal forerunner cell
DIX domain	Dishevelled, Axin
DV	Dorsoventral, Dorsal-to-ventral
dYSL	Dorsal yolk syncytial layer
EMT	Epithelial-to-mesenchymal transition
EpiSC	Epiblast stem cells
ES	Embryonic stem
EVL	Enveloping layer
FGF	Fibroblast growth factor
GPCR	G protein-coupled receptor
HMG	High mobility group
ICM	Inner cell mass
MAPK	Mitogen-activated protein kinase
MBT	Mid-blastula transition
MPF	Maturation promoting factor
PCP	Planar cell polarity
PDZ domain	Postsynaptic density protein (PSD95), Disc large tumor suppressor (Dlg1), and zonula occludens1 protein (ZO-1)
PMZ	Posterior marginal zone
TALEN	TAL-effector nuclease
TE	Trophectoderm
Tgfb	Transforming growth factor beta
UV	Ultraviolet irradiation

6.1 Introduction

Bilaterality is a central feature of animal body organization. In certain invertebrates, such as some insects and cephalopods, this feature is determined by the structure of the egg itself (Wilson 1928), but vertebrates and many other animals define the plane of bilateral symmetry de novo in each embryo. In vertebrates, this plane is ultimately defined by the formation of the generalized vertebrate tissues, the dorsal neural tube, notochord and somites. The vertebrate body axis fully forms during gastrulation, following the internalization of axial mesendoderm at the future dorsal midline of the embryo (Fig. 6.1). This event initiates at the dorsal (upper) lip of the forming blastopore, the importance of which was first clearly recognized by

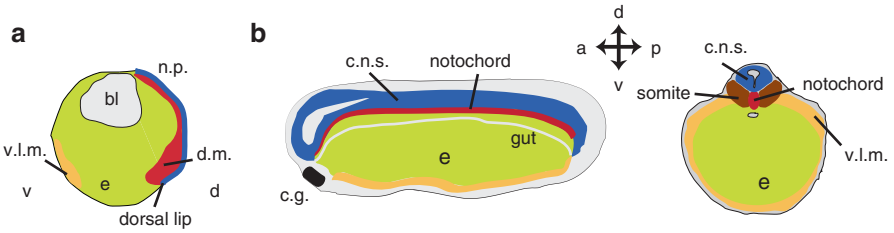

Fig. 6.1 Vertebrate axial organization. (**a**) Diagram of a sagittal section through a *Xenopus* gastrula, showing the involution of the dorsal mesoderm (d.m., *dark red*) at the dorsal lip. The neural plate (n.p., *blue*) overlies the dorsal mesoderm. *bl* blastocoel, *v.l.m.* ventrolateral mesoderm (*orange*), *e* endoderm (*yellow*). (**b**) Sagittal (*left panel*) and coronal (*right panel*) diagrams of a tailbud-stage *Xenopus* embryo showing the elongated anterior-to-posterior axis and organization of tissues within. The neural tube is located dorsally and will form the entire central nervous system (c.n.s.). The dorsal mesoderm gives rise to the notochord and somites, ventrolateral mesoderm (v.l.m.) will form the kidneys, body wall muscles and vascular system. The endoderm forms the gut and its derivative organs. The cement gland (c.g.), a larval amphibian anchoring structure, is shown at the anterior end. After Hausen and Riebesell (1991)

Spemann in amphibians, and was defined as the "organizer" of axis formation (Spemann 1918; Spemann and Mangold 1924). Spemann performed transplantation experiments in salamanders, demonstrating that the dorsal lip could induce and organize a normally patterned second body axis when grafted to the opposite (ventral) side of a host gastrula. In this "secondary embryo," the organizer cells contributed mostly to notochord themselves, whereas host cells populated the bulk of the induced axis, which included neural tube, somites, intermediate mesoderm and gut endoderm. Additional experiments showed that organizer might also contribute to anteroposterior patterning of the embryo, demonstrating a central role for the organizer tissue in controlling cell interactions during development.

Although these main findings were firmly established by the 1930s, it was not until the 1990s that the cellular and molecular mechanisms underlying the action of the organizer were revisited, resulting in the identification of conserved growth factor antagonists and transcription factors. The background and history of this work has been written about exhaustively by Spemann and his contemporaries and later by modern authors (Spemann 1938; Waddington 1940; Hamburger 1988; Grunz 2004). As outlined later in this chapter, the conservation of the organizer extends to the cellular and genetic levels and largely defines the core mechanisms of early vertebrate body plan formation.

In contrast to the conservation of the organizer and its components, the ultimate origins of axial bilateral symmetry in vertebrates are seemingly more diverse. Axis formation was first extensively studied using amphibians and was linked to cytoplasmic localizations in the egg. This was evident in the formation of a natural marker of the future dorsal side, what came to be called the "gray crescent" (Roux 1888). Early mechanistic studies suggested the crescent formed by rotation of the outer cortex over the yolky inner cytoplasm (reviewed in Clavert 1962; Ancel and Vintemberger 1948). This "cortical rotation" was verified by later authors and found to involve the

organization and polarization of microtubules dorsally and the transport of dorsalizing determinants (Gerhart et al. 1989). Similar overall patterns are seen in primitive fish (Clavert 1962), suggesting that axis specification through cortical rotation in the fertilized egg is an ancestral condition in vertebrates.

By contrast, sauropsids (birds and reptiles) and more derived fish (teleost and selachiians/dogfish) lack an obvious physical marker of dorsoventral polarity. These eggs contain abundant yolk and undergo discoidal cleavage, and axis formation occurs after significant cleavage in the blastoderm. In birds and reptiles, evidence suggests that rotation of the egg during passage through the oviduct affects axis formation in the blastoderm. Similar gravitational mechanisms were originally thought to exist in dogfish and teleosts (Clavert 1962), although recently, mechanisms involving cytoskeletal polarization in the cortex, analogous to the amphibian cortical rotation have been found in teleosts (zebrafish and medaka).

With the exception of the egg-laying monotremes, which undergo discoidal cleavage and are likely similar to reptiles with regard to axial patterning, mammals represent a significant divergence from this broad trend. The eggs of therian mammals have lost yolk, reverted to holoblastic cleavage (secondary holoblastic cleavage) and evolved the blastocyst structure to facilitate implantation. Consequently, the first cell fate decisions are centered on distinguishing the embryo proper from extraembryonic lineages rather than on establishing bilateral symmetry. Axial patterning is thus rather late, only becoming apparent after implantation, about a week into development. Early blastomeres retain pluripotency for an extended time and axis formation requires multiple reciprocal interactions with extraembryonic tissues.

Although there was evidence that formation of the organizer depended on polarization of the egg, the mechanisms connecting the two were totally unknown to early embryologists. Studies in amphibians unexpectedly found that the organizer was itself formed through induction, rather than by inheriting gray crescent material. This organizer-inducing activity was predominantly found in dorsovegetal cells of the blastula, later termed the “Nieuwkoop center” after its discoverer, and its formation depended on cortical rotation (Gerhart et al. 1989). These experiments were a critical link in the chain of causality from egg to organizer and were represented in various three- and four-signal models familiar to developmental biologists (Slack 1991). The cortical rotation → Nieuwkoop center → organizer model has been a useful conceptual tool and has directed much of the research into the molecular basis for these processes and their conservation across vertebrates. It is now appreciated that cortical rotation results in dorsal Wnt/beta-catenin signaling, activating Transforming growth factor beta (Tgfb)/Nodal signaling in the vegetal cells, which induce and pattern the organizer in the overlying equatorial cells. Analogous mechanisms have been found acting in the teleost dorsal yolk syncytial layer (dYSL) of the egg and in the avian posterior marginal zone (PMZ) epiblast, based on molecular and functional data, suggesting deep conservation of these processes in the early vertebrate lineage.

Recent cellular and molecular characterization of axis formation and patterning has produced a wealth of examples of such deeply conserved vertebrate developmental mechanisms. Vertebrate embryology has historically been a comparative science, with investigations encompassing a wide range of diverse organisms. More recently,

the use of specialized molecular and genetic approaches has largely limited the study of axis formation to a few tractable vertebrates, notably the mouse, chicken, *Xenopus*, and zebrafish. However, these species are all fairly evolutionarily derived representatives of their respective groups, making inference of primitive vertebrate characters problematic. With the growing ease of high-throughput genome analyses, stem cell technology and programmable genome editing, the barriers to performing comparative molecular and genetic studies are becoming increasingly reduced, potentially heralding a return to a broad comparative approach to vertebrate development.

In the context of the egg-to-embryo transition, the formation of the body axis is perhaps a defining process, since early developmental processes become organized into a unit comprising an individual. Indeed the idea of individuality in twinned embryos was an inspiration for Spemann to begin studying the embryological mechanisms of axis formation (Hamburger 1988), and remains relevant in current bioethics arguments regarding human embryos. This chapter shall review the core concepts relating to the origins and patterning of the axis, focusing on recent advances in understanding intracellular reorganizations, intercellular signaling events and cellular migrations. Emphasis has been given to recent molecular advances in the context of first discoveries and initial functional studies. Many of the ideas in this chapter have been extensively reviewed separately in the context of certain organisms, molecules or individual processes, but this chapter will attempt to tie these together to generate a more unified picture of axial development throughout the vertebrates.

6.2 Origins of Axial Polarity in the Egg and Early Embryo

The process of determining the initial plane of bilaterality and axis formation was first examined closely in the amphibians, where the gray crescent served as marker of the future axis (Fig. 6.2). Like most vertebrate eggs, amphibian eggs are initially symmetrical about the animal–vegetal axis (axisymmetrical), with the animal pole being the site of polar body extrusion, by definition, and more darkly pigmented. The vegetal pole is less pigmented and more concentrated with yolk. Using localized fertilization of frog eggs (*Rana* (*Lithobates*) spp.), Newport and Roux showed that the meridian of sperm entry coincided with the embryonic midline (and often the first cleavage plane), with the dorsoanterior axis forming opposite the sperm entry side (Newport 1851, 1854; Roux 1885, 1887). Importantly, Roux noted an apparent shifting of the animal–vegetal axis toward the sperm entry point, along with the appearance of a lightened area in the pigment on the opposing side. This feature formed before first cleavage and indicated the axial plane of the embryo, irrespective of the cleavage plane, which could be highly variable relative to sperm entry, depending on species (Roux 1887, 1888). Later studies confirmed these observations, further showing that the position of this "gray crescent"[1] strongly

[1] The nomenclature of the gray crescent has been quite variable. Roux (1888) referred to this feature as a "crescent-shaped gray seam" (*halbmondförmigen grauen Saumes*). Morgan and Tsuda

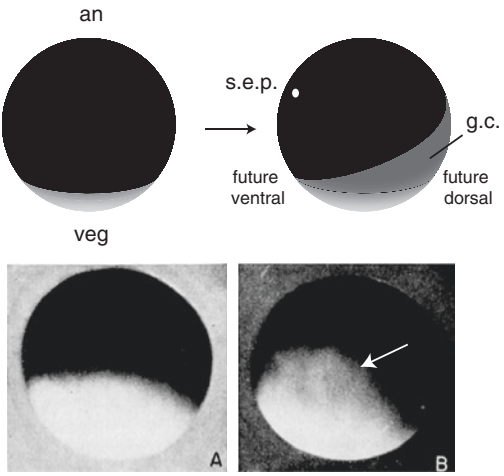

Fig. 6.2 Gray crescent formation in amphibians. *Top panel*, diagram of an amphibian egg (e.g., *Rana*) before (*left*) and after fertilization (*right*). The heavily pigmented animal pole (an) and the paler vegetal pole (veg) are indicated. After fertilization, corticocytoplasmic movements opposite to the sperm entry point (s.e.p.) result in the appearance of the gray crescent (g.c.) on the prospective dorsal side. *Bottom panel*, images of a *Rana* egg at fertilization (**a**), and at 20 min post-fertilization, showing the gray crescent (**b**; dorsal view, *arrow*). *Bottom panel* reproduced from Rugh (1951)

predicts the area in which the future dorsal lip of the blastopore would form (Morgan and Tsuda 1894; Schultze 1899; Roux 1903; Morgan and Boring 1903; Brachet 1904). These classical embryological observations established that the dorsal axis and bilateral symmetry were determined upon fertilization of the egg and likely occurred prior to the first division, thus disputing the long-held idea that the cleavage itself was determinative.

Despite the important nature of the connection between the gray crescent and the dorsal axis, the mechanisms of gray crescent formation and function remained elusive for many years. In the first comprehensive effort to understand the cellular changes underlying gray crescent formation, Ancel and Vintemberger (1948; reviewed in Clavert 1962) examined the movement of electrocautery wounds that were made either in the deep yolk or on the surface of frog eggs. The motion of these markers revealed that outer egg cortex moves dorsally relative to the stationary deeper yolky cytoplasm. Although other models were also considered, such as asymmetric cortical contraction, a later series of marking and egg manipulation studies in *Xenopus* largely substantiated and clearly documented the cortical rotation model (Vincent et al. 1986; Vincent and Gerhart 1987).

(1894) used the term "white crescent," whereas Morgan and Boring (1903) used "grey crescent" [sic], translated as "*graue Feld*" (gray field). Later this became universally referred to as the gray crescent/*grauer Halbmond/croissant gris*.

Measurements of relative cortical displacement using superficially or deeply placed fluorescent dyes found that cortical rotation begins about halfway though the first cell cycle, eventually covering an average 30° of arc over the dense yolky cytoplasm. Also, cortical movement progressed along an animal–vegetal meridian, and toward the future dorsal side of the egg (generally away from the sperm entry point), with the region of greatest movement correlating with the position of the axial midline (Vincent et al. 1986; Gerhart et al. 1989). It is thought that changes in fluid dynamics in the fertilized egg result in a low viscosity/high elasticity shear zone in the subcortical region as well as an increase in firmness in the deep cytoplasm, allowing this differential movement between two parts of the egg (Elinson 1983; Gerhart et al. 1989).

In an extensive comparison of axis forming mechanisms, Clavert (1962) indicates that, in addition to amphibians, primitive fish including lampreys, lungfish, and chondrostean fish (sturgeons and paddlefish) likely form gray crescents, suggesting that these organisms also likely undergo cortical rotation. With these older comparative studies and more recent molecular data taken together, it is apparent that the basics of the amphibian cortical rotation model are conserved throughout the anamniotes (icthyopsids). And vestiges may exist even in some amniotes. It is also now generally appreciated that cortical rotation establishes a self-organizing, transient microtubule polarity in the zygote that is critical for the transport of cortical cytoplasmic dorsal determinants and activation of Wnt/beta-catenin signaling (reviewed in Gerhart 2004; Weaver and Kimelman 2004; Houston 2012). Wnt/beta-catenin signaling (see Sect. 6.3.5) also plays a key role in bird and mammal axis formation, but this is likely controlled by mechanisms other than cytoplasmic localization.

6.2.1 Mechanisms of Amphibian Cortical Rotation

6.2.1.1 Cortical Rotation in Anurans

A number of studies have shown that cortical rotation is primarily controlled by the assembly of parallel microtubule arrays in the vegetal cortex (Fig. 6.3). Treatment of fertilized eggs during the middle of the first cell cycle with microtubule-depolymerizing agents such as ultraviolet irradiation (UV), exposure to anti-microtubule drugs, cold and high pressure, can inhibit gray crescent formation and/or block axis formation in both frog and salamander eggs (Malacinski et al. 1975; Manes et al. 1978; Manes and Elinson 1980; Scharf and Gerhart 1983; Vincent and Gerhart 1987). Correspondingly, impressive arrays of parallel microtubules are assembled in the vegetal cortical region during the period of cortical rotation in *Xenopus* and *Rana* (*Lithobates*) (Elinson and Rowning 1988).

Microtubules are completely disassembled in the egg at fertilization, but progressively repolymerize over the first 35 min, approximately when relative cortical movement begins. Microtubules in the cortical region initially form a disorganized network that gradually becomes organized as cortical rotation progresses. By mid-cortical rotation, microtubules are predominantly oriented with the plus ends

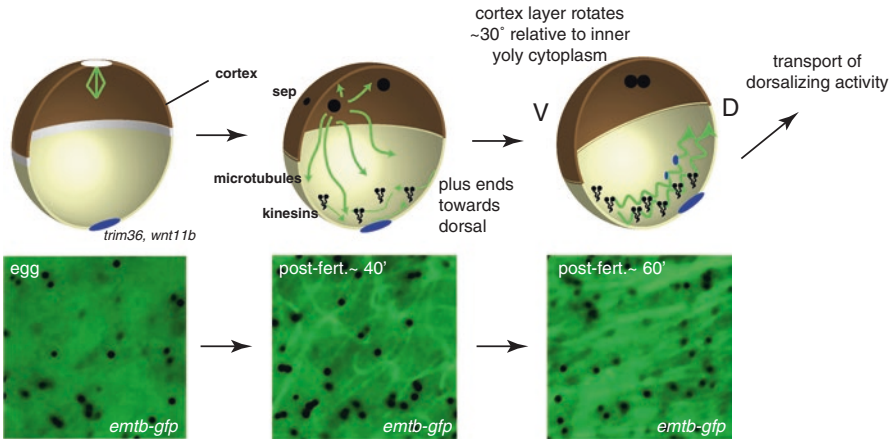

Fig. 6.3 Events of cortical rotation in *Xenopus*. Microtubules are disassembled during oocyte maturation, and are absent from the egg cortex (*left panels*). Certain RNAs are localized to the vegetal cortex during oogenesis (*blue*) and encode proteins critical for cortical rotation and dorsalization (e.g., *trim36*, *wnt11b*). After fertilization, the incoming sperm pronucleus and associated centrosome initiate astral microtubule assembly. Cortical microtubule assembly also begins, forming a network by 40 min post-fertilization. A shear zone forms and microtubules associate with the yolky cytoplasmic core (not shown) and cortical rotation begins, under the action of kinesin-like proteins (kinesins). Relative cortical movement occurs dorsally, possibly the result of nudging by ventrally positioned astral microtubules, and rapidly orients microtubule plus ends dorsally (Olson et al. 2015) (*middle panel*). Microtubule assembly and organization becomes robust by 60 min post-fertilization and full cortical rotation commences, continuing until first cleavage. Rapid transport of dorsalizing activity occurs along parallel microtubule arrays using kinesin-like motors (*right panel*). The corresponding bottom panels show live images of microtubules labeled with Enconsin microtubule-binding domain tagged GFP (EMTB-GFP), showing progressive assembly and alignment during cortical rotation (Olson et al. 2015)

towards the dorsal side (Houliston and Elinson 1991; Olson et al. 2015). Live imaging studies indicate that the arrays associate and move with the deeper cytoplasm (Houliston 1994; Larabell et al. 1996; Olson et al. 2015). These microtubule arrays are transient and are progressively depolymerized upon first cleavage under the control of MPF activation (Marrari et al. 2003), thus terminating cortical rotation.

Surprisingly little is known about the regulation of microtubule activity during cortical rotation. Generalized kinesin-related protein activity in the cortex proper is thought to tether the microtubule array to the cortex and facilitate movement (Marrari et al. 2003). This has been assessed using function-blocking antibodies but specific roles for individual kinesins have not been identified. Kinesin1/Kif5b appears dispensable in *Xenopus* (Marrari et al. 2000, 2003), and Dynein has been shown to act early in rotation, as shown by injection of the antagonistic Dctn2 (Dynamitin/p50; Marrari et al. 2004). Recently, a suite of mRNAs localized to the vegetal pole in oocytes has also been implicated in regulating microtubule assembly. Maternal mRNA depletion experiments show that reductions in *perilipin2* (*plin2*; Chan et al. 2007), *tripartite motif containing 36* (*trim36*; Cuykendall and Houston 2009), and *dead end homolog 1* (*dnd1*; Mei et al. 2013) lead to abnormal

microtubule array formation and failure of cortical rotation. Trim36 can function as a single RING-finger-type ubiquitin ligase, and this activity is essential for its role in microtubule assembly (Cuykendall and Houston 2009). Dnd1 is an RNA-binding protein required to tether *trim36* mRNA to the cortex, facilitating locally enriched Trim36 protein levels (Mei et al. 2013). Dnd1 is typically associated with germline specification (Weidinger et al. 2003), and it is not known whether these functions are related. The role of Plin2 is unclear. The protein is associated with lipid droplets (Chan et al. 2007), but a structural role for the *plin2* RNA has also been suggested (Kloc 2009). A different set of localized mRNAs are involved in vegetal microtubule organization and transport in the zebrafish zygote (Nojima et al. 2010; Ge et al. 2014), although with slightly different functions (see Sect. 6.2.2). It remains to be determined how these localized molecules interact with microtubule regulatory proteins and motor proteins to control microtubule assembly in cortical rotation.

The initial cue for the direction of cortical rotation in normal development is thought to be sperm entry, as this site is generally opposite the direction of movement. The central model for orientation of the array is a reciprocal positive feedback loop, during which random asymmetry in microtubule growth is refined and amplified by rotation of the cortex (Gerhart et al. 1989; Gerhart 2004). Microtubules growing into the cortex, originating from the sperm aster and within the cortex may provide the initial movement cue (Houliston and Elinson 1991; Schroeder and Gard 1992). Cortical movement then serves to progressively stabilize microtubule growth and formation in the same direction. High-resolution live imaging of microtubule assembly and orientation has verified that cortical rotation begins before there is visible bias in plus end directionality or microtubule alignment (Olson et al. 2015), an observation that was suggested from earlier studies but never directly shown (Larabell et al. 1996). Additionally, plus end orientation occurs almost as soon as cortical rotation begins, indicating that directionality is determined in a punctuated manner rather than progressively (Olson et al. 2015).

In vivo, sperm entry or slight asymmetry with respect to gravity could be sufficient to initiate cortical movement, although a "vector summation" of microtubule polymerization forces, as initially proposed (Gerhart et al. 1989) cannot be ruled out. The shear-induced alignment of organelles (endoplasmic reticulum) may also play a role in perpetuating alignment, since ER and microtubules are often interdependent (Terasaki et al. 1986). Because cortical movement can have a role in determining the orientation of microtubules, the overall role of cortical rotation may be thought of as twofold; first to generate relative displacement of the cortex, and second to align the microtubule array facilitating the faster and longer range transport of determinants.

Evidence for these determinants came from 90° egg tipping experiments, which cause the axis to form in the uppermost part of a tipped egg (Ancel and Vintemberger 1948; Kirschner et al. 1980; Gerhart et al. 1981). Also, tipping can rescue axial development following UV-irradiation (Scharf and Gerhart 1980; Chung and Malacinski 1980). In amphibian eggs, denser yolk accumulates in the vegetal pole, which when tipped off axis, results in a tendency to "fall" downward against the cortex, which is immobilized in these experiments, creating relative displacement. Tipping does not restore microtubules (Ziskind and Elinson 1990), further suggesting that the relative

displacement of cortical dorsal determinants is essential, whether achieved normally by microtubule motive force or experimentally by gravitational force.

Other studies indicated the existence of an essential, transplantable dorsalizing activity associated with the cortex/subcortical cytoplasm (Yuge et al. 1990; Hainski and Moody 1992; Holowacz and Elinson 1993; Kikkawa et al. 1996; Kageura 1997). And, live imaging studies have shown various substances moving dorsally within the shear zone during cortical rotation. These include a subset of pigment granules and organelles, fluorescent beads, and certain GFP fusion proteins (Miller et al. 1999; Weaver et al. 2003). Their movement is rapid (~50 μm/min) and saltatory, consistent with generalized kinesin-based transport along microtubules. Transport can be measured from 30°–120° of arc from the vegetal pole, equal to and greater than the overall relative cortical displacement (Rowning et al. 1997; Miller et al. 1999; Weaver et al. 2003). Interestingly, this distribution matches that of dorsalizing cytoplasm taken from the egg (Fujisue et al. 1993; Holowacz and Elinson 1993). Additionally, stimulation of microtubule assembly with deuterium can hyperdorsalize embryos, potentially through wide-spread distribution of this dorsalizing material along many egg meridians (Scharf et al. 1989; Miller et al. 1999). The identity of the molecules responsible for the activity of this cytoplasm in vivo is unclear but are likely related to Wnt/beta-catenin signaling (see Sect. 6.3.2).

Cortical rotation can thus be considered a robust self-organizing symmetry-breaking process that integrates cytoskeletal and physical forces to generate a single direction for the short-range relative displacement of the cortex and for the long-range distribution of molecules and putative determinants towards the presumptive dorsal side.

6.2.1.2 Cortical Rotation in Urodeles

Although much of the recent cell and molecular characterization of cortical rotation has been done in anurans (*Xenopus* and *Rana* (*Lithobates*)), urodeles *Triturus* and *Ambystoma* are known to form gray crescents (Bánki 1927; Clavert 1962). However, urodele eggs are normally polyspermic and the relationships between the site of sperm entry or male pronucleus formation and the site of the gray crescent are unclear. Recently, relative cortical displacements analogous to those in *Xenopus* have been observed in *Cynops* (Fujisue et al. 1991), which also exhibits vegetal microtubule array assembly during the period of cortical rotation (Iwao et al. 1997). Curiously, although some species are ventralized by UV-irradiation (see above), irradiation of *Cynops* eggs dorsalizes embryos (Doi et al. 2000), suggesting that putative dorsal determinants are more widely dispersed in these eggs and would remain so in the absence of microtubule assembly and cortical rotation. This situation may possibly mimic the random dispersion of determinants occurring in deuterium-treated *Xenopus* eggs. Thus, the basic mechanisms of microtubule-dependent cortical rotation and dorsal determinant transport are conserved in amphibians.

Urodeles are thought to lack vegetal cortical localization of RNAs (Elinson and del Pino 2011; Houston 2013), which is interesting given the connection between

localized RNAs and cortical rotation in *Xenopus* and zebrafish. However, it has not been specifically demonstrated whether the exact RNAs implicated, including *trim36* and *syntabulin* (*sybu*), are in fact unlocalized in urodeles. Since these RNAs are partially associated with the germ plasm, which is not found in urodeles (Nieuwkoop and Sutasurya 1979), one might expect an absence of localization. Urodeles may however localize important components posttranslationally. Nevertheless the basic mechanisms of polarizing the egg and distributing dorsal determinants appear conserved, but are not well understood in either case.

6.2.2 *Cortical Rotation and Dorsal Determinant Transport in Zebrafish*

Axis formation in zebrafish similarly relies on asymmetric localization of dorsal determinants and activation of Wnt/beta-catenin signaling. The polarizing mechanisms and the similarity of these to classical amphibian cortical rotation are only now becoming apparent, however. It has been traditionally thought that typical cortical rotation does not occur in teleost fish. The origin of this assumption is mysterious, but likely can be attributed to initial observations on the importance of a polarized dYSL in teleost axial patterning rather than formation of a gray crescent-like clear crescent, which does occur in non-teleosts (Long 1983; Ho 1992). There do not appear to have been any classical embryological studies directly addressing either relative displacement of cytoplasm and cortex or the existence of transplantable vegetal cortical cytoplasm.

However, parallel microtubule arrays have been noted at the vegetal pole cortex of the early post-fertilization (~20 min) medaka and zebrafish egg (Jesuthasan and Stähle 1997). By 30 min post-fertilization, this array is offset from the vegetal pole, giving bilateral symmetry to the egg. During cleavage of the blastoderm, a second set of microtubule arrays forms along the animal–vegetal axis (Strähle and Jesuthasan 1993; Solnica-Krezel and Driever 1994), and polarized transport of fluorescent beads has been observed to move animally into the YSL and marginal blastomeres (Jesuthasan and Stähle 1997). Disruption of either set of microtubules with UV, cold, or nocadazole disrupts axis formation as well as epiboly (Strähle and Jesuthasan 1993; Solnica-Krezel and Driever 1994; Jesuthasan and Stähle 1997).

Thus a two-step transport pathway is proposed (Fig. 6.4). Asymmetry is initially established by the localization of determinants to the future dorsal vegetal side of the egg, followed by generalized upward movement of material into the YSL and marginal blastoderm cells (the dorsal determinants being carried along only on the dorsal side). In support of this idea, a recent live imaging study has demonstrated for the first time, as was tacitly assumed, that the plus ends of zebrafish vegetal cortical microtubules are oriented dorsally as in frogs (Tran et al. 2012). Given the relationship between movement of the cytoplasmic core and microtubule orientation in *Xenopus* eggs (Olson et al. 2015), it is likely that relative cortical movement, at least locally, is involved in orienting these microtubules in teleost eggs as well.

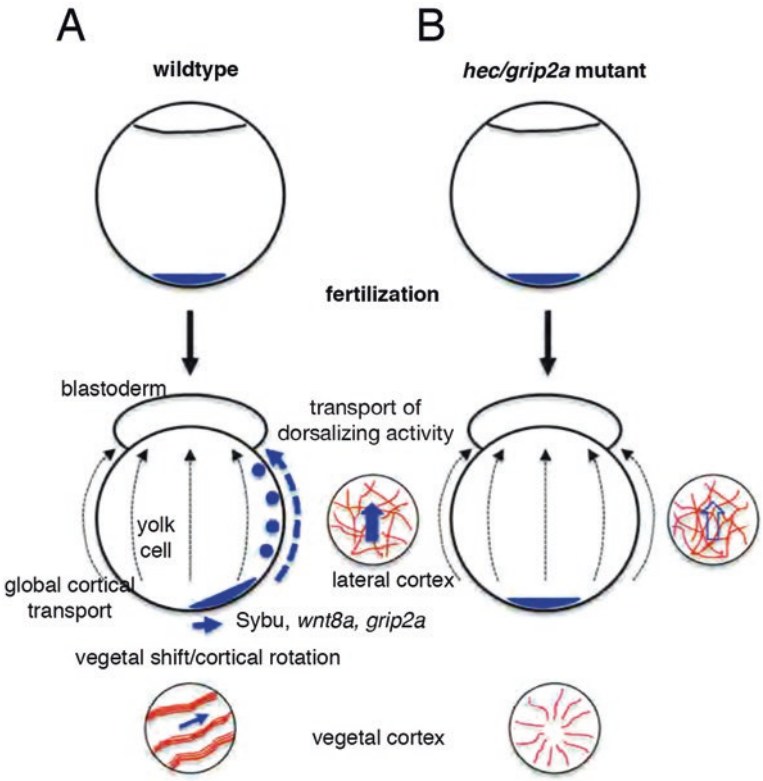

Fig. 6.4 Dorsal determinant transport in zebrafish. (**a**) Sequence of events in wildtype embryos. RNAs and other dorsal determinants are localized vegetally during oogenesis (*blue*). After fertilization, cytoplasm streams to the animal pole, forming the future blastoderm. Microtubule assembly initiates ~20 min post-fertilization at the vegetal pole of the yolk cell; localized RNAs and Syntabulin protein (Sybu) are shifted toward the future dorsal side. Microtubule networks in the lateral cortex facilitate global transport animal-ward, which on the dorsal side would contain axis determinants. (**b**) In *hecate* (*hec*) mutants lacking Grip2a, maternal vegetal localization occurs, but cortical rotation and microtubule assembly are deficient post-fertilization. This image is reproduced and modified from, Ge X, Grotjahn D, Welch E, Lyman-Gingerich J, Holguin C, Dimitrova E, et al. (2014) Hecate/Grip2a acts to reorganize the cytoskeleton in the symmetry-breaking event of embryonic axis induction. PLoS Genet 10(6): e1004422. doi:10.1371/journal.pgen.1004422, under the terms of the Creative Commons Attribution License (CC BY 4.0)

Additional insight into microtubule assembly in zebrafish and further support for the dual-range model of transport in the yolk cell has recently come from analysis of maternal-effect mutants in zebrafish (Nojima et al. 2004, 2010; Lyman Gingerich et al. 2005; Ge et al. 2014). The mutants *hecate* (*hec*; Ge et al. 2014) and *tokkaebi* (*tkk*; Nojima et al. 2010) are ventralized with near complete penetrance and harbor disruptions in *syntabulin* (*sybu*) and *glutamate receptor interacting protein 2a* (*grip2a*) loci, respectively. Parallel microtubule assembly at the vegetal pole is normal in *tkk* mutants but disrupted in *hec* mutants. Upward movement to the YSL/

blastoderm margin is normal in *hec* eggs, underscoring the independence of these two transport systems. Sybu encodes a potential cargo linking protein for Kif5b, suggesting a role in microtubule transport of dorsal determinants vegetally. Grip2a encodes a scaffolding protein important for subcellular localization in mammalian neurons.

Both *sybu* and *grip2a* mRNAs localize to the vegetal cortex, along with *wnt8a* mRNA (see below), and these RNAs all undergo an off-center "shift," mirroring that of the microtubule array (Nojima et al. 2010; Lu et al. 2011; Ge et al. 2014). Sybu protein fails to localize to the prospective dorsal side in nocodazole-treated eggs, suggesting it is trafficked by microtubules. The exact role of Grip2a is not known but it may recruit protein complexes to vesicles or organelles that attach to and help align microtubules. Interestingly, *grip2* mRNA is localized in *Xenopus*, but follows a germ plasm-like pattern and is not thought to play a role in axis formation (Tarbashevich et al. 2007). Similarly, *sybu* is localized to the germ plasm in *Xenopus* and may play an undefined role in axis formation, possibly in transport or in Wnt activation (Colozza and De Robertis 2014).

In zebrafish, maternal loss-of-function mutants have implicated *kif5ba* in vegetal microtubule formation and axis formation (Campbell et al. 2015), although its role is complex. Organized vegetal microtubules fail to form and *wnt8a* does not shift dorsally. However, *grip2a* asymmetric translocation still occurs and *sybu* RNA is not maintained vegetally (Campbell et al. 2015). It is unclear to what extent these phenotypes reflect roles for Kif5ba in localizing components vegetally during oogenesis or more acute roles during microtubule organization and transport.

6.2.3 Asymmetry in Early Amniote Embryos

Initial axis formation in fish and frogs occurs in the fertilized egg; the dorsal determinants are either inherited directly by dorsal cells (frogs, primitive fish) or transmitted from the uncleaved yolk cell to peripheral dYSL and overlying dorsal marginal blastomeres (teleost fish). The axis in amniotes (birds, reptiles and mammals) relies on mainly on reciprocal interactions between upper embryonic and lower extraembryonic tissue layers (epiblast and hypoblast, respectively) and asymmetric cell movements, with only hints that early asymmetry in the egg or early embryo are involved. Additionally, the links to localized activation of growth factor signaling pathways are much less clear.

6.2.3.1 The Role of Gravity in Axis Formation in Sauropsids

Classic experiments in the chick have suggested that axis specification occurs in response to gravity as the egg rotates as it passes through the oviduct (Fig. 6.5). Axis formation in reptile embryos is thought to occur in a similar fashion, although is less thoroughly studied in this regard. Bird and reptile embryos are both highly polyspermic, making it unlikely that sperm entry plays a role in axis formation

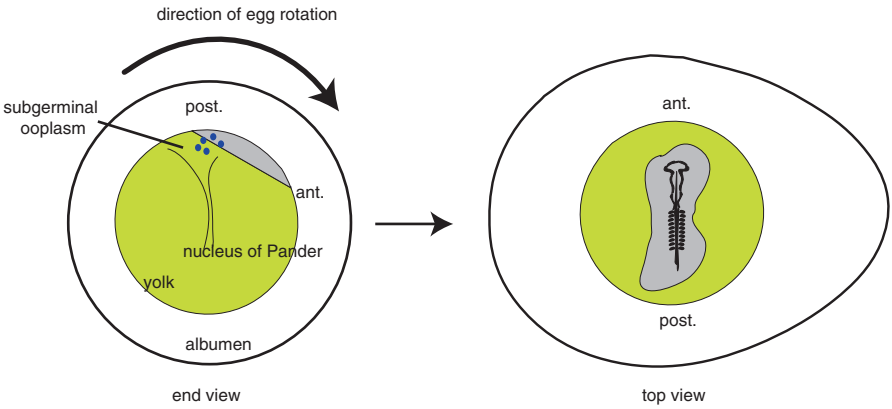

Fig. 6.5 Model for establishment of asymmetry in bird eggs. *Left*, sectional view of a uterine chicken egg viewed from the sharp end. The direction of rotation is indicated; because of this rotation, the lighter blastoderm cytoplasm is maintained off angle as it continually floats to the highest point. The blastoderm is exposed to the subgerminal cytoplasm, which is hypothesized to contain axis determinants (*blue*). At this stage, the blastoderm is several thousand cells and has not formed the area pellucida epiblast. *Right*, top view of 2–3 day embryo showing anterior-to-posterior axial polarity. This embryo would conform to von Baer's rule, with head oriented away with the blunt end positioned left. *ant.* anterior, *post.* posterior

(Waddington 1956). The long-noted von Baer's rule of thumb suggested a relationship between the axis of the egg and that of the embryonic axis (von Baer 1828). This axis is most often (~70 %) situated perpendicular to the long axis of the egg, with the left side of the embryo oriented towards the blunt end. Eggs rotate clockwise about the long axis as they travel through the oviduct (~0.2 rpm) and continue rotating in the uterus, where they acquire the shell membranes and shell. As the egg rotates, the blastoderm is maintained at an angle of ~45°, balancing the inertia of the rotation with the tendency of the buoyant blastoderm to float on the dense yolk (Clavert 1962). In this arrangement, the anterior of the embryo forms at the lower end. Most eggs enter the uterus and are laid sharp-end first and thus end up following von Baer's rule. The minority of cases where the blunt end enters first are thus truly exceptions that prove the rule, since it is only the embryo's orientation with respect to the external egg shape that is changed; the posterior of the embryo still forms at the upper end of the blastodisc.

Both in utero and in vitro experiments have defined a critical period for axis establishment in the uterus (Vintemberger and Clavert 1959; Clavert 1961; reviewed in Clavert 1962). Egg orientation was manipulated at different times prior to egg-laying and the orientation of the embryonic axis was altered if the presentation of the egg was changed at least six hours prior to laying. Additionally, eggs could be removed and incubated in vitro in a rotating cylinder and the orientation of the embryo was determined by the relative direction of rotation. Similar to the experiments in frogs, the effects of earlier rotations could be overridden by later manipulations up to the critical period of axis formation. This period correlates with the time

when the chick blastoderm thins to a single layer, forming the *area pellucida* epiblast. Another set of studies showed that uterine eggs could be incubated in a variety of orientations without rotation, or even hung from a chalaza without shells, and the axis always formed along the gravitational axis with the posterior end uppermost (Kochav and Eyal-Giladi 1971; Eyal-Giladi and Fabian 1980). Thus, it is the response to gravity that is critical, not the stress of movement or effect of rotation per se.

Axial polarity and bilateral symmetry in the blastoderm is evident both morphologically and molecularly prior to primitive streak formation. The future posterior half of the embryo, in which the primitive streak forms, can first be distinguished by the formation of a ridge of cells in the deep layer of the posterior *area opaca*, Koller's/Rauber's sickle (Callebaut and Van Nueten 1994). Additionally, the hypoblast layer (analogous to the anterior visceral endoderm, see below) begins to form in this region, coalescing from isolated hypoblast islands delaminating from the epiblast in a posterior-to-anterior progression. The hypoblast is then replaced by the endoblast (posterior visceral endoderm), derived from Koller's sickle, in the same progression (Stern 1990; Stern and Downs 2012). Molecular analyses have also identified early differential gene expression in this region, including *Gdf1* (*alias cVg1*; Seleiro et al. 1996; Shah et al. 1997) in the PMZ of the epiblast and *Goosecoid* (*Gsc*) in Koller's sickle (Izpisúa Belmonte et al. 1993). Transplantation experiments showed non-cell/tissue autonomous induction of ectopic axes, sparking parallels between the PMZ and the amphibian Nieuwkoop center (see Sect. 6.3.5).

The mechanisms leading to these developmental events in the posterior are unknown. The prevailing hypothesis for the initiation of this posterior polarity is the differential exposure of presumptive areas to maternal cytoplasm during cleavage. In the chicken egg, a particular cytoplasmic layer, the subgerminal ooplasm (gamma- and delta-ooplasm) is contained in a central region below the blastodisc and overlying the latebra and Nucleus of Pander (Callebaut 2005). With the blastoderm offset from the animal pole by the inertia of the rotating egg (see above), this cytoplasm would have more prolonged contact with cells arising in the upper (future posterior end) of the embryo. The subgerminal cytoplasm may also be differentially inherited by primordial germ cells, which form in response to cytoplasmic determinants (germ plasm) in birds (Tsunekawa et al. 2000). It is thus possible that unknown axial determinants might also be localized to this region, as in amphibians.

6.2.3.2 Early Polarization of the Mammalian Embryo

The evolution of implantation in therian mammals resulted in many changes in the structure of the egg and early embryo, including a loss of yolk, the reemergence of holoblastic cleavage and the early segregation of embryonic and extra embryonic tissues, forming a preimplantation blastocyst (see Chap. 4). In light of these major alterations in life history, it has long been of interest to determine the extent that axes in the mammalian embryo are determined by cytoplasmic asymmetries as in other vertebrates.

Early authors concluded that this must be the case, although these conclusions were admittedly based on a few cases of poorly characterized abnormal embryos generated following blastomere perturbations (Waddington 1956). However, in contrast to amphibians, the separation of early mammalian (rodent and rabbit) blastomeres does not result in complementary embryos either having or lacking dorsal structures (Seidel 1956; Tarkowski 1959; Tarkowski and Wróblewska 1967). Additionally, early mammalian blastomeres demonstrate a high degree of developmental plasticity, with each of the four-to-eight cell blastomeres contributing to all cell lineages in chimeric embryos (Tarkowski 1961; Mintz 1964; Kelly 1977). Furthermore, cell fate specification with respect to epiblast/primitive endoderm/trophectoderm is largely dependent on cell polarity related to inside or outside cell position within the morula, as well as on the timing of asymmetric cell division in generating inside cells (Hillman et al. 1972; Ziomek and Johnson 1980; Pedersen et al. 1986; Morris et al. 2010) (see also Chap. 4). Ablation experiments have found that removal of the animal or vegetal poles from fertilized eggs and early blastomeres is fully compatible with normal development (Zernicka-Goetz 1998; Ciemerych et al. 2000), unlike the case in amphibians. It is therefore generally concluded that segregation of maternal determinants in the egg is unlikely to direct axis formation or cell fate patterning in mammals, or that if such a bias exists it can be readily overridden by other cellular interactions.

Nevertheless, axis specification requires that symmetry breaking occur at some point prior to gastrulation. When this asymmetry is established and to what extent it depends on earlier developmental bias or is more or less random has been a recurring debate. There have been various attempts to correlate cleavage patterns in the early embryo with asymmetries in the blastocyst and conceptus and with the eventual anteroposterior axis of the embryo. A preponderance of the evidence however suggests that much of this observed "bilateral symmetry" likely results from physical constraints imposed by the *zona pellucida* (vitelline membrane) or other external constraints and is not connected to the orientation of the anteroposterior axis (for detailed reviews of this literature, please see Takaoka and Hamada 2012; Zernicka-Goetz 2013; Bedzhov et al. 2014 and references therein).

The most compelling evidence for an early cell fate bias is the observation that the order and orientation of rotational cleavages in the mouse embryo can influence blastomere fate in the blastocyst (Fig. 6.6). In particular, the vegetal blastomere (distal to the polar body) that arises from a particular tetrahedral cleavage pattern (which occurs in a subset of cases), will disproportionately contribute to the trophectoderm in normal embryos (Piotrowska-Nitsche et al. 2005; Torres-Padilla et al. 2007a). Furthermore, chimeras composed exclusively of vegetal blastomeres fail to survive (Piotrowska-Nitsche et al. 2005), likely because these cells cannot generate sufficient numbers of pluripotent epiblast cells to support development (Morris et al. 2012b).

Lineage labeling studies of individual or all four cells have found a similarly biased contribution of four cell-stage blastomeres to either inner cell mass (ICM) or trophectoderm (TE) fates in a subset of embryos (Fujimori et al. 2003; Tabansky et al. 2013). Importantly, this developmental bias was reflected in cell lineage but not in relative cell positioning toward the embryonic or abembryonic poles of the

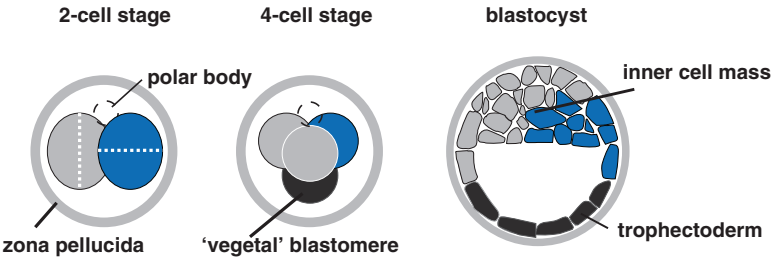

Fig. 6.6 Early bias of mouse blastomeres towards lineage fate but not axial polarity. Two-cell blastomeres undergo rotational cleavage (*dotted white lines* indicate cleavage planes), generating a fraction of embryos with a tetrahedral cell arrangement. In this formation, vegetal blastomeres are biased towards contributing to the trophectoderm (*dark gray*) in the blastocyst. The corresponding animal blastomeres are biased towards contributing to the inner cell mass (*blue*). After Zernicka-Goetz et al. (2009)

blastocyst. For technical reasons however, the specific contribution of vegetal blastomeres could not be assessed in these studies. Mechanisms underlying this bias may include epigenetic regulation of cell polarity (Parfitt and Zernicka-Goetz 2010), decreased pluripotency transcription factor occupancy at target genes (Plachta et al. 2011) or a combination of factors. How this differential regulation is initiated is unknown, but the lack of maternal influences and a lack of differential gene expression in two- and three-cell blastomeres (VerMilyea et al. 2011) suggest that this bias is either an emergent property or a positioning effect in the four-cell stage embryo.

It is unclear at present whether any bias in early blastomere fate can be connected to axis specification in the mammalian embryo. Although this will be discussed further in Sect. 6.5.1 and similar to the bird embryo, the proximal events in mammalian axis formation involve the asymmetric migration of cells in the extraembryonic anterior visceral endoderm/hypoblast.

6.3 Initiation of Axis Induction by Dorsal Determinant Signaling

Numerous models have been suggested for how early asymmetries in the egg and embryo can lead to the specification of the organizer and ultimately to axis formation. Classical views, perhaps influenced by the importance of cytoplasmic localizations in invertebrates, suggested that the amphibian gray crescent contained precursors or determinants of the organizer (Wilson 1928). Another influential idea was that of a dorsal "cortical field" intersecting with a vegetal yolk gradient to determine the position of the organizer (Dalcq and Pasteels 1937). Later experiments showed that mesoderm in general, and the organizer in particular, required inductive cell–cell signaling by the vegetal prospective endoderm (see Chap. 7; Boterenbrood and Nieuwkoop 1973; Gimlich and Gerhart 1984; Dale et al. 1985), suggesting that cell-autonomous inheritance of organizer determinants was subordinate

to mesoderm induction. Importantly with respect to axis formation, Nieuwkoop and colleagues showed that the blastula vegetal mass is dorsoventrally patterned, with only the dorsovegetal cells being able to induce dorsal mesoderm/organizer. This dorsal signaling center, or "Nieuwkoop center" as it became known (Gerhart et al. 1989), was also demonstrated by transplantation of dorsal vegetal cells into UV-ventralized hosts (or ventrally into normal hosts), resulting in largely non-cell-autonomous organizer and axis induction (Gimlich and Gerhart 1984; Gimlich 1986; Kageura 1990).

Cortical rotation emerged as the candidate upstream event leading to Nieuwkoop center formation in dorsovegetal cells, as embryos ventralized by UV-irradiation lack both Nieuwkoop center and organizer activity (Smith et al. 1985; Gerhart et al. 1989). Also, because the extent of mesoderm induction is unchanged in ventralized embryos (Cooke and Smith 1987), a hypothesis was formed that the Nieuwkoop center generates a distinct dorsalizing signal or a competence modifying signal, which acts along with a general mesoderm inducer to induce the organizer. This idea became enshrined in the influential three-signal models of axis formation (Smith et al. 1985; Smith 1989; Heasman 1997). It is now recognized, owing to the work of many labs over many years, that this "dorsal signal" is not a unique signal at all, but represents an early and elevated wave of Nodal-related Tgfb signaling that is regulated by dorsally enriched Wnt/beta-catenin signaling and other maternal factors (see below).

Although many of these studies were conducted using *Xenopus* embryos, transplantation experiments have shown that localized regions in the blastula-equivalent stages of the zebrafish and chicken embryo can induce axes non-cell autonomously (dYSL, Mizuno et al. 1999; PMZ epiblast, Bachvarova et al. 1998). These regions also ultimately act through elevated Nodal signaling, either downstream of or in concert with Wnt/beta-catenin signaling, suggesting that the mechanisms of axis induction are widely conserved vertebrate development. In mammals however, Nodal signaling likely precedes obvious Wnt asymmetry and is the main determinant of axis formation, albeit in conjunction with Wnt signaling. In this section, the roles of early Wnt signaling in establishing dorsal fates in amphibians and fish are reviewed, along with the conserved but divergent roles of Wnt and Nodal signaling in regulating organizer formation across vertebrates.

6.3.1 Basic Wnt Signaling Mechanisms

Since its initial discovery as a mammalian oncogene (Nusse and Varmus 1982), signaling by the deeply conserved *Wnt1* (*int-1*/*wingless* (*wg*)) family of growth factors has emerged as a central feature of many aspects of animal development and disease. The reception of Wnt signals and intracellular signal transduction mechanisms has been extensively studied in vivo in both vertebrate and invertebrate organisms as well as in tissue culture cells. Although there are many variations that are important in specific tissues and disease states, three main arms of the pathway are widely implicated in vertebrate axis formation. These are: (1) the regulation of Ctnnb1

protein stability (beta-catenin protein hereafter), nuclear localization and transcriptional activity, (2) the regulation of cytoskeletal organization and cell polarity, and (3) the release of calcium from intracellular stores. With the caveats in mind that much of Wnt signaling entails complex, context-dependent and networked interactions, it remains useful to understand the basic features of Wnt pathways involved in early axis development. There are numerous comprehensive reviews on different aspects of Wnt signaling (MacDonald et al. 2009; Hikasa and Sokol 2013); here the key evidence of Wnt signaling in axis formation and the core signal transduction mechanisms most involved in axis formation and patterning will be briefly reviewed.

6.3.1.1 Wnt/Beta-Catenin Signal Transduction Mechanisms

In Wnt-unstimulated cells, beta-catenin protein is constitutively turned over by the activity of a multiprotein "destruction" complex (Fig. 6.7). This complex contains Axin1, Adenomatosis polyopsis coli (Apc), Glycogen synthase kinase 3 beta (Gsk3b), and Casein kinase 1 alpha (Ck1a/Csnk1a1), and serves to regulate the phosphorylation of cytoplasmic beta-catenin by Gsk3b and Ck1a (reviewed in MacDonald et al. 2009; Clevers and Nusse 2012; Hikasa and Sokol 2013). These phosphorylations in the beta-catenin N-terminus allow recognition by members of the beta-transducin repeat containing E3 ubiquitin protein ligase family (Btrc, Jiang and Struhl 1998; Liu et al. 1999a) and target phospho-beta-catenin for ubiquitylation, resulting in degradation by proteasomes (Aberle et al. 1997). Axin is thought to be a key limiting component of this complex, regulating the assembly of the destruction complex (Lee et al. 2003) and recruiting the beta-catenin kinases (Ck1a and Gsk3b) for the priming and processive phosphorylation, respectively, of beta-catenin (Liu et al. 2002; Amit et al. 2002).

Wnt stimulation inactivates the destruction complex through a little understood mechanism involving recruitment of the cytoskeletal adaptor protein Dishevelled (Dvl homologs 1–3). This blocks the activity of Gsk3b (Siegfried et al. 1992, 1994; Cook et al. 1996) and prevents beta-catenin phosphorylation, thereby allowing cytoplasmic accumulation and subsequent nuclear localization of beta-catenin (MacDonald et al. 2009; Clevers and Nusse 2012; Hikasa and Sokol 2013). Nuclear beta-catenin interacts with the LEF/TCF family of High Mobility Group (HMG) domain transcription factors (Brunner et al. 1997, see below) either to activate or to "derepress" transcription of Wnt-responsive genes (Fig. 6.7).

Signaling is initiated by binding of Wnts to one of seven-transmembrane domain Frizzled (Fzd) receptors plus a coreceptor, lipoprotein receptor-related protein Lrp5 or Lrp6 (Lrp5/6; reviewed in He et al. 2004). Fzds are heterotrimeric G protein-coupled receptors that are activated in response to Wnts (Slusarski et al. 1997b; Katanaev and Buestorf 2009). Wnts bind to Fzd through the receptor's extracellular cysteine-rich domain (CRD), with key contacts being made between the Wnt lipid moiety and a separate hydrophobic "index finger" interacting with grooves in the CRD (Janda et al. 2012). Wnts also interact with the Lrp6 extracellular domain, leading to clustering of the receptors and coreceptors (Tamai et al. 2000; Mao et al. 2001; Kato et al. 2002;

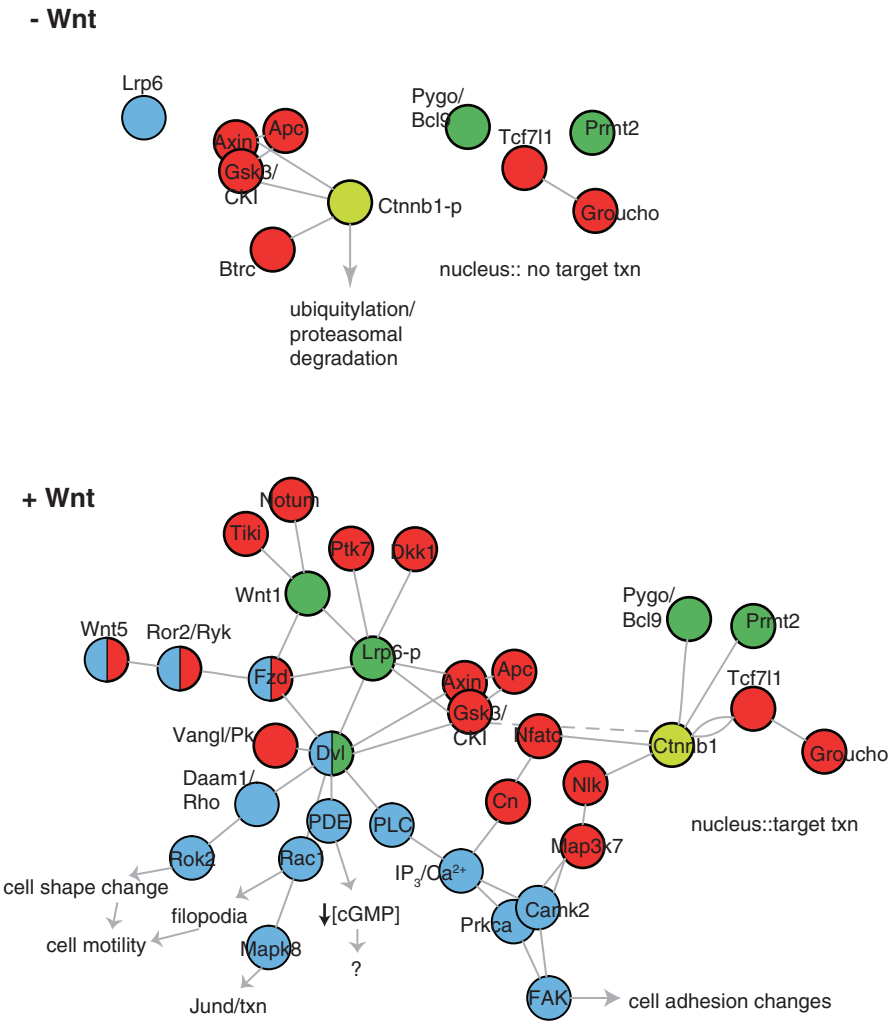

Fig. 6.7 Generalized Wnt signaling networks. In the absence of activating Wnt ligands (*top panel*, -Wnt), beta-catenin protein (Ctnnb1) is phosphorylated by destruction complex components and tagged for proteasomal degradation. In the nucleus, Tcf7l1/Tcf3 represses Wnt target promoter activity through recruitment of Groucho. Upon stimulation with Wnt ligand, a variety of pathways are activated (see text for details). Predominantly positive-acting components with respect to beta-catenin regulation are shown in *green*, negative components in *red*, beta-catenin-independent components are *light blue*. Beta-catenin is shown in *yellow. Circles* indicate component nodes, *lines* indicate edges, or interacting components. This arrangement is not meant to convey specific exact binding relationships or stoichiometry. Wnt1 is shown as a beta-catenin-activating ligand, whereas Wnt5 is shown as a Wnt/PCP and Wnt/Calcium-stimulating ligand. Plot was generated with iGraph in R (Csardi and Nepusz 2014). *txn* transcription

Liu et al. 2003; Itasaki et al. 2003). The induced proximity of the intracellular domains of Fzd and Lrp6 is necessary and sufficient to initiate downstream signaling and inhibition of beta-catenin phosphorylation (Tolwinski et al. 2003).

The inhibition of beta-catenin degradation following Wnt receptor activation remains incompletely understood. Recent observations together with extensive data on biochemical interactions have suggested that Wnt-Fzd binding activates Dvl, possibly through GPCR activation, to recruit Axin/Gsk3b/Ck1a complexes to the Lrp6 intracellular domain, resulting in phosphorylation by Gsk3b and Ck1a (Mao et al. 2001; Tolwinski et al. 2003; Cliffe et al. 2003; Tamai et al. 2004; Davidson et al. 2005; Zeng et al. 2005, 2008; Egger-Adam and Katanaev 2010; Jernigan et al. 2010). Lrp6 phosphorylation occurs at PPPSPxS motifs, which serve as sites for additional Axin complex recruitment and are thought to directly inhibit destruction complex Gsk3b activity (Piao et al. 2008; Cselenyi et al. 2008; Wu et al. 2009). Furthermore, Axin itself is a Gsk3b substrate (Yamamoto 1999) and Gsk3b inhibition results in Axin dephosphorylation and its dissociation from phospho-Lrp6 and beta-catenin (Kim et al. 2013). Axin is then free to be either phosphorylated again to reconstitute beta-catenin destruction complexes or degraded. These data are consistent with a kinetic analysis of beta-catenin regulation, which suggests that Wnt signaling results in partial inhibition of both Gsk3b and Ck1a activities (Hernández et al. 2012). It is possible that this effect could be explained by the inactivation of a subset of limiting and finite destruction complexes through Axin dephosphorylation, which would then depress overall beta-catenin phosphorylation at the population level in a distributed manner.

In a separate but not necessarily mutually exclusive model, Dvl recruitment leads to multimerization of phospho-Lrp6-Fzd complexes, followed by accumulation of Dvl aggregates, leading to positive feedback recruitment and inactivation of destruction complexes (Metcalfe and Bienz 2011; Dobrowolski and De Robertis 2012). There is also evidence that these receptor complexes are incorporated into signaling endosomes (Lrp6 signalosomes) to stabilize and amplify signaling (Bilic et al. 2007). Other data suggest that these signalosomes are eventually sequestered into multivesicular bodies, leading to the longer-term removal of Gsk3b activity and the inability to phosphorylate newly synthesized beta-catenin (Taelman et al. 2010).

6.3.1.2 Transcriptional Regulation by Wnt/Beta-Catenin Signaling

Transcriptional responses in response to beta-catenin are mediated by binding to Lymphoid Enhancer-binding Factor 1 (Lef1)/Transcription factor 7 (T-cell specific, HMG-box; Tcf7) proteins. These proteins are constitutively nuclear and typically repress target genes by recruiting Groucho family repressors (Roose et al. 1998; Barker et al. 2000). Beta-catenin accumulation can lead to displacement of Groucho and activation of target genes, through a combination of derepression and transcriptional activation, mediated by distinct Lef1/Tcf7 proteins. Tcf7l1 (Tcf3 hereafter) likely exclusively acts as a transcriptional repressor during early development, with Lef1 and Tcf1 proteins serving as activators, and Tcf7l2 (Tcf4) exhibiting

spliceform-dependent activator and repressive functions (Pukrop et al. 2001; Gradl et al. 2002; Wöhrle et al. 2007; Weise et al. 2010).

This protein family has diverged in function, with Tcf3 primarily performing a repressive role during early development and others acting as beta-catenin-dependent co-activators later in development (see Sect. 6.5.3). Tcf3 constructs lacking the ability to interact with beta-catenin, by deletion of the N-terminal beta-catenin-binding domain (deltaNTcf3), have been used to inhibit Wnt/beta-catenin-regulated transcription, as these cannot be derepressed or activated by beta-catenin. Expression of deltaNTcf3 during the cleavage stages efficiently ventralizes embryos (Molenaar et al. 1996; Pelegri and Maischein 1998), but fails to inhibit ventrolateral development or to block a late Wnt overexpression effect in *Xenopus* (i.e., anterior truncations; Hamilton et al. 2001). Additionally, experimental depletion of Tcf3 is sufficient to activate Wnt target gene expression during vertebrate axis development (Kim et al. 2000; Houston et al. 2002; Dorsky et al. 2003; Merrill et al. 2004) and in embryonic stem cells (Yi et al. 2008). Recent data from mouse studies in which the mutant deltaNTcf3 was knocked into the endogenous *Tcf7l1* locus have substantiated the idea that Tcf3-mediated repression is critical for its role in early development (Wu et al. 2012a). Gastrulation proceeded normally in these mice, suggesting that the proper amount of transcriptional derepression of Tcf3 targets can occur in the absence of beta-catenin-Tcf3 interactions during axis formation. However, beta-catenin interactions with Tcf3 and with other Lef1/Tcf7 proteins are required later in development and in cancer cells (Wu et al. 2012a; Shy et al. 2013).

Derepression of Tcf3 is sufficient for Wnt target gene activation, although co-activators are also required for normal development, suggesting both likely operate in vivo. Beta-catenin recruits a number of co-activators including p300 and the conserved nuclear complex containing Pygopus and Bcl9 proteins (Kramps et al. 2002; Parker et al. 2002; Belenkaya et al. 2002). Pygo/Bcl9 are thought to be dedicated to Wnt signaling and may regulate the extent that Tcfs and beta-catenin associate with chromatin (Hoffmans et al. 2005; Fiedler et al. 2008; Mieszczanek et al. 2008). Also, beta-catenin has also been implicated in establishing poised chromatin architecture prior to major zygotic gene activation. Evidence in *Xenopus* suggests that beta-catenin recruits Histone H3 Arginine 8 Methyltransferase (Prmt2; Blythe et al. 2010) to modify chromatin at target loci prior to the onset of target gene expression. Thus, Wnt target genes are regulated both by direct transcriptional activation following beta-catenin recruitment and by beta-catenin-regulated changes to chromatin, modes of regulation that may be temporally uncoupled. It is unclear, however to what extent the Pygo-regulated mechanisms and Prmt2 chromatin modifications are interrelated or instead exhibit overlapping or redundant regulation of Wnt targets genes.

6.3.1.3 Wnt/Planar Cell Polarity (PCP) Signaling

The first studies on Wnt signaling focused on the regulation of beta-catenin in development and cancer. Subsequent work found additional roles for a subset of Wnt ligands other components in controlling cell movements during axis organization

and/or antagonism of the Wnt/beta-catenin pathway (Rauch et al. 1997; Rothbächer et al. 2000; Wallingford et al. 2000; Veeman et al. 2003a) (Fig. 6.7). In vertebrates, these beta-catenin-independent Wnt pathways (often referred to, malapropos, as the "noncanonical" Wnt pathways) were shown to act through conserved *Drosophila* planar cell polarity (PCP) homologs and/or through release of intracellular calcium (Wnt/PCP and Wnt/Calcium pathways). Although these different pathway designations are convenient conventions, there is likely a large degree of overlap and interaction among them in vivo, particularly in the case of the Wnt/PCP and Wnt/Calcium pathways, and the ultimate outcome of signaling is likely dependent on the complement of Fzd receptors and coreceptors present on a given cell. This important point was exemplified early on by experiments showing that Wnt5a, traditionally considered a beta-catenin-independent Wnt ligand, could induce second axes in *Xenopus* when co-expressed with its cognate receptor Frizzled 5 (He et al. 1997), and recently by studies demonstrating Wnt5a-mediated regulation of beta-catenin-dependent and -independent Wnt signaling in mammals (Mikels and Nusse 2006; van Amerongen et al. 2012).

PCP signaling in vertebrates involves a set of components largely homologous to those mediating planar cell polarity signaling during imaginal disc development in insects (Vinson and Adler 1987; Krasnow and Adler 1994). This core set of proteins controls asymmetric Fzd1 localization (Strutt 2001) independently of Wnt ligands (Lawrence et al. 2002) and have been characterized genetically and biochemically in *Drosophila* (reviewed in Maung and Jenny 2011; Jose Maria Carvajal-Gonzalez 2014). These proteins include Fzd, Dishevelled, Flamingo (Fmi, a seven transmembrane pass cadherin), Prickle (Pk, a LIM and PET domain protein), strabismus/Van Gogh (Stbm/Vang, a four transmembrane protein with a PDZ motif), and Diego (Dgo, an ankyrin repeat protein). Homologous proteins also control epithelial cell and tissue polarity in vertebrates, notably in the inner ear (reviewed in Veeman et al. 2003a; Bayly and Axelrod 2011). Additionally, vertebrate PCP proteins are critical for controlling cell shape and cell migration in mesenchymal-type cells. Cell intercalation and cell migration during vertebrate gastrulation and neurulation in particular are dependent on Wnt/PCP signaling (reviewed in Solnica-Krezel and Sepich 2012).

The mechanisms of signal transduction during Wnt/PCP signaling in vertebrates are more varied and less well characterized than those of the beta-catenin-dependent pathway. Activation of the Wnt/PCP pathway in vertebrates is dependent on certain Wnt-Fzd combinations and a different set of coreceptors instead of Lrp5/6, including Ryk (Kim et al. 2008; Yoshikawa et al. 2003), Ror2 (Schambony and Wedlich 2007; Gao et al. 2011), and various Glypican proteoglycans (Topczewski et al. 2001; Ohkawara et al. 2003). Additionally, the transmembrane protein encoding Protein tyrosine kinase 7 (Ptk7) has been characterized as a novel regulator of PCP signaling (Lu et al. 2004; Yen et al. 2009). The role of Ptk7 is unclear, but it may represent an additional Wnt coreceptor modulating beta-catenin inhibition and activation with PCP signaling (Peradziryi et al. 2011; Hayes et al. 2013; Bin-Nun et al. 2014; Linnemannstöns et al. 2014). Dvl involvement is also critical for beta-catenin-independent Wnt signaling, although different domains are important for each function by controlling protein complex assembly and subcellular localization (Axelrod

et al. 1998; Boutros and Mlodzik 1999; Rothbächer et al. 2000). The N-terminal DIX domain (Dishevelled, Axin) is critical for beta-catenin regulation whereas the C-terminal DEP (Dvl, Egl-10, Pleckstrin) domain regulates PCP and calcium signaling. In *Drosophila*, Dvl associates with Fzd1 and localizes to the distal cell margin. This complex inhibits the distal accumulation of Vang/Pk complexes, which are restricted proximally.

In vertebrates, similar complexes are implicated but the assembly and asymmetry of these is less understood. Fzd/Dvl association likely occurs following GPCR activation, and Dvl and Fzds accumulate in asymmetric puncta in cells in various vertebrate tissues undergoing PCP. In the zebrafish gastrula, Dvl-GFP is localized to the posterior membrane of cells whereas injected *Drosophila* Prickle-GFP localizes to the opposite, anterior edge (Ciruna et al. 2006; Yin et al. 2008). Additionally, in the mouse posterior notochord/node, Prickle2 and Vangl1 colocalize at the anterior edge of cells (Antic et al. 2010) and Dvl-GFP localizes posteriorly (Hashimoto et al. 2010). However, in other tissues such as the cochlea, Vangl2 and Fzd3 colocalize (Wang and Nathans 2007), but Prickle2 and Fzd6 localize to opposite sides (Deans et al. 2007). Celsr1 (a vertebrate Fmi homolog; cadherin, EGF LAG seven-pass G-type receptor 1) may also play a role in recruiting Dvl/Fz complexes to adherens junctions in the neural plate and mediating subsequent signaling (Nishimura et al. 2012). Thus in vertebrates, the roles of the different core PCP components may have diverged following gene duplication and the acquisition of Wnt ligand dependence and may have taken on tissue- or cell type-specific roles.

Dvl recruitment in the context of Wnt/PCP signaling is implicated in the control of cytoskeletal dynamics through the activation of small GTPases. Dvl can recruit the Formin-related Daam1 protein to activate Rho in an Wnt-dependent manner and regulate actin dynamics (Habas et al. 2001). Additionally, Rho activation can lead to Rho kinase (Rok2) activation to control cell shape (Marlow et al. 2002; Tahinci and Symes 2003). In a separate and parallel pathway, Dvl can directly activate Rac downstream of Wnt, leading the stimulation of filopodial extensions and Mapk8 (Jun N-terminal kinase, JNK) activation (Habas et al. 2003; Tahinci and Symes 2003). The coordinate activity of Rho and Rac, and potentially other small GTPases, is required for cell intercalation and convergent extension morphogenesis in many developing tissues.

6.3.1.4 Wnt/Calcium Release Signaling

Certain Wnt-Fzd combinations can stimulate the release of intracellular calcium stores (reviewed in Veeman et al. 2003a; Kohn and Moon 2005) and signal independently of beta-catenin. The regulation of this pathway also begins with Fzd-mediated heterotrimeric G protein activation and involves well-characterized GPCR responses, namely phosphoinositide turnover (Slusarski et al. 1997b), activation of cGMP-phosphodiesterase (Ahumada et al. 2002), as well as Calmodulin-dependent protein kinase 2 (Camk2) and Protein kinase C (Prkca) activation (Sheldahl et al. 1999; Kuhl et al. 2000). Many of the same coreceptors involved in Wnt/PCP

signaling are also critical for the Wnt/calcium pathway, suggesting that these pathways overlap considerably (Fig. 6.7). In line with this idea, overexpression of Dvl can initiate calcium flux and activate Camk2 and Prkca in fish and frog embryos (Sheldahl et al. 2003). Similarly, overexpression of Prickle1 indirectly regulates calcium dynamics (Veeman et al. 2003b). Also, recruitment of Dvl to the membrane during PCP signaling requires a calcium-regulated PKC isoform, Prkcd (Kinoshita et al. 2003). Wnt/PCP and Wnt/Calcium are likely to be tightly integrated, owing to shared components and shared roles in regulating morphogenesis during gastrulation and beta-catenin antagonism.

Evidence suggests that Wnt/Calcium signaling is essential for inhibiting beta-catenin activation during axis formation. Loss of maternal Wnt5b in zebrafish eliminates calcium flux in the blastula and triggers ectopic beta-catenin activity, resulting in dorsalized embryos (Westfall et al. 2003). This effect was partially rescued by Camk2, suggesting that calcium-mediated activation of this pathway is sufficient to suppress beta-catenin activity. Wnt/Calcium is also implicated in activating Nemo-like kinase (Nlk) (Ishitani et al. 1999, 2003; Meneghini et al. 1999) and Nfatc nuclear translocation (Saneyoshi et al. 2002) to antagonize beta-catenin activity.

6.3.1.5 Wnt Secretion and Extracellular Regulation

Wnts are secreted and are modified by glycosylation (Brown et al. 1987; Papkoff et al. 1987) and lipidation (Willert et al. 2003). Efficient secretion of Wnts requires glycosylation and palmitoleoylation, the latter of which is mediated by the Porcupine (Porcn) family of acyl transferases (van den Heuvel et al. 1993; Kadowaki et al. 1996; Hofmann 2000; Tanaka et al. 2000). Tyrosine sulfation has also been observed and may be necessary for activity in some cases (Cha et al. 2009). Wnt secretion also requires trafficking of Wnt from the Golgi apparatus to the plasma membrane by the Wntless Wnt ligand secretion mediator (Wls; *alias* Evi/Gpr177/Wingful) as well as efficient recycling of Wls through the endosome-retromer system (Bartscherer et al. 2006; Coudreuse 2006). Interestingly, *Wls* is a direct Wnt/beta-catenin target gene in mouse and is required for extracellular Wnt signaling during mouse axis formation (Fu et al. 2009), indicating that Wnt activity potentiates its own signaling. Additional evidence suggests Wnt proteins may also be packaged into lipoprotein particles and/or exosome vesicles (Panáková et al. 2005; Gross et al. 2012). Wnts can act as both long-and short-range signaling molecules in the extracellular space, acting as developmental morphogens (Zecca et al. 1996). Wnt signaling gradients can also interact with those of a Wnt antagonist, Dkk1, to establish hair follicle spacing through a Turing-like reaction-diffusion mechanism (Sick et al. 2006), illustrating one of the complex ways this pathway can used to establish tissue patterns in development.

Wnt signaling can be tightly regulated in the extracellular space by a host of different Wnt antagonists. Many of these proteins belong to large protein families and have redundant and tissue-specific functions throughout development (Cruciat and Niehrs 2013). The main secreted Wnt antagonists involved in axial patterning are the

Secreted Frizzled-related proteins (Sfrps), which bind directly to Wnts and antagonize different Wnt ligands, and Dickkopf1 (Dkk1), which acts at the level of the Wnt/Lrp6 receptor complex. In addition, the secreted Notum pectinacetylesterase homolog was identified as a Wnt antagonist in *Drosophila* (Giraldez et al. 2002; Gerlitz and Basler 2002) and is thought to act by promoting membrane shedding of Glypican Wnt coreceptors (Kreuger et al. 2004). Recent data from flies and vertebrates also suggests that Notum acts as a Wnt deacylase, cleaving the Wnt palmitoleate moiety, resulting in Wnt ligand oxidation and inactivation (Kakugawa et al. 2015; Zhang et al. 2015). Notum is conserved and is involved in feedback regulation of Wnt signaling body axis patterning in Planaria (Petersen and Reddien 2011), and recent data suggest a role in dorsoventral neural tube pattering in zebrafish (Flowers et al. 2012).

Transmembrane antagonists have recently been identified as well. Those with roles in axis formation include the leucine-rich repeat protein Trophoblast glycoprotein (Tbgp/Waif1) and Tiki1 (Trabd2a). Tbgp is thought to act as a feedback Wnt inhibitor, acting in Wnt-receiving cells to alter Lrp6 subcellular localization (Kagermeier-Schenk et al. 2011). Trabd2a/Tiki1 is a transmembrane metalloproteinase enriched in the organizer that can cleave a subset of Wnt ligands, causing their abnormal oxidation and oligomerization and reduced receptor binding (Zhang et al. 2012).

6.3.2 Wnt/Beta-Catenin Signaling in Early Axis Formation

The central role of Wnt/beta-catenin signaling in axis formation was initially demonstrated largely through simple overexpression experiments in *Xenopus* and zebrafish embryos. The first of these was the induction of axis duplications in *Xenopus* by injected mouse *Wnt1* mRNA (McMahon and Moon 1989). *Xenopus wnt8a* (*Xwnt-8*; Christian et al. 1991; Sokol et al. 1991, Smith and Harland 1991), and several other Wnt ligands (Wolda et al. 1993; Du et al. 1995; Kelly et al. 1995a) can also induce secondary axes and rescue UV ventralization. Importantly, beta-catenin also exhibits axis inducing activity (Funayama et al. 1995; Guger and Gumbiner 1995), and both Wnt and beta-catenin can induce axial structures non-cell autonomously when expressed in vegetal blastomeres, suggesting that this activity acts analogously to a Nieuwkoop center (Smith and Harland 1991; Guger and Gumbiner 1995).

Interestingly, later overexpression of *wnt8a* during gastrulation (from injected plasmid DNA as opposed to mRNA) causes a loss of anterior structures, indicating roles for Wnts in patterning of the axis as well as its induction (Christian and Moon 1993). Other Wnts, including Wnts 4, 5a and 11b, do not elicit axis duplications but disrupt gastrulation movements and cell adhesion when overexpressed (Moon et al. 1993; Ku and Melton 1993; Du et al. 1995). Additionally, these Wnts can be antagonistic to the axis-inducing Wnts in some cases (Torres et al. 1996). The same ligands were also shown to trigger intracellular calcium release when expressed in the early zebrafish embryo (Slusarski et al. 1997a, b).

Loss-of-function experiments have established that Wnt/beta-catenin signaling is essential for axis formation in vertebrates. The first evidence for this came from

antisense oligonucleotide mRNA depletion of maternal *ctnnb1* mRNA in *Xenopus* oocytes, leading to embryos lacking axial structures and dorsal-specific gene expression (Heasman et al. 1994). Additionally, in Nieuwkoop conjugate experiments, late blastula vegetal masses from *ctnnb1*-depleted embryos fail to induce dorsal mesoderm in animal caps, suggesting that maternal Wnt/beta-catenin is essential for the generation of the Nieuwkoop signal and acts upstream of other axis-inducing molecules (Wylie et al. 1996). Analysis of heterochronic Nieuwkoop conjugates pre-and post-midblastula transition also showed that dorsal and general mesoderm induction are primarily zygotic events (Wylie et al. 1996). Beta-catenin is present in dorsal nuclei prior to major zygotic genome activation in the *Xenopus* morula and blastula as well as in the zebrafish dYSL and dorsal marginal blastomeres (Schneider et al. 1996; Jesuthasan and Stähle 1997; Kelly et al. 2000; Dougan et al. 2003) demonstrating that Wnt/beta-catenin signaling is active in the relevant region of the embryo.

In addition to theses data, genetic studies in zebrafish identified a requirement for a maternally expressed beta-catenin in normal axis formation (*ctnnb2*/*ichabod*; Kelly et al. 2000). Furthermore, in the mouse, genetic deletion of *Ctnnb1* results in embryos lacking axial structures and anteroposterior polarity, resulting from lack of all mesoderm and failure to form the anterior visceral endoderm (AVE) (Haegel et al. 1995; Huelsken et al. 2000; Morkel et al. 2003). Mouse beta-catenin is predominantly required in the epiblast, as shown in chimeric embryo experiments (Huelsken et al. 2000) and is not required maternally (De Vries et al. 2004), reflecting a different mode of activation in mammals. The main Wnt ligand expressed at this time, *Wnt3*, is primarily expressed in the posterior epiblast and is required for embryonic axis and mesoderm formation (Liu et al. 1999b). Interestingly, the formation of the AVE is normal in *Wnt3* null mice, indicating a differential role for beta-catenin in the development of this tissue. A Wnt ligand-independent role for beta-catenin in anteroposterior patterning has been proposed (Morkel et al. 2003), possibly through regulation of *Tdgf1* (*teratocarcinoma-derived growth factor 1*, *alias Cripto/frl1*) expression and subsequent effects on Nodal activity (see Sect. 6.5). Although genetic manipulations are less tractable in the chicken, studies using extracellular Wnt inhibitors suggested that Wnt signaling is required for experimental axis induction (Skromne and Stern 2001).

6.3.3 Asymmetric Activation of Wnt/Beta-Catenin Signaling in Early Amphibian and Fish Embryos

6.3.3.1 Xenopus

Despite the well-documented roles for beta-catenin in axis formation in *Xenopus*, and more recently in zebrafish, it remains relatively unclear how Wnt/beta-catenin signaling is initiated in early embryos, as well as the extent that these activating mechanisms are conserved. Cytoplasmic transplantation studies in *Xenopus* identified the presence of a cytoplasmic, transplantable dorsalizing activity in the vegetal

cortical region (Darras et al. 1997; Marikawa et al. 1997; Marikawa and Elinson 1999). By correlating its activity with various axis-inducing molecules, this vegetal cortical cytoplasm was found to mimic intracellular activation of the Wnt/beta-catenin signaling pathway (Marikawa and Elinson 1999). Curiously, UV-irradiation experiments in *Xenopus* oocytes indicated that this cytoplasm showed cell cycle-dependent sensitivity to UV Irradiation of the egg disrupts microtubule assembly and cortical rotation, although the activity of the vegetal cortical cytoplasm itself is not affected. By contrast, UV-irradiation of full-grown oocytes effectively does eliminate the dorsalizing ability of vegetal cytoplasm and ventralizes embryos (Holwill et al. 1987; Elinson and Pasceri 1989). Eggs irradiated as oocytes undergo normal cortical rotation and are not rescued by tipping (Elinson and Pasceri 1989), suggesting that a critical component of axis induction is absent.

The target of UV irradiation in the oocyte is not known, but either its action is completed by oocyte maturation or it is subsequently sequestered and no longer susceptible to irradiation. These features may be useful in identifying potential candidate molecules. In apparent support of a direct cytoplasmic beta-catenin activation model, particles of exogenous Wnt-activating proteins, Dvl2-GFP (Miller et al. 1999) and Frat1-GFP (Weaver et al. 2003) were shown to undergo dorsal translocation during cortical rotation, suggesting that the dorsalizing activity might be composed of beta-catenin stabilizing agents. These molecules might then directly stabilize beta-catenin, or act by sensitizing dorsal cells to Wnt signals. Further indications of potential Wnt ligand-independent dorsalizing mechanisms came from observations that overexpression of secreted Wnt antagonists were unable to suppress endogenous axis formation (Hoppler et al. 1996; Leyns et al. 1997; Wang et al. 1997).

More recent studies in *Xenopus* suggest a more typical Wnt signaling model, with maternal Wnt11b acting to induce beta-catenin activity. Maternal *wnt11b* is localized to the vegetal cortex during oogenesis via a mitochondrial cloud-dependent pathway (see Chap. 8) and was initially considered a prime candidate for the vegetal dorsally activity, based on its activity in UV-rescue experiments. In light of the evidence favoring the direct activation model (see above), and other experiments showing that Wnt11b can regulate beta-catenin-independent signaling, a role for *wnt11b* in axis formation was later discounted. However, a reinvestigation using antisense oligo mediated maternal mRNA depletion showed that maternal *wnt11b* is indeed required for axis formation (Tao et al. 2005).

Wnt11b is though to act in concert with uniformly expressed Wnt5a (Cha et al. 2008), forming extracellular complexes with each other and with other proteins, including heparin sulfate proteoglycans and the Nodal coreceptor Tdgf1 (Tao et al. 2005; Cha et al. 2008, 2009). The activity of this Wnt complex can be antagonized by maternal Dkk1, suggesting a model in which cortical rotation tips the balance of Wnt activity to overcome generalized Wnt antagonism (Cha et al. 2008). The mechanism of Wnt11b enrichment dorsally following cortical rotation is unclear, as both enrichment of total *wnt11b* RNA (Tao et al. 2005) or enhanced polyadenylation (Schroeder et al. 1999) have been proposed. A similar mechanism, albeit with different Wnts and antagonists, has been proposed in zebrafish (see below). However, Wnt11b can also regulate beta-catenin levels in an autocrine fashion in fully grown

oocytes (Kofron et al. 2007), providing the possibility that Wnt activation may wholly or partially occur before fertilization. Possible mechanisms for Wnt activation in *Xenopus* are shown Fig. 6.8 (top panels).

Although there is good evidence for secreted Wnt11b activity, it is unclear when Wnt11b is required. Also, this role for Wnt11b has not been reconciled with the cytoplasmic activation model. One potential unifying model has been proposed; that ongoing Wnt signaling in the oocyte generates activated Lrp6 signaling endosomes that are transported dorsally (Dobrowolski and De Robertis 2012). However, it is not known to what extent these Lrp6-containing endosomes are formed in the oocyte. Lrp6 is phosphorylated in eggs but becomes dephosphorylated following egg activation (Davidson et al. 2009), suggesting that stable signaling complexes may actually be inactivated prior to cortical rotation. Furthermore, analyses of the relative activity of vegetal cortical cytoplasm suggests it acts at the level of the destruction complex and does not mimic the activity of activated receptors (Marikawa and Elinson 1999).

6.3.3.2 Zebrafish

The regulation of Wnt signaling in zebrafish also has intriguing parallels and differences to the situation in *Xenopus* (Fig. 6.8, bottom panels). Maternal/zygotic mutants of *wnt11* form the axis normally in zebrafish (Heisenberg et al. 2000), suggesting that fish use other mechanisms or other Wnts. Maternal *wnt8a* has been proposed to act as a dorsal determinant in zebrafish (Kelly et al. 1995a; Lu et al. 2011). *Wnt8a* is the only vegetally localized *wnt* transcript in the yolk cell, and is shifted asymmetrically following cortical rotation. Vegetal localization also occurs through a mitochondrial cloud dependent mechanism, similar to frog *wnt11b* (Lu et al. 2011). In the fish however, injection of a dominant-negative Wnt8a construct was able to reduce the expression of *chrd* and *dharma*, the latter being a direct Wnt target gene. Full-length Wnt8a also rescues to some extent embryos ventralized by nocodazole (Lu et al. 2011). Also, depletion of Sfrp1 or Frzb hyperdorsalizes embryos, indicating dorsal enrichment of a maternal Wnt, Wnt8a in the case of fish, may be a trigger to overcome generalized Wnt antagonism in the embryo. While this and the similar mechanism proposed to act in frog are intriguing (Cha et al. 2008), it remains to seen whether relatively small changes in Wnt levels involved are responsible for dorsalization.

Evidence in fish is also suggests that other mechanisms of Wnt antagonism are critical in suppressing beta-catenin activation. Notably, maternal/zygotic mutants for *wnt5b* are hyperdorsalized, owing to defective Wnt/calcium signaling and failure to repress Wnt/beta-catenin signaling (Westfall et al. 2003). A similar but not well-characterized pathway may exist in frogs (Saneyoshi et al. 2002). In *Xenopus*, the maternal role of Wnt5b has not been assessed, however Wnt5a is proposed to activate instead of repress Wnt/beta-catenin signaling in frogs (Cha et al. 2008). Axis formation is largely normal however in *wnt5a*, *wnt5b* double mutant mice (Agalliu et al. 2009; spinal cord formation), suggesting that the roles of Wnt5 paralogues may be

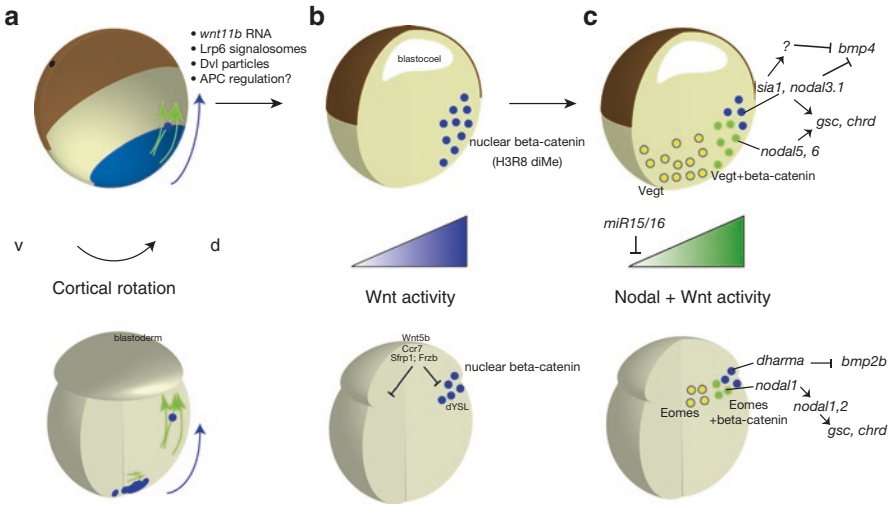

Fig. 6.8 Models for Wnt/beta-catenin activation in *Xenopus* and zebrafish. (**a**) During cortical rotation in *Xenopus* (*top*) and zebrafish (*bottom*), beta-catenin stabilizing dorsalizing activity is transported into the equatorial region of the embryo by microtubule-mediated rotation of the cortex and through transport along microtubule arrays. Candidates for this activity include *wnt11b* and Lrp6/Dvl particles in *Xenopus* and *wnt8a* in zebrafish. (**b**) By the cleavage stages (16–128-cell stage), beta-catenin be-comes activated and enriched in dorsal vegetal and marginal nuclei until MBT. In *Xenopus*, priming of Wnt target genes occurs through dimethylation of Histone3 at arginine 8 (H3R8). In zebrafish, beta-catenin accumulates in dorsal marginal and dYSL nuclei, and is antagonized by multiple antagonists and calcium signaling mediators. (**c**) During the peri-MBT stages, beta-catenin activates direct Wnt targets and cooperates with maternal T-domain proteins (Vegt, Eomes) to activate nodal initially on the dorsal side. The combination of nodal and BMP antagonism induced by beta-catenin induce the formation of the organizer (*gsc*, *chrd*)

species-specific. Recent data suggest that calcium transients downstream of the chemokine receptor Ccr7 GPCR signaling are also involved in suppressing beta-catenin activity (Wu et al. 2012b). The prominence of calcium regulation mechanisms suggests tight control over beta-catenin stabilization in the zebrafish blastula.

6.3.4 *Beta-Catenin Activity Dorsalizes the Primary Germ Layers*

Beta-catenin stabilization in the blastula following cortical rotation is the central mechanism for establishing early dorsal fates across all three primary germ layers (Fig. 6.8). In the vegetal prospective endodermal cells, beta-catenin is critical for the dynamic regulation of Nodal expression and signaling, which is directly involved in mesendoderm induction and patterning and likely constitutes what is referred to as the Nieuwkoop center (see Chap. 7). In *Xenopus*, the typical *nodal homolog* genes (e.g., *nodal homologs 1*, *2*, *4*, *5*, and *6*) are expressed in a temporally and spatially

graded fashion in the late blastula/early gastrula. These genes depend on vegetally localized maternal *vegt* activity, showing high dorsovegetal expression at the onset of gastrulation, followed by a shift ventrolaterally by late gastrulation, mirrored by Smad2 activity (Agius et al. 2000; Faure et al. 2000; Lee et al. 2001). Maternal beta-catenin directly patterns this activity first by contributing to early *nodal* paralogue expression (*nodal5*, *nodal6*) in dorsovegetal blastomeres prior to and immediately after the onset of zygotic transcription at the mid-blastula transition (MBT; see Chap. 9) (Takahashi et al. 2000; Rex et al. 2002; Xanthos et al. 2002; Hilton et al. 2003; Blythe et al. 2010). This role of beta-catenin may be related to its function in recruiting Prmt2 to prime gene expression during the cleavage stages (Blythe et al. 2010). Beta-catenin also synergizes with Nodal activity to generate higher *nodal1* expression dorsally in the early gastrula, although mid-late gastrula expression becomes uniform and is independent of beta-catenin (Agius et al. 2000; Lee et al. 2001).

In addition to overlapping with Vegt, beta-catenin might also functionally interact with Gdf1 (*alias* Vg1), which is encoded by another maternally localized mRNA (Rebagliati et al. 1985; Melton 1987; Weeks and Melton 1987). Gdf1 is a Nodal-related Tgfb family ligand and activates Smad2 signaling through Nodal receptors/coreceptors. Wnt/beta-catenin activity synergizes with *gdf1* overexpression to induce axial structures in *Xenopus* (Cui et al. 1996). Maternally depleted *gdf1* embryos lack anterior structures and are deficient in the expression of certain organizer markers, including secreted antagonists *nog*, *chrd*, *dkk1*, and *cer* (Birsoy et al. 2006). Early Smad2 activation is also compromised, indicating that Gdf1 contributes to overall Nodal signaling on the dorsal side of the late blastula (Birsoy et al. 2006). Thus, beta-catenin activity can enrich Nodal expression and activity dorsally at multiple regulatory levels.

This temporal control of Nodal activity is functionally significant. In Nieuwkoop conjugate experiments, late blastula vegetal explants from *ctnnb1*-depleted *Xenopus* embryos fail induce dorsal fates in early equatorial or animal cap tissue (Xanthos et al. 2002; Wylie et al. 1996). However, if *ctnnb1*-depleted vegetal explants from late gastrula embryos are used, which now express *nodal* genes, dorsal mesoderm is induced (Xanthos et al. 2002). Additionally, pre-MBT Nodal activity regulated by beta-catenin is essential for normal axis formation, possibly for perpetuating *nodal* expression through autoinduction (Skirkanich et al. 2011). Beta-catenin also likely contributes to Nodal ongoing autoregulation, both directly and indirectly. Dorsal beta-catenin activity is required to repress *microRNAs 15* and *16* (*miR15/16*) through an unknown mechanism (Martello et al. 2007). These miRs target and downregulate an essential component of the Nodal receptor complex, *activin A receptor, type IIA* (*acrv2a*) (Martello et al. 2007), which contributes to the early dorsal bias in Nodal activity.

In zebrafish, maternal beta-catenin signaling similarly regulates early *nodal homolog* gene expression. Two paralogues are expressed in the early dYSL (*nodal1/squint* and *nodal2/cyclops*); *nodal1* is likely a direct beta-catenin target (Kelly et al. 2000) and *nodal2* is autoregulated by Nodal signaling itself. Genetic and other loss-of-function experiments support a role for the early Nieuwkoop center *nodal* genes in axis formation. Single and double mutants for *nodal1*, *nodal2* (*squint*, *cyclops*) exhibit axial patterning and mesendoderm defects (Rebagliati et al. 1998; Feldman

et al. 1998; Sampath et al. 1998; Erter et al. 1998), and these abnormalities can be rescued by YSL-specific expression of Nodal ligands. Additionally, *nodal1* is maternally expressed in zebrafish and its mRNA localized to the presumptive dorsal side at the 4-cell stage, through an uncharacterized mechanism involving microtubules (Gore et al. 2005). The role of this maternal transcript is not fully clear; it may lead to enhanced Nodal activity dorsally, although there are hints that the 3′UTR may help promote dorsal nuclear beta-catenin accumulation by acting as a noncoding RNA (Lim et al. 2012).

In addition to stimulating higher levels of Nodal activity, dorsal beta-catenin directly initiates a set of gene regulatory interactions that integrate with Nodal signaling in the prospective mesendoderm (Fig. 6.8). In *Xenopus*, Wnt/beta-catenin signaling directly activates the paired homeobox paralogues *siamois1* (*sia1*) and *siamois2* (*twin/sia2*) in dorsovegetal cells in the blastula (Lemaire et al. 1995; Brannon and Kimelman 1996; Brannon et al. 1997). Sia1/2 integrate with Nodal/Tgfb signaling in activating additional genes in the anterior endoderm and organizer, including *cerberus*, *hhex*, and *goosecoid*, whose promoters are bound by Sia1 (Ishibashi et al. 2008; Rankin et al. 2011; Sudou et al. 2012). Maternal beta-catenin signaling is also required to repress early *bmp4* expression dorsally in *Xenopus* (Baker et al. 1999), and this is also mediated through Sia1/2. These proteins are required to repress Bone Morphogenetic Protein (BMP) expression and activity dorsally, which is essential for dorsal mesoderm formation (see Sect. 6.4.1). Sia1/2 activate the expression of several BMP antagonists, including *noggin* (*nog*) and *chordin* (*chrd*) (Ishibashi et al. 2008), and also indirectly repress *bmp4* expression, through an unknown intermediate. *Nog* and *chrd* expression are maintained by Nodal signaling, but initial expression depends only on maternal beta-catenin (Wessely et al. 2001). Although Sia1/2 are predominantly expressed in mesendodermal precursors, their expression can extend into some equatorial precursors that escape mesoderm induction (Kuroda et al. 2004), where they specify early neural fate and the prospective anterior neuroectoderm (Ishibashi et al. 2008; Klein and Moody 2015).

Similarly, in zebrafish maternal beta-catenin activates expression of *dharma* in dorsal marginal blastomeres and dYSL. Dharma is a paired homoebox gene somewhat functionally analogous to *sia1*, but unrelated by descent (Fekany et al. 1999; Kelly et al. 2000). Dharma has numerous roles in dorsoventral patterning, including direct repression of *bmp2b* (Koos and Ho 1999; Leung et al. 2003a). Interestingly, both *sia1* and *dharma* are expressed prior to major zygotic gene activation in frogs and fish (Yang et al. 2002; Leung et al. 2003b; Blythe et al. 2010) suggesting very early and direct roles for these proteins in dividing the embryo into prospective BMP-expressing or -absent territories. Additionally in fish, the accumulation of nuclear beta-catenin occurs concomitantly with a change in cell division patterns relative to the body axis as well as slower cell division in the presumptive dorsal shield region, but a causal relationship between these events has not been established (Keller et al. 2008).

Although the activation of Nodal and Sia1/2 appears to mediate many of the functions of maternal beta-catenin signaling, other less characterized pathways are likely required as well. One other beta-catenin target gene, *nodal homolog 3.1*

(*nodal3.1*; Smith et al. 1995), likely functions in controlling morphogenesis during organizer formation. Nodal 3.1 is an atypical member of the Nodal protein family present only in anuran amphibians (Smith et al. 1995). This protein has BMP antagonist activity (Hansen et al. 1997) and, during gastrulation, is restricted to the superficial epithelial layer of the organizer (Glinka et al. 1996), a region with discrete morphogenetic regulatory ability (Shih and Keller 1992a). Loss-of-function experiments suggest that Nodal3.1 regulates convergent extension, acting as a Fibroblast growth factor (FGF) receptor ligand (Yokota et al. 2003), although this mechanism is not well understood.

In summary, beta-catenin function is critical at many different regulatory levels to specify dorsal fate across all prospective germ layers in the early blastula in *Xenopus* and zebrafish. These general functions would include generating dorsal-inducing signals, mediating competence to respond to those signals, reducing the ability of dorsal cells to respond to ventralizing BMP signals, and regulating dorsal morphogenesis. In this context, the strong expression of *nodals* in the dorsovegetal blastomeres is likely responsible for the Nieuwkoop center phenomenon, although beta-catenin activity is required in prospective organizer mesoderm and ectoderm as well. In amniotes, however, axis formation is also governed by the dynamic regulation of Nodal signaling in concert with Wnt/beta-catenin activity. In these cases, the initial establishment of restricted Nodal signaling occurs without early maternal Wnt signaling and the role of a Nieuwkoop center analog in inducing the organizer is less clear.

6.3.5 Wnt/Beta-Catenin and Nodal Signaling During Axis Formation in Amniotes

6.3.5.1 Chick

The role of concerted Wnt and Tgfb signaling in the dynamic regulation of Nodal expression and activity is conserved during amniote axis formation. In contrast however to the case in fish and frogs, axis formation in birds and mammals is driven by spatially restricted Nodal activity overlapping with more generalized Wnt/beta-catenin signaling in the marginal zone of the epiblast (Fig. 6.9a). In the chicken, there is no evidence for localized Wnt expression in the egg or early cleavage stages. *Wnt8a* (*alias*, *CWnt8C*) is the predominant Wnt expressed prior to gastrulation and is enriched in the PMZ epiblast, but is also found in a decreasing gradient around the marginal zone from posterior-to-anterior (Hume and Dodd 1993; Skromne and Stern 2001). By contrast, the Nodal-related gene *Gdf1* is specific to the PMZ at the same stage (Shah et al. 1997; Skromne and Stern 2001) and represents one of the earliest asymmetrically expressed genes in the chick blastoderm (and the first to be discovered).

Grafting of Gdf1-expressing cells induces an ectopic axis anywhere in the marginal zone (Shah et al. 1997; Skromne and Stern 2001). Interestingly, ectopic expression of Wnt alone is not a robust axis inducer in chicken (Joubin and Stern 1999). Gdf1 and Wnt8a can synergize in axis induction, and the axis-inducing abil-

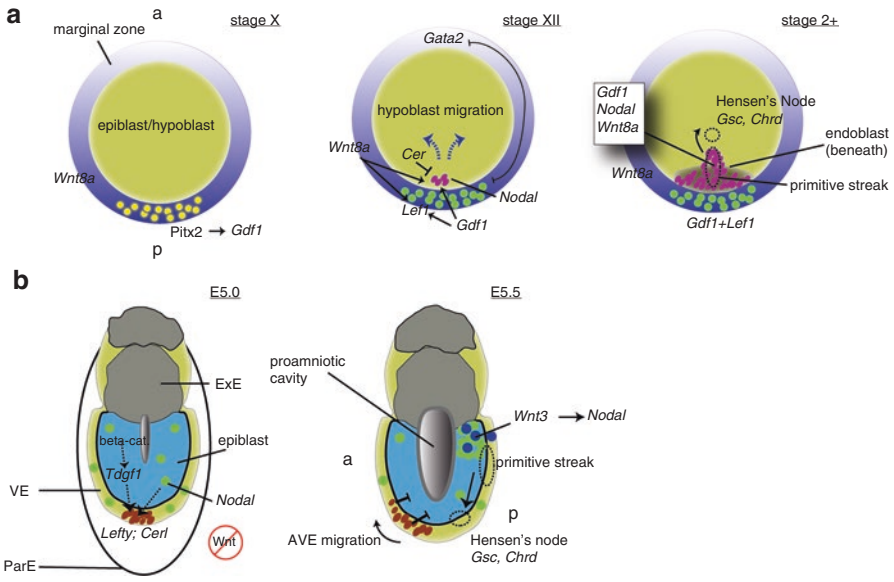

Fig. 6.9 Models for axis induction signaling in chick and mouse. (**a**) In the chick blastoderm (*left panel*, top/dorsal view, ~stage X, Eyal-Giladi and Kochav 1976) the outer marginal zone of the epiblast expresses *Wnt8a* in a posterior-to-anterior gradient (purple shading). In the PMZ, Pitx2 (*yellow*) activates *Gdf1* expression (*green*). Subsequently (*middle panel*), the newly formed hypoblast (below the plane of the page) begins anterior migration and Gdf1 + Wnt8a cooperate to induce *Lef1* in the PMZ and Nodal in the adjacent epiblast. *Gata2* is expressed in the anterior marginal zone and antagonizes Gdf1 long-range. Nodal (*magenta*) is antagonized by Cerberus (Cer), which is expressed in the hypoblast. By the initial primitive streak stage (*right panel*, stage 2+, Hamburger and Hamilton 1951), the anterior migration of the hypoblast and migration of the endoblast beneath the posterior epiblast removes the inhibition of Nodal and allows feed-forward signaling leading to primitive streak formation. The same signaling molecules are ex-pressed in the primitive streak and induce organizer genes in Hensen's node (*dotted circle*, *Gsc*, *Chrd*) at the anterior tip of the streak. (**b**) In the mouse, the earliest asymmetries are the expression of *Lefty1* and *Cerl* (*red-brown*) at the tip of the postimplantation AVE (*left panel*, ~E5.0). These genes are regulated by Nodal (*green*) and Tdgf1, and Tdgf1 is regulated by beta-catenin in the absence of secreted Wnt ligand activity (stop symbol). Lefty1 and Cerl antagonize Nodal and feedback regulation drives AVE migration towards the proximal egg cylinder on one side (*right panel*, ~E5.5). Nodal activity is restricted to the posterior epiblast and is responsible for *Wnt3* expression (*blue*), which in turn maintains Nodal. These signals cooperate to induce the primitive streak, which induces Hensen's node toward the distal tip later in gastrulation. *a* anterior, *p* posterior, *ExE* extraembryonic ectoderm, *VE* visceral endoderm, *ParE* parietal endoderm

ity of Gdf1 is blocked by co-expression with Wnt antagonists (Skromne and Stern 2001). These Gdf1 and Wnt8a signals cooperate to induce *Lef1*, a Wnt signaling transcriptional activator, in the PMZ (Skromne and Stern 2001) coincident with the activation of Nodal in the adjacent area pellucida epiblast (Skromne and Stern 2002). Nodal itself is required for primitive streak and axis formation (Bertocchini and Stern 2002). Based on shared embryological and molecular characteristics, it has been speculated that the PMZ epiblast in chick represents the equivalent of the Nieuwkoop center (Bachvarova et al. 1998). However, in birds and likely in mammals

as well (see below), the middle regions of the primitive streak also express *Gdf1*, *Wnt8a*, and *Nodal* and may be more relevant for inducing the anterior streak and Hensen's node/organizer (Joubin and Stern 1999; Bertocchini and Stern 2002). The Nieuwkoop center in chicks may thus be considered a primitive streak-inducer, as opposed to its more traditional organizer-inducing amphibian role (also see Sect. 6.7). As compared to mammalian axis formation, which depends initially on Nodal asymmetry (see below), the chick may represent an intermediate condition, where Wnt signaling is widely active prior to gastrulation but the role of Nodal-related proteins in inducing the axis is becoming predominant.

The mechanisms controlling localized *Gdf1* expression in the PMZ remain largely unknown. In contrast to the strict requirement for maternal factors in fish and amphibians, there is only a presumptive early influence of maternal determinants in birds, and axis formation is highly self-organizing (i.e., regulative). Classic embryological studies showed that partitioning of the blastoderm results in axis initiation in each individual fragment (Lutz 1949; Spratt and Haas 1960; Callebaut and Van Nueten 1995), suggesting that axis determinants are not uniquely restricted to one region. In recent reinvestigations of this phenomenon, *Gdf1* expression reinitiates stochastically at the "new posterior" pole of these blastoderm explants and regulates formation of the new primitive streak (Bertocchini et al. 2004). The *Wnt8a* gradient presumably reforms around the new site of *Gdf1*, suggesting that *Wnt8a* is likely downstream or independent of Gdf1. There is no evidence to suggest whether this regulation is direct or indirect. Data also show that the transcription factor *Gata2* is expressed earlier than *Gdf1*, and in a roughly complementary anteriorly biased gradient (Bertocchini and Stern 2012). Both Gata2 and Gdf1 control each other's expression indirectly through a global signaling gradient, likely mediated by BMP signaling downstream of Gata2. Additionally, recent microarray screening and functional analyses identified the Pitx2 homeodomain protein as an essential upstream activator of Gdf1 expression in the PMZ (Torlopp et al. 2014). This Pitx/Gdf1/Nodal regulatory relationship is deeply conserved in several well-known developmental processes, including sea urchin axis patterning, amphibian mesendoderm induction and left-right pattering (Torlopp et al. 2014 and references therein), indicating these genes form a robust module can be readily redeployed for novel functions. However, there are as yet no clues to any mechanisms that would potentially activate Pitx2 or connect this pathway to any maternal asymmetries. Interestingly, recent data suggest that chick embryos form a yolk syncytial layer (Nagai et al. 2015), similar to that of teleosts. This is likely the result of independent convergent evolution and it will be exciting to learn to what extent this structure might function similarly in axis induction.

6.3.5.2 Mouse

In contrast to the case in chick, in which the graded expression of *Wnt8a* in the marginal zone still plays some role in normal axis development, several lines of evidence in the mouse suggest that active Wnt/beta-catenin signaling is not part of the early axial polarization mechanism (Fig. 6.9b). Many Wnt genes are expressed in the preimplantation blastocyst (Kemp et al. 2005), although studies in Wnt reporter

mice initially suggested that Wnt signaling is not active in the blastocyst prior to implantation (Mohamed et al. 2004; Na et al. 2007). Recent studies with more sensitive reporter constructs have detected some beta-catenin-dependent activity in the ICM/epiblast of peri-implantation blastocysts (E3.0–4.5; Granier et al. 2011; ten Berge et al. 2011). Similarly, transient nuclear beta-catenin has been observed by immunolocalization at the same stage (Chazaud and Rossant 2006). However, the extent of activity is unclear, since overexpression of stabilized beta-catenin does not ectopically induce Wnt target genes before gastrulation (Kemler et al. 2004). Thus, although there are indications of early but non-polarized beta-catenin activity in the preimplantation mammalian embryo, the role of this signaling appears to be dispensable or easily compensated for, at least with regard to axis formation.

Correspondingly, mutants for the major Wnts, Wnt/beta-catenin coreceptors, as well as regulators of Wnt secretion and activity all undergo normal preimplantation development, including anteroposterior patterning in the AVE. These mutants also fail to form mesoderm and do not gastrulate. These Wnt/beta-catenin pathway mutants include (but are not limited to) *Wnt3* (Liu et al. 1999b), *Lrp5/6* (Kelly et al. 2004), *Mesdc1* (Hsieh et al. 2003), *Wntless* (Fu et al. 2009), and *Porcn* (Biechele et al. 2011, 2013; Barrott et al. 2011). Since beta-catenin mutants exhibit these same mesoderm defects but additionally fail to form the AVE, a Wnt ligand-independent role for beta-catenin in anteroposterior patterning has been proposed (Morkel et al. 2003), possibly mediated by activation of Tdgf1 expression and subsequent effects on Nodal activity (see Sect. 6.5.2). These results suggest possible similarities to the potential role of Wnt ligand independent signaling in *Xenopus* axis formation (see Sect. 6.3.3), but too little is known about either mechanism to draw meaningful comparisons. There are other agonist ligands of Wnt/beta-catenin signaling, such as Norrin and R-spondin (Cruciat and Niehrs 2013), but these also rely on Lrp5/6. Other potential mechanisms of regulating beta-catenin activity may exist, such as regulation through Hippo signaling (Varelas et al. 2010) or through Seven in abstentia homologs (Siah) (Topol et al. 2003), although these have not been extensively investigated in the context of axis formation.

Rather than relying on polarized Wnt activity, the earliest asymmetries in the mouse blastocyst are the expression of Nodal antagonists Cerl and Lefty1 in the primitive endoderm of the peri-implantation blastocyst (~E4.0; Torres-Padilla et al. 2007b). Expression of these genes likely depends on FoxH1-mediated Nodal signaling (Takaoka et al. 2006; Torres-Padilla et al. 2007b), initiating a cascade of both positive and negative feedback regulation of Nodal signaling in the embryo. Cerl/Lefty1 asymmetry is likely to arise stochastically at this early stage, as the blastocyst contains a rather small number of cells and it is unlikely that the few Cerl/Lefty1 expressing cells would arise precisely in the center. These cells are then thought to lead and propagate AVE migration toward the future anterior side, i.e., towards the side of initial asymmetry (Takaoka et al. 2011; Morris et al. 2012a), restricting Nodal activity to the prospective posterior. This aspect will be discussed further in Sect. 6.5. Additionally, *Nodal* is expressed at low levels in the ICM prior to implantation, but there is no evidence that Wnt/beta-catenin signaling activates this expression. Later in development, prior to and during gastrulation, *Nodal* expression is maintained by Wnt3 signals in the posterior epiblast and in the forming primitive

streak (Brennan et al. 2001; Ben-Haim et al. 2006), indicating that Wnt regulation of *Nodal* remains conserved, but is deployed modularly in development.

Interestingly, although Wnt may not activate early *Nodal* expression in the blastocyst, data suggest that Tcf3 repression of *Nodal* is a conserved feature of vertebrate development. Deletion of mouse *Tcf3* (Merrill et al. 2004) results in the formation of secondary axes (Hensen's nodes and notochord) and in upregulation of Nodal target genes (e.g., *Foxa2*; Hoodless et al. 2001; Yamamoto et al. 2001) in the pregastrula embryo. And, *Nodal* is upregulated in *Tcf3* null embryonic stem (ES) cells (Cole et al. 2008). In fish and frogs, early-localized Wnt signals provide an initial signal to relieve repression by Tcf3 (Kim et al. 2000; Houston et al. 2002; Dorsky et al. 2003). Mammals must have evolved different mechanisms to overcome this repression in the absence of pervasive early Wnt activity. Recent data suggest that *Nodal* initiation and potentiation may be controlled by a distinct enhancer bound by pluripotency transcription factors Oct4 and Klf4, in addition to ongoing Smad2-dependent signaling (Papanayotou et al. 2014). Interestingly, Tcf3 null ES cells also exhibit derepression of other genes, in addition to *Nodal*, that are co-regulated by pro-pluripotency factors (Cole et al. 2008), suggesting that in normal ES cells, these proteins may help overcome Tcf3-mediated repression in the absence of strong activation of Wnt/beta-catenin signaling.

Is there a mammalian Nieuwkoop center? In line with a lack of asymmetry in the mammalian egg, clonal analyses and transplant experiments performed in the pregastrula mouse embryo have not identified a region with the requisite Nieuwkoop properties; indeed transplanted epiblast cells typically change fate according to their new position (Lawson et al. 1991; Lawson and Pedersen 1992; Parameswaran and Tam 1995). Also, there is a high degree of cell mixing in the epiblast during normal development (Gardner and Cockroft 1998), which would be inconsistent with a lineage-restricted Nieuwkoop center. However, there is overlap of Nodal and Wnt3 in the posterior proximal epiblast and subsequently throughout the primitive streak (Tam et al. 2006) and high levels of Nodal signaling are required for organizer formation (Vincent et al. 2003). The early proximal epiblast may have some hallmarks of a Nieuwkoop center, although the region of highest and most complete axis-inducing activity is the anterior primitive streak itself, prior to Hensen's node formation (Kinder et al. 2001), similar to the case in chicken. Interestingly, these cells do not significantly contribute to the organizer/Hensen's node or notochord, instead making contributions to the anterior mesendoderm (Kinder et al. 2001). Thus, organizer induction in mammals may still require non-cell-autonomous induction by Wnt and Nodal signals, with the signals being generated from the nascent mesendoderm, as opposed to strictly extra-mesodermal Nieuwkoop center-like mechanisms.

6.4 The Organizer and Dorsoventral Patterning

Axis formation and patterning are largely determined by a discrete zone of midline mesoderm in the prospective dorsal region of the gastrula. The collective cell signaling and morphogenetic properties of these cells and their descendants during

normal development and under experimental conditions led to their designation as the "organization center" or "organizer" (*Organisationszentrum/Organisator*) (Spemann 1921; Spemann and Mangold 1924). During experiments to test the extent of cell fate determination in the gastrula, Spemann and Mangold showed that the dorsal (upper) lip of the newt gastrula could retain its fate when transplanted to the ventral side, typically forming a normally patterned "secondary embryo" that matched the axial organization of the host. By grafting dorsal lips from albino embryos into pigmented hosts (Fig. 6.10), they assessed the contribution of donor cells, and strikingly demonstrated that the secondary dorsal tissues including the neural tube were formed from induced host cells, with donor cells forming notochord and somites (Spemann 1921; Spemann and Mangold 1924). The term organizer was thus coined by Spemann to reflect the ability of the dorsal (upper) blastopore lip to direct the development of the characteristic embryonic structures in vertebrates (notochord, somites, dorsal neural tube).

Spemann's characterization of the organizer was highly influential and rapidly led to the identification of homologous regions in other vertebrates: birds (chick and duck, Hunt 1929; Waddington 1930), mammals (chick into rabbit, Waddington 1934; rabbit into rabbit/chick, Waddington 1936, 1937; mouse into frog, Blum et al. 1992; and mouse into mouse, Beddington 1994), lamprey (Bytinski-Salz 1937; Yamada 1938), and teleost fish (minnow, perch, and trout, Oppenheimer 1934a, b; Luther 1935). Similar dorsalizing and neuralizing activities have also been found for the prospective notochordal cells of invertebrate cephalochordates (Tung et al. 1962), suggesting the organizer is a basal feature of the chordates.

In bird and mammalian embryos, organizer activity is limited to the anterior primitive streak, including the discrete anterior tip (Hensen's node, "*Knoten*," Viebahn 2001; Blum et al. 2007).[2] In teleosts, the organizer corresponded to a thickened area of the blastoderm on the dorsal side, termed the embryonic shield. The initial characterization of the organizer identified several embryological properties of the organizer that are central to its functions and are still studied in the context of axial development. These are: the tissue-autonomous involution and convergent extension of the dorsal lip and the differentiation into axial mesoderm (notochord), the induction and regionalization of the neural plate, and self-organization/developmental plasticity (i.e., regulation) (Spemann 1938).

As intriguing as these observations were, the organizer remained largely a phenomenon until the application of molecular biological methods to developmental biology in the 1980s–1990s. These were first used to characterize the neural inducing activity of the organizer. This aspect was the historical readout of organizer

[2] In this chapter, Hensen's node is used to refer to the anterior tip of the primitive streak in all birds and mammals, and is considered equivalent to the dorsal lip/organizer. Often the mouse organizer is referred to as the "node" without the eponym. However, the node can also refer to the posterior notochord "node" involved in left-right patterning, which lacks organizer activity. This terminology can cause confusion since the latter structure is embryologically distinct from Hensen's node. In human embryology, Hensen's node is referred to as the primitive node/knot or Hensens' knot (Gray 1918; Larsen et al. 2009).

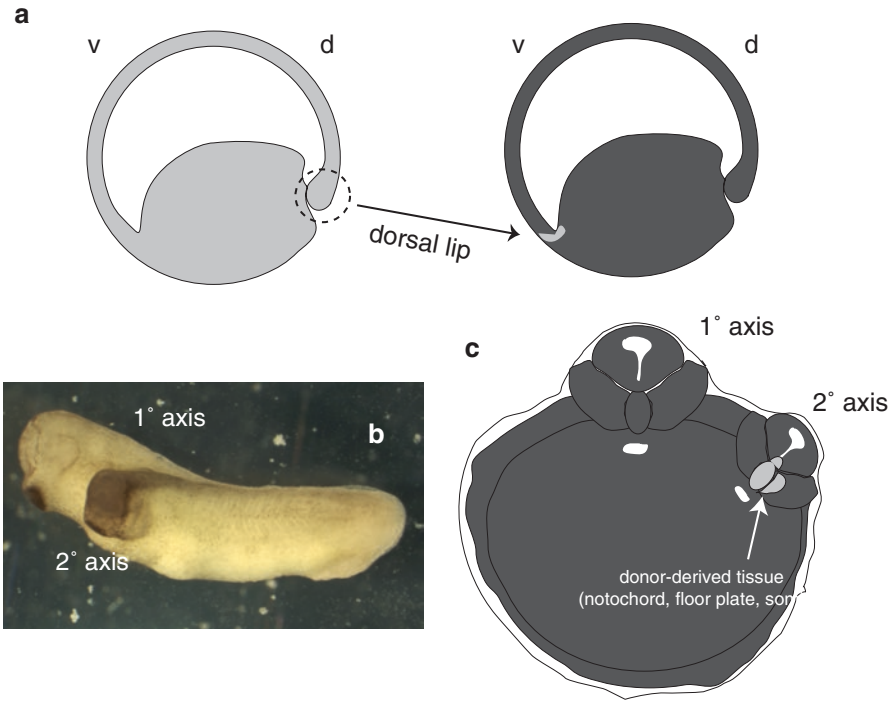

Fig. 6.10 The organizer experiment of Spemann and Mangold. (**a**) Diagrammatic model of Spemann and Mangold's dorsal lip transplantation from a lightly pigmented species (*light gray*) to the ventral region of a darker species (*dark gray*). (**b**) Image of a *Xenopus* tadpole following the successful grafting of an early gastrula dorsal lip, showing the endogenous axis (1° axis) and the induced partial axis (2° axis). The dark pigment in the head of the 2° axis is related to abnormal head development in the induced axis. (**c**) Diagram of a cross section through an embryo resulting from a dorsal lip transplant as in (**a**). The typical lineage contribution of the donor lip is lightly shaded reflecting the species origin, indicating contributions to the notochord, floor plate and medial somite (**c** is after Spemann and Mangold 1924). *v* ventral, *d* dorsal

function prior to the molecular era, owing to ease of identifying induced neural plates and nervous system tissue in sectioned material. Beginning with the observations that dissociation and delayed reaggregation of amphibian animal cap ectoderm could cause neural differentiation (Grunz and Tacke 1989, 1990; Saint-Jeannet et al. 1990; Godsave and Slack 1991), and the cloning of organizer-specific transcription factors (Cho et al. 1991; Dirksen and Jamrich 1992; Taira et al. 1992), a series of experiments from many labs identified antagonism of Bone Morphogenetic Protein (BMP) signaling activity as a key activity of the organizer in vertebrate axial development (Smith and Harland 1992; Sasai et al. 1994, 1995; Wilson and Hemmati-Brivanlou 1995; Zimmerman et al. 1996). The early history of the molecular characterization of the organizer and its impact on the fields of developmental biology and the evolution of development has been extensively reviewed

over the years by many of the key participants (Harland 1994, 2008; Elinson and Holowacz 1995; De Robertis and Sasai 1996; Harland and Gerhart 1997; Chang and Hemmati-Brivanlou 1998; De Robertis et al. 2000; De Robertis 2006, 2009; Harland and Grainger 2011).

6.4.1 *The Differentiation of Axial Mesoderm and the Role of BMP Antagonism*

It is clear from numerous studies that the formation of a ventral-to-dorsal BMP activity gradient through extracellular antagonism underlies the main functions of the organizer in dorsoventral axial patterning (Fig. 6.11a). BMPs are members of the Tgfb superfamily of secreted growth factors. These function as dimers and activate heteromeric cell surface serine/threonine kinase receptors, resulting in the phosphorylation of BMP-specific Smad transcription factors (Smad 1/5/8) (Chap. 7; reviewed in Little and Mullins 2006; Ozair et al. 2012). BMPs are expressed in non-organizer tissue, namely the ventrolateral regions in amphibians and posterior primitive streak of the epiblast in chick and mouse. These molecules function in a

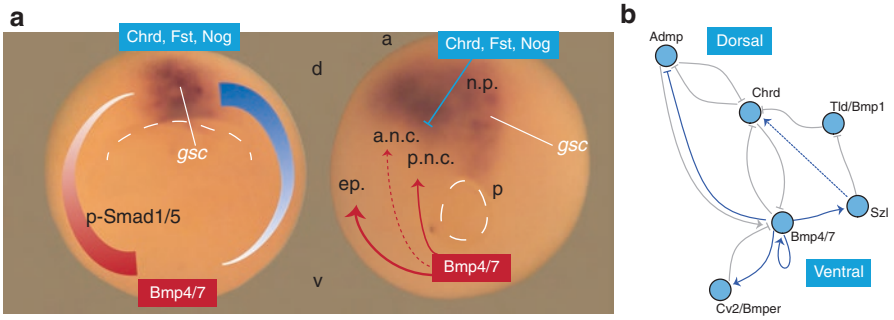

Fig. 6.11 Dorsoventral patterning of the gastrula. (**a**) Image of an early gastrula *Xenopus* embryo (*left image*) showing *gsc* mRNA expression in the organizer region (*purple*). This area expresses BMP antagonists Chrd, Fst, and Nog in a dorsoventral gradient (*blue shading*), and is complementary to a gradient of BMP signaling (*red shading*), resulting in a graded pattern of phospho-Smad1/5 (p-Smad1/5). On the right is a late gastrula showing continued ventroposterior pattering by BMP signaling, specifying in progressively later fashion (differing line thicknesses) the anterior neural crest (a.n.c), posterior neural crest (p.n.c.) and epidermis (ep.), in addition to the underlying germ layers (not shown). (**b**) Simplified network model of secreted protein interactions acting in dorsoventral patterning. Chrd secreted by the organizer antagonizes Bmp activity, mediated by Bmp4/7 ventrally and Admp within the organizer. Bmp activity inhibits *chrd* expression. Tld/Bmp1 acts as a Chrd inhibitor via proteolysis; Szl is a Tld inhibitor, indirectly promoting Chrd activity in the extreme ventral region (dotted arrow). The critical reciprocal control interactions responsible for self-organization are indicated with blue lines; Bmp4/7 positively controls its own expression but inhibits *admp* expression. Bmps also upregulate *cv2/bmper*, a ventral Bmp antagonist, and inhibit *chrd* dorsally. Model after De Robertis (2009)

dose-dependent fashion to promote ventrolateral mesendoderm and epidermis, and can prevent neural development of dissociated animal caps and inhibit dorsal mesendoderm formation in explant assays (Jones et al. 1992; Dale et al. 1992; Fainsod et al. 1994; Suzuki et al. 1994; Graff et al. 1994; Wilson and Hemmati-Brivanlou 1995; Hammerschmidt et al. 1996b; Streit et al. 1998).

The first insights into the molecular biology of the organizer came from the isolation of conserved homeobox and forkhead transcription factors expressed in the organizer: *goosecoid* (*gsc*), *lim homeobox 1* (*lhx1*), and *forkhead box a4* (*foxa4*) (Cho et al. 1991; Taira et al. 1992; Dirksen and Jamrich 1992). Importantly, ectopic expression of *gsc* in ventral cells caused second axes with notochordal tissue, indicating that *gsc* was not merely a marker of, but a critical functional component of the organizer (Cho et al. 1991). Studies in other organisms subsequently found a deeply conserved role for these proteins in regulating axial development in both protostomes and deuterostomes and even to some extent in diploblasts (Martindale 2005).

Although it was clear that overexpression of these proteins, notably Gsc, could induce most aspects of organizer function and regulate cell–cell signaling during axis formation (Niehrs et al. 1993), the nature of these signals was not immediately apparent. In a series of seminal experiments, expression cloning and differential cDNA screening in *Xenopus* identified secreted molecules encoded by *noggin* (*nog*; Smith and Harland 1992; Lamb et al. 1993), *chordin* (*chrd*; Sasai et al. 1994), and *follistatin* (*fst*; Hemmati-Brivanlou et al. 1994) as potent dorsalizing and neuralizing molecules specifically expressed in the organizer. These genes turned out to encode extracellular BMP antagonists that bind to secreted BMPs and inhibit the activation of BMP receptors (Zimmerman et al. 1996; Piccolo et al. 1996). Importantly, overexpression of these BMP antagonists mimicked the action of the organizer in nearly every respect.

Functional interference and genetic manipulations confirmed the requirements for BMP signaling and BMP antagonism in dorsoventral patterning. Of particular importance, genetic mutations in zebrafish *bmp7* and *bmp2b*, BMP receptor *acvr1* and *smad5* (*snailhouse*, *swirl*, *lost-a-fin*, and *somitabun* mutants, respectively) result in dorsalized embryos (Kishimoto et al. 1997; Nguyen et al. 1998; Hild et al. 1999; Dick et al. 2000; Schmid et al. 2000; Mintzer et al. 2001), whereas embryos lacking *chrd* function (*chordino*; Hammerschmidt et al. 1996a, b) are ventralized. Results using antisense oligo-based loss-of-function in *Xenopus* similarly showed dorsalization upon reduction of BMPs and ventralization following inhibition of Nog, Chrd, Fst (Oelgeschläger et al. 2003; Khokha et al. 2005; Reversade et al. 2005). In the mouse and the chick, the roles of BMPs are more complex owing to their functions in extra embryonic tissues and in controlling cell proliferation (Hogan 1996). Nonetheless, *Nog* and *Chrd* are expressed in the anterior primitive streak and Hensen's node (Connolly et al. 1997; Streit et al. 1998; Streit and Stern 1999; Bachiller et al. 2000), and *Nog*, *Chrd* double mouse mutants exhibit dorsoanterior truncations (Bachiller et al. 2000). Additionally, genetic deletion of *Bmp4* in mouse results in ventral mesoderm defects (Winnier et al. 1995).

A host of other BMP antagonists as well as antagonists of other ligands were also discovered and a commonly accepted model emerged; namely that dorsoventral patterning across germ layers is mediated by a gradient of BMP activity originating

ventrally and antagonized dorsally by organizer molecules. Additional support for this model came from characterization of patterns of BMP activity, finding that levels of phosphorylated active Smad1 were enriched ventrally and absent from the organizer (Faure et al. 2000; Schohl and Fagotto 2002).

BMP antagonism derived from the organizer also patterns the convergent extension movements of the axial mesoderm (also see Sect. 6.6). High levels of BMP inhibit convergent extension behavior in *Xenopus* (Graff et al. 1994), and in fish there is evidence that BMP signaling inhibits expression of convergent extension-promoting Wnt/PCP ligands, *wnt11* and *wnt5b* (Myers et al. 2002). BMP signaling may also inhibit dorsal mesoderm involution but this has not been extensively studied (Nakayama et al. 1998). And, BMPs can promote epithelial-mesenchymal transitions and ingression behavior, which occurs in more ventrolateral (non-organizer) mesoderm. Thus, the production of secreted BMP antagonists is essential and largely sufficient for the self-differentiation and morphogenesis of the organizer into notochord mesoderm and for the patterning of the surrounding germ layers.

6.4.2 *The Role of the Organizer and BMP Antagonism in Neural Induction*

Because of the historical link to early organizer studies and a general interest in the development of the central nervous system, the role of the organizer in neural induction has received considerable attention. Also, conceptually, neural induction is thought of differently from axis and mesendoderm induction. Mesoderm and endoderm are induced from ectoderm, whereas ectoderm develops if inducers are absent, and can thus be considered the "default" germ layer. Surprisingly and perhaps counter-intuitively, experiments in *Xenopus* suggested that neural, rather than epidermal, was the default state of the ectoderm (Hemmati-Brivanlou and Melton 1997; Chang and Hemmati-Brivanlou 1998; Stern 2006; Ozair et al. 2012). Inhibition of mesendoderm-inducing Tgfb signaling using dominant-negative receptors or endogenous antagonists (Hemmati-Brivanlou and Melton 1994; Chang and Harland 2007) or depletion of maternal *vegt* mRNA (Zhang et al. 1998) results in neuroectoderm formation in all presumptive germ layers. Critical evidence for the neural default model includes experiments indicating that BMPs induce epidermis in dissociated cells that would otherwise become neuralized, and that Nog, Chrd and Fst act as direct neural inducers in ectoderm tissue by blocking BMP signaling. Interestingly, these antagonists elicited only anterior neural fates, which hinted at possible mechanisms of anteroposterior patterning by the organizer (discussion of this aspect will be deferred to the following section).

Whereas the central requirement for BMP antagonism is clear, the extent that BMP inhibition alone is sufficient for neural induction is unclear. Studies in chick and *Xenopus* have indicated that neural induction also requires Wnt antagonism (Wilson et al. 2001; Pera et al. 2003; Fuentealba et al. 2007) as well as ongoing FGF signaling (Linker and Stern 2004; Delaune et al. 2005; Marchal et al. 2009). Subsequent experiments using different methodologies have suggested that FGF

signaling is particularly involved in establishing "pre-neural" ectodermal fate in the chick and is not sufficient for neural induction, whereas in *Xenopus*, BMP antagonism is largely sufficient in vivo (Wills et al. 2010; Pinho et al. 2011). Experiments in mammalian embryonic stem cells indicate that culture under low density or Tgfb-inhibited conditions can lead to neural development (Tropepe et al. 2001; Chambers et al. 2009), largely supporting the default model in mammalian cells.

The relative roles of FGF signaling and/or Wnt antagonism may reflect species level differences in the function of the organizer. The *Xenopus* organizer expresses *shisa2*, an antagonist of Fzds and FGF receptors (Yamamoto et al. 2005). Shisa homologues are not expressed in the organizer in chicken and mouse, although they are expressed in anterior regions (Furushima et al. 2007; Hedge and Mason 2008). Thus, FGF signaling may be less critical in *Xenopus* neural induction because it is normally inhibited in the presumptive neural region. FGF signaling may contribute to neural induction by inhibiting Smad1, through MAPK-mediated linker domain phosphorylation (Pera et al. 2003), or by inducing the expression of various pre-neural genes (Sheng et al. 2003; Pinho et al. 2011). In chick, non-organizer Wnt signaling can promote BMP activity, both indirectly by inhibiting the ability of FGF activity to repress *Bmp* expression and by acting more directly to promote BMP activity (Wilson et al. 2001). This latter activity may occur at the level of C-terminally phosphorylated active Smad1. FGF/MAPK and Gsk3b activity (i.e., absence of Wnt signaling) can phosphorylate the linker region of active Smad1 leading to its turnover, whereas limiting MAPK activity of Wnt treatment can prolong active Smad1 signaling (Fuentealba et al. 2007).

In general, FGF may be more critical in cases where neural development occurs over a longer time course and many rounds of cell divisions, as in the chick and mouse. Additionally, FGFs may have different roles in the context of an epiblast epithelial architecture (i.e., pseudostratified, interdigitating columnar epithelia). A similar idea has come from experiments in embryonic stem cells, where FGF signaling and low Wnt activity are thought to promote a pluripotent stem cell state resembling the postimplantation epiblast (EpiSC) (reviewed in Ozair et al. 2012). Thus FGFs may not just promote a "pre-neural" state, but a "pre-germ layer differentiation" state, which would encompass neural fate.

6.4.3 Self-Organization and Developmental Plasticity in the Organizer

In addition to the neural inducing and mesoderm patterning functions, the developmental plasticity of the organizer can be largely explained by BMP regulation. Early experiments by Spemann showed that partial constriction of the gastrula along the midline led to double-headed tadpoles, each with a normally formed anterior axis (Spemann 1903). Thus, half an organizer can restore normal bilateral symmetry locally and scale its effects accordingly. Similarly, rather normal embryos develop from dorsal half explants as opposed to hyperdorsalized embryos, which would be the expected result in the absence of self-organization (Reversade and De Robertis 2005).

Recent experiments in *Xenopus* have shown that this self-organization ability relies on the feedback regulation of Chrd flux in the embryo (De Robertis 2009) (Fig. 6.11b). The stability and activity of Chrd is regulated in the extracellular space by proteolytic degradation, mediated by Bmp1, a member of the Tolloid family of metallopeptidases. The activity of Bmp1 can be inhibited by the Sizzled (Szl) protein (Lee et al. 2006), a secreted Frizzled related protein. Szl is expressed ventrally in *Xenopus* as well as zebrafish, and becomes restricted to an extreme ventral domain in the ventral mesoderm (Salic et al. 1997; Yabe et al. 2003; Collavin and Kirschner 2003; Martyn and Schulte-Merker 2003). Szl therefore promotes Chrd activity, creating a relatively shallow gradient of dorsalizing activity, which can explain the apparent paradox that *szl* loss-of-function embryos are ventralized despite its expression ventrally (Yabe et al. 2003; Collavin and Kirschner 2003; Martyn and Schulte-Merker 2003; Lee et al. 2006). Chrd and BMPs proteins can form long-range gradients within Brachet's cleft, the narrow intraembryonic space in the gastrula separating the ectoderm from the mesendoderm, possibly using the fibronectin-rich extracellular matrix to facilitate distribution (Plouhinec et al. 2013).

Self-organization becomes possible because key dorsal and ventral genes encode proteins with opposing molecular activities, but are under differential transcriptional control. Although the organizer expresses many growth factor antagonists, this region also contains several BMP receptor agonists, *admp* (*anti-dorsalizing morphogenetic protein*) and *bmp2* (Moos et al. 1995; Joubin and Stern 1999; Lele et al. 2001; Inomata et al. 2008). A subset of BMP antagonists are expressed in a ventral domain, including *bmper*/*crossveinless2* (Ambrosio et al. 2008) and *bambi* (Onichtchouk et al. 1999). A central element of this regulatory network is the positive feedback control of the dorsal BMP agonists (De Robertis 2009): The prominent ventrally expressed BMPs (*bmp4/7*) are positively regulated by BMP signaling itself, whereas *admp/bmp2* agonists are inhibited by BMP activity. Similarly, the ventrally expressed BMP antagonists and *szl* are induced by BMPs, whereas *chrd* is repressed. Additionally, all BMPs are inhibited by binding to Chrd and can be released for action by Bmp1-mediated cleavage of Chrd.

Thus, in a dorsal half explant, loss of BMPs and Szl would lead to enhanced Chrd cleavage and inhibition resulting in higher *admp/bmp2* expression. These ligands would then accumulate in the area of highest Bmp1/lowest Chrd activity, activating *bmp4/7* expression. Increasing levels of these "new" ventral BMPs would limit the extent of *chrd* and *admp* expression as well as establish a new *szl* domain, resetting the Chrd gradient to the size of the new "embryo" (Reversade and De Robertis 2005). Analogous regulatory networks have been uncovered in zebrafish (Lele et al. 2001; Xue et al. 2014) and are likely to exist in chick, as orthologues of the main genes are present. Mammals appear to lack *Admp* and *Szl* (although *Bmp2* is present), making it unclear whether similar axial self-organization occurs or to what extent mechanistically similar processes have evolved. As the appreciation of network effects in biology is growing, it will be interesting to discover whether these principles of embedded antagonistic proteins under complementary transcriptional control are general features of self-organization in development.

6.4.4 Molecular Interpretation of BMP Gradient Signaling

Accumulated data has provided insight into the mechanisms through which cells interpret different BMP signaling levels to achieve different dorsoventral cell fates. Typical models invoke a positional information mechanism, in which the graded concentration of a morphogen determines cell fate. However, it is becoming clear that there are spatiotemporal aspects to growth factor signaling gradients in general, and to BMP gradients in particular. Recent experiments have shown that dorsalized maternal-zygotic BMP receptor (*acvr1*) mutant fish (*lost-a-fin*) are rescued by inducible *acvr1* expression only if expression is initiated prior to the midgastrula stage (Tucker et al. 2008). Similarly, inducible expression of *chrd* after the middle of gastrulation fails to dorsalize wildtype embryos. Analogous results were seen in *Xenopus* and in fish using timed application of an anti-BMPR drug (Wawersik et al. 2005; Kwon et al. 2010), further supporting the idea that early but not late exposure to BMP signals is critical for inhibiting dorsal fates and promoting ventral fates.

Temporal regulation was particularly evident in timed Chrd induction experiments. Dorsoanterior markers become progressively refractory to BMP inhibition, whereas more lateral-posterior tissues (neural crest and pronephros) became progressively more sensitive (Tucker et al. 2008). These studies thus suggest that BMP signaling is active later and longer in ventroposterior tissues and never active in prospective organizer. Additionally, dorsoventral and anteroposterior patterning occurred along a similar time course, indicating these processes are coordinated (see also Sect. 6.5.2). Interestingly in these studies, embryos do not seem to sense cumulative BMP exposure, as ventroposterior fates could still be induced with a late burst of BMP signals (Tucker et al. 2008). As discussed above, the BMP gradient is self-organizing, therefore a small pulse of BMP activity could potentially regenerate the activity gradient to regulate different cell fates. Threshold dose and temporal aspects of exposure to BMP signals thus cooperate to specify axial patterning.

The nature of interpretation of the BMP gradient at the transcriptional level may also partly underlie this temporally progressive dorsoventral patterning. In *Xenopus* ectoderm cells, competence to respond to BMP is maintained throughout gastrulation (Simeoni and Gurdon 2007). And transcription can be triggered rapidly and requires the continuous receptor activation to maintain appropriate steady-state levels of Smad1 in the nucleus (Simeoni and Gurdon 2007). These responses are distinct from those elicited during Activin/Tgfb-mediated mesoderm induction (Chap. 7), which are characterized by a more limited period of competence, extending signaling from active endocytic complexes, delayed onset of transcription, and cumulative sensing of ligand levels (Dyson and Gurdon 1998; Shimizu and Gurdon 1999; Bourillot et al. 2002; Piepenburg et al. 2004; Jullien and Gurdon 2005). The molecular bases for the differences between Tgfb and BMP signaling, despite similar pathway architecture, is not known but may underlie the general tendency of ventral signals to limit dorsal ones. These differences may also help explain the temporal aspects of BMP responses, as discussed above, as well as the importance of Smad1 as a center of signaling integration.

BMP-mediated transcriptional regulation is controlled by the formation of phosphorylated Smad1/Smad4 complexes and subsequent nuclear translocation and association with target promoters. In general, Smad-responsive cis-regulatory elements are thought to mediate low affinity interactions and Smad-associated cofactors are therefore required for specific target promoter interactions. Smad1-binding transcriptional cofactors Znf423 (Oaz; Seoane et al. 2000 and Hivep1 (Human immunodeficiency virus type I enhancer binding protein 1/Schnurri1; Yao et al. 2006) have been characterized and facilitate binding of BMP-activated Smads to BMP-responsive enhancer elements. These cofactors likely recruit general transcriptional activators or repressors depending on the cellular or epigenetic context (Blitz and Cho 2009).

6.4.5 *Complexity of Cross-Regulation Between BMP Signaling and the Organizer*

BMP signaling activates a conserved cascade of gene regulation involving several immediate response genes, including the homeobox *ventx* genes, as well as *msx1* and *wnt8a* (Gawantka et al. 1995; Schmidt et al. 1996; Ault et al. 1996; Ladher et al. 1996; Onichtchouk et al. 1996; Suzuki et al. 1997; Hoppler and Moon 1998), and a secondary target, *even-skipped homolog 1* (*evx1/xhox3*) (Ruiz i Altaba et al. 1991). Additionally, Ventx and Gsc proteins cross-repress each other's expression, mediating part of the negative feedback regulation of BMP and organizer gene expression (Fainsod et al. 1994; Gawantka et al. 1995; Onichtchouk et al. 1996; Trindade et al. 1999; Sander et al. 2007). In the mesoderm, Brachyury homolog (T) provides an essential input to *ventx* expression (and hence *gsc* repression) through its interaction with Smad1 (Messenger et al. 2005). Interestingly, double inhibition of Ventx and Gsc results in normal embryos (Sander et al. 2007) indicating that these proteins are only strictly required to regulate each other, and that redundant mechanisms pattern the axis in their absence. Multiple such independent and redundant cross-regulatory interactions between Gsc-Ventx and other protein pairs likely would provide robustness to dorsoventral patterning mechanisms.

Similar cross-regulatory loops exist in zebrafish (Nikaido et al. 1997; Melby et al. 2000; Kawahara et al. 2000; Imai et al. 2001), although the Ventx genes have undergone a unique evolutionary trajectory in other organisms (Scerbo et al. 2014). These genes appear to have been lost in rodents and are only expressed in a limited set of hematopoietic cells in humans (Rawat et al. 2010). The Ventxs bear ancient homology to Nanog, a gene implicated in pluripotency and primitive endoderm formation (Kozmik et al. 2001). Nanog may have been co-opted for some of the functions of Ventx in mammals, including possibly Gsc repression (Vallier et al. 2009), whereas Ventx may have substituted for Nanog function in fish and amphibians (Camp et al. 2009; Scerbo et al. 2012). This case is particularly likely in the anuran lineage, which appears to have secondarily lost Nanog (Scerbo et al. 2014). In spite of this reduced dependency on Ventx in mammals, cross-antagonism between BMP signaling and Gsc in patterning the axis/primitive streak has been

evolutionarily maintained, as this relationship exists as reciprocal repression of EVX1 (activated by BMP signaling) and GSC (Kalisz et al. 2012) in human ES cells. Thus, although the involvement of the well-characterized Ventx proteins is variable throughout evolution, possibly related to the timing and requirement for ventral mesoderm in hematopoiesis in early development (Kozmik et al. 2001), there remains an ancient and conserved network of reciprocal repression between BMPs and organizer genes.

6.5 Anteroposterior Axis Patterning

In general, within the dorsal blastopore/anterior primitive streak, the sequence of internalizing mesoderm determines anteroposterior character, with anterior mesendoderm (anterior definitive endoderm and prechordal plate) being followed by the chordamesoderm (notochord). Signals from the organizer have long been implicated in establishing anteroposterior fates along the body axis. This effect was seen in Spemann and Mangold's early experiments in which second axes induced by organizer transplantation often showed varying degrees of completeness at the anterior end (Spemann and Mangold 1924). These results were roughly correlated with the stage of gastrula from which the donor dorsal lip was taken; earlier lips induced more complete axes with well-formed heads and later lips induced truncated axes or second tails (Spemann 1931). These experiments were interpreted to suggest that the organizer comprised a spatially distinct "head organizer" that would induce forebrain in the early gastrula, and a "trunk organizer" that would induce hindbrain and spinal cord in more posterior ectoderm during later gastrulation. The remaining region, the midbrain, is formed largely by interactions between forebrain and hindbrain.

Work by Nieuwkoop and others later suggested a different model. Implantation of folded strips of competent animal cap ectoderm at various anteroposterior levels in the prospective neuroectoderm generated a graded pattern of neural fates in the implant, with anterior neural/neural plate border tissue forming distally in the implant and more posterior neural forming proximally (Nieuwkoop 1952, 1999) (Fig. 6.12a). These observations were interpreted to suggest a neural "activation" step that initiates a tendency toward forebrain differentiation, followed by a "transforming" event that converts activated anterior neural tissue to more posterior fates (Nieuwkoop 1952, 1999). Earlier experiments had shown that artificial neural induction in urodele animal caps using nonspecific chemical and physical methods invariably generated forebrain (Holtfreter 1944). Also, explants taken from early prospective neuroectoderm showed progressive waves of activation followed by transformation, occurring from posterior to anterior and coincident with mesoderm internalization (Eyal-Giladi 1954). These different activities were found to depend on the nature of the underlying axial mesoderm, with activation predominating in the prechordal mesoderm and anterior notochord and transformation predominating in the posterior notochord (Sala 1955). Experimental data have at various times favored one model or the other,

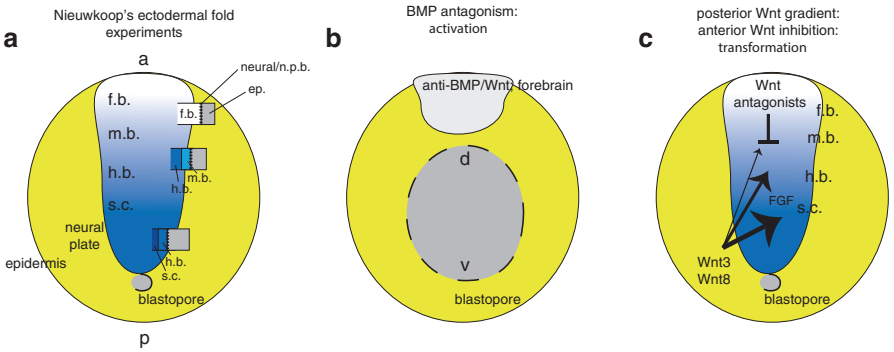

Fig. 6.12 Models for anteroposterior axis patterning in vertebrates. (**a**) Depiction of Nieuwkoop's ectodermal fold implantation experiments (Nieuwkoop 1952; Nieuwkoop and Nigtevecht 1954). Dorsal posterior view of a neurula stage amphibian embryo; neural fate is represented as a gradient from light-to-dark with darker color indicating more posterior fates; the epidermis is yellow. The implanted folds are shown as boxes, divided to show the approximately position of induced neural fates. Each fold is characterized by a distal epidermal portion (ep.) bounded by general neural/neural plate border (activated tissue); this is followed proximally by graded neural fates, reflecting the hypothesized influence of a transforming gradient (as opposed to a distinct inducer at each AP level). (**b**) Molecular interpretation of Nieuwkoop's model. In the gastrula, neural induction is accomplished by BMP antagonism, which induces neural tissue with forebrain character. (**c**) During later gastrulation, the expression of Wnts directly induces posterior fates in anterior neural-fated tissue in a dose-dependent fashion. FGF signaling is required in a permissive role. Wnt antagonists expressed in the anterior mesendoderm limit the extent of Wnt signaling and the anterior remains forebrain

but accumulating molecular evidence supports a modified version of the Nieuwkoop model (Fig. 6.12b and c). This support is based on three major findings:

1. that BMP antagonists, Noggin, Chordin and Follistatin act as endogenous neural inducers, and induce exclusively anterior neural fate in competent ectoderm (see Sect. 6.4, Smith and Harland 1992; Hemmati-Brivanlou et al. 1994; Sasai et al. 1994),
2. the identification of "transforming" molecules, such as FGFs, Wnts, Nodals, and retinoids, which posteriorize anterior tissue (reviewed in Stern 2005) but do not directly neuralize ectoderm per se, and,
3. the elucidation of proposed "anterior stabilizing" signals (Fraser and Stern 2004). These are mediated by continuing BMP antagonism (Hartley et al. 2001), Wnt antagonism by inhibitors such as Frzb1 and Dickkopf1 (Leyns et al. 1997; Wang et al. 1997; Glinka et al. 1998), multifunctional antagonism by members of the Cerberus/DAN family of proteins (Bouwmeester et al. 1996) and the feedback Nodal antagonism by Lefty1 (Thisse et al. 2000; Cheng et al. 2000). These molecules are expressed prior to gastrulation in the extraembryonic endoderm (anterior visceral endoderm (AVE)/hypoblast/foregut endoderm), and during gastrulation in the anterior definitive endoderm and prechordal plate, providing signals that initiate and stabilize anterior pattern, respectively.

6.5.1 Cellular and Tissue Basis of Anteroposterior Patterning

6.5.1.1 The Hypoblast/Anterior Visceral Endoderm in Anterior Patterning

Comparative studies have suggested that the role of the hypoblast/AVE in anterior patterning is widely conserved, based on analogous gene expression patterns in the AVE and later anterior definitive endoderm, cell migration and anterior signaling activities (rabbit hypoblast, Knoetgen et al. 1999; chicken hypoblast, Foley et al. 2000; teleost YSL, Ho et al. 1999; amphibian foregut/anterior endoderm, Bouwmeester et al. 1996; Jones et al. 1999). Interestingly, long before it was molecularly characterized, the hypoblast received considerable attention as a potential regulator of anteroposterior patterning through the control of cell movements. Classical cell tracking experiments in the chicken showed that the pregastrula epiblast undergoes bilaterally symmetrical "polonaise-like" or "double vortex" cell movements, which head anteriorly along the midline before circling back and converging at the posterior, where the primitive streak will form (Wetzel 1929; Gräper 1929). This pattern of cell movements was shown to be controlled by the hypoblast (Waddington 1930, 1933). In these studies, which were repeated and extended by later authors (e.g., Azar and Eyal-Giladi 1981; Foley et al. 2000), changing the orientation of the underlying hypoblast changed the orientation and placement of the forming primitive streak. Although this effect of the hypoblast on cell movements was clearly recognized by Waddington at the time (Waddington 1933), later experiments focused mainly on presumed signaling/inductive properties of the hypoblast in head and mesoderm induction (Azar and Eyal-Giladi 1981).

In mammals, the AVE has been extensively studied at the cellular and genetic level. The AVE forms prior to gastrulation and is characterized by cells with a distinct columnar morphology (rabbit, Viebahn et al. 1995; mouse, Kimura et al. 2000; Rivera-Pérez et al. 2003) as well as specific gene expression patterns (VE-1 antigen, Rosenquist and Martin 1995; *Hesx1*, Thomas and Beddington 1996; *Otx2*, Acampora et al. 1995; *Hhex*, Thomas et al. 1998; *Lhx1*, *Gsc*, *Foxa2*, *Cerl*, Belo et al. 1997). In mouse, these genes are expressed in visceral endoderm cells at the distal tip of the forming egg cylinder (Thomas et al. 1998). Cell labeling with DiI (Thomas et al. 1998) or with transgenic methods (Kimura et al. 2000; Rivera-Pérez et al. 2003; Srinivas et al. 2004), showed that these *Hhex*-expressing AVE cells unilaterally migrate towards the future anterior region of the embryo. Physical ablation of patches of AVE compromised development of the underlying forebrain (Thomas and Beddington 1996), demonstrating a requirement for the AVE in anterior patterning. Also, gene targeting analysis of chimeric embryos showed that a number of transcription factors are required in the AVE for its proper formation and migration and for anterior specification (*Otx2*, Acampora et al. 1995; Rhinn et al. 1998; Perea-Gomez et al. 2001; *Foxa2*, Dufort et al. 1998; *Lhx1*, Shawlot et al. 1999 and *Hhex*, Martinez Barbera et al. 2000 among others).

Despite this requirement for anterior development, several experiments have revealed that the hypoblast and related tissues largely lack head organizing properties. Einsteck implantation of the prospective foregut endoderm in *Xenopus* fails to result in ectopic head induction (Bouwmeester et al. 1996). Similar results were seen in chick and mouse regarding the function of the AVE/hypoblast, which likewise fail to induce anterior structures in transplant assays (Tam and Steiner 1999; Foley et al. 2000). Additionally, removal of the hypoblast/AVE results in ectopic primitive streak and mesoderm formation and anteriorization of the embryo, demonstrating that these tissues are repressors rather than inducers of mesoderm (Bertocchini and Stern 2002) as well as permissive regulators of anterior development (Yamamoto et al. 2004). Thus a more complex picture of anteroposterior patterning is emerging in which the hypoblast/AVE/anterior endoderm self-organizes its own morphogenesis and engages in reciprocal signaling with the epiblast/prospective definitive mesendoderm to control cell fate and morphogenesis in the embryo.

6.5.1.2 Formation and Migration of the Hypoblast/AVE

The initial formation of the hypoblast/AVE has been studied with considerable detail in the mouse but is little understood in the chicken. In the latter case, hypoblast cells delaminate from the early epiblast, forming isolated clusters, which merge together from posterior-to-anterior (Stern 2004). While it has never been studied as such, it is likely that this process is analogous to the cell sorting of primitive endoderm from epiblast in the mouse blastocyst. In the mouse, the primitive endoderm in contact with the epiblast (embryonic visceral endoderm) differentiates into the visceral endoderm after implantation, under the control of BMP signaling (Yamamoto et al. 2009). Continuing BMP signals from the epiblast and extraembryonic ectoderm likely inhibit the formation of the AVE at the proximal end of the mouse egg cylinder (Rodriguez 2005; Mesnard et al. 2006). The AVE is ultimately specified through the activation of Nodal/Smad2 signals from the epiblast (Waldrip et al. 1998; Brennan et al. 2001) and the inhibition of BMP/Smad1 signals (Yamamoto et al. 2009).

In the presumptive AVE, Nodal indirectly and directly induces a number of AVE transcription factors as well as the expression of Nodal antagonists *Lefty1* and *Cerberus-like* (*Cerl*) and the Wnt antagonists *Dickkopf1* and *Sfrp5* (Brennan et al. 2001, see below). The expression of Lefty1 and Cerl in the AVE inhibits the autoregulatory maintenance of Nodal expression in the distal epiblast (Norris et al. 2002). As AVE migration commences and is replaced by posterior visceral endoderm, Nodal and Nodal target gene expression are gradually restricted to the posterior epiblast, mediating mesoderm induction and primitive steak formation (Ding et al. 1998; Brennan et al. 2001; Perea-Gomez et al. 2002; Yamamoto et al. 2004). Thus, anterior migration of the AVE has several consequences for anteroposterior patterning: the maintenance of ectoderm germ layer and anterior neural in the anterior epiblast, and the removal of inhibitory signals for primitive streak/mesendoderm formation (Fig. 6.13).

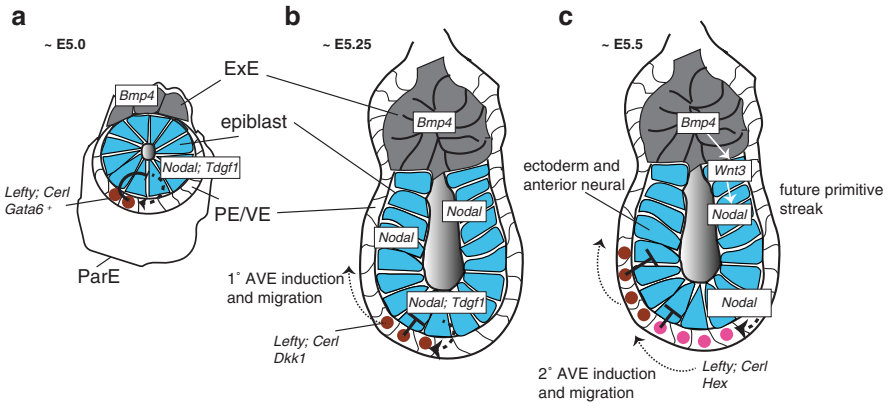

Fig. 6.13 Model for origin and role of the AVE in anteroposterior pattering in the mouse. (**a**) In the peri-implantation blastocyst (E5.0), a subpopulation of primitive endoderm (PE) expressing *Lefty1* and *Cerl* arise stochastically positioned asymmetrically in the distal egg cylinder. This population requires Nodal and Tdgf1 in the epiblast and is inhibited by Bmp4 in the extraembryonic ectoderm. (**b**) As the conceptus grows after implantation (E5.25), the AVE begins to also express Wnt antagonists and is repelled by Nodal and Wnt signals; the action of BMP is limited to the proximal epiblast and PE, allowing migration of AVE in the distal region. (**c**) As AVE migration proceeds, a second set of *Lefty1*; *Cerl*-expressing cells is induced in the distal VE (2°AVE) by Tdgf1-independent Nodal signaling. These cells and the 1°AVE migrate in a coherent stream toward the presumptive anterior, inhibiting Nodal and Wnt signaling in the epiblast and specifying the anterior neuroectoderm. Progressive loss of Nodal from the anterior limits activity to the posterior, where Nodal and Wnt maintain and amplify each other's expression through BMP4, inducing the primitive steak (PS) in the prospective posterior. *ParE* parietal endoderm, *PE/VE* primitive/visceral endoderm, *ExE* extra-embryonic ectoderm. Images were modified and adapted from: Bedzhov I, Graham SJL, Leung CY, Zernicka-Goetz M (2014) Developmental plasticity, cell fate specification and morphogenesis in the early mouse embryo. Philosophical Transactions of the Royal Society B: Biological Sciences 369:20130538. doi:10.1098/rstb.2013.0538 under the terms of the Creative Commons Attribution License CC BY 3.0

Recent experiments have identified considerable complexity in the control of AVE specification and migration. Gain-of-function studies in mouse embryos showed that *Cerl/Lefty1* and *Dkk1* can act as attractants for AVE migration, whereas Nodal and Wnts can act as repellants (Yamamoto et al. 2004; Kimura-Yoshida et al. 2005). Additionally, it is also now better appreciated that the AVE is heterogeneous, arising from an early sub-population of primitive endoderm in the preimplantation blastocyst, in addition to a later population established from naïve distal visceral endoderm (Takaoka et al. 2006). Importantly, the primary AVE cells (*alias* DVE, in some literature) originate asymmetrically in the visceral endoderm and are necessary to "lead" the migration of the secondary AVE in unilateral anterior migration (Takaoka et al. 2011; Morris et al. 2012a). This complexity of the AVE was first hinted at in mice mutant for the Nodal coreceptor *Tdgf1*, which fail to express early AVE markers (Ding et al. 1998). Although these do become expressed later, the AVE nevertheless fails to migrate. Reporter gene analyses showed that the *Cerl* and

Lefty1-expressing primary AVE arises asymmetrically within the primitive endoderm (E4.0–5.0; Takaoka et al. 2006; Torres-Padilla et al. 2007b), with live imaging of cultured embryos revealing a second population gaining *Cerl*-expression at E5.0 (Torres-Padilla et al. 2007b). Genetic lineage labeling of *Lefty1*-expressing cells confirmed that the secondary AVE arises from progenitors that lack early *Lefty1*-expression (Takaoka et al. 2011).

The leading role of the primary AVE was suggested through microsurgical ablation of the primary AVE, which resulted in a wider defect in remaining VE migration (Miura and Mishina 2007). Importantly, inducible genetic lineage ablation of *Lefty1*-expressing cells clearly showed that the secondary AVE does not migrate anteriorly if early *Lefty1*-expressing primitive endoderm is eliminated (Takaoka et al. 2011). Similar results were seen in live imaging of embryos cultured on a collagen-coated polyacrylamide matrix to mimic implantation (Morris et al. 2012a). In this case, laser ablation of leading "pioneer" cells expressing the highest levels of *Cerl* prevented the migration of the AVE. Interestingly, ablation of an immediate "follower" cell also inhibited migration, suggesting that a coherent stream of interacting cells is necessary for anterior migration (Morris et al. 2012a).

This behavior conceptually resembles "follow-the-leader" collective cell migration strategies evident in epithelial wound healing, lateral line and neural crest cell migration, cancer metastasis and other paradigms (Friedl and Gilmour 2009; Wynn et al. 2012; Cheung et al. 2013; Dalle Nogare et al. 2014; Yamaguchi et al. 2015). However, many of the well-characterized cases involve mesenchymal cells or epithelial-to-mesenchymal transitions, whereas AVE migration occurs by directional intercalation and neighbor exchange within the simple epithelium of the visceral endoderm (Srinivas et al. 2004). Trinkaus performed seminal work on coordinated migration in epithelial sheets and coupled protrusive behavior over 40 years ago (Vaughan and Trinkaus 1966), but the mechanisms controlling follow-the-leader behavior are only beginning to be studied. Recent data in *Xenopus* suggest that cell–cell adhesion between leaders and followers results in mechanical stimulation of cadherins and formation of a cadherin-keratin-plakoglobin complex, which is critical for stimulating protrusive behavior opposite the site of cell–cell contact (Weber et al. 2012). This maintenance of cellular tension as well as chemoattraction by the leading cells is likely to play key roles in this coordinated chain migration.

The initiation of AVE migration is similarly not well understood. Nodal-dependent cell proliferation in the epiblast is required (Stuckey et al. 2011), suggesting that the egg cylinder may need to reach a critical size, possibly to extend the AVE outside the reach of inhibitory signals. Thus, an initial asymmetry in the AVE, established when the embryo is small, would be amplified during growth and would determine the direction of subsequent migration, which is maintained by coordinated cell movements.

6.5.2 Molecular Regulation of Anteroposterior Patterning

6.5.2.1 Nodal and Wnt Antagonists as Regulators of Anterior Patterning

Clues to the molecular regulation of anteroposterior patterning first came from the characterization of conserved Nodal and Wnt growth factor antagonists expressed in the amphibian organizer endoderm region and in the amniote hypoblast/AVE (see below). *Xenopus cerberus* (*cer*) was identified through differential screening of dorsal lip-specific molecules (Bouwmeester et al. 1996). Cer overexpression on the ventral side could induce multiple second axes consisting only of heads (*Kerberos* was the multi-headed guard dog of the underworld in Greek mythology; Hesiod and Evelyn-White 1914). This head-inducing activity was attributed to the ability of Cer to antagonize multiple developmental signaling molecules extracellularly, including BMP, Wnt and Nodal proteins (Piccolo et al. 1999), although Nodal antagonism is likely paramount. The activity of Cer could largely be recapitulated by co-expression of the Cerberus Nodal antagonist domain (Cerberus-short, CerS) with Chrd. Additionally, overexpression of *nodal1* (*nodal homolog 1*) after the mid-blastula transition (i.e., from a plasmid vector) resulted in anterior truncations (Piccolo et al. 1999). Additional studies with amniote Cerl suggested the anti-Wnt activity is minimal in mouse and chick and that Cerl represents mainly a Nodal and BMP antagonist (Marvin et al. 2001). These and studies in zebrafish using Lefty, another Nodal antagonist, established that inhibition of Nodal activity is critical for proper patterning of anterior fate in the axial mesendoderm (Thisse et al. 2000).

In addition to anti-Nodal signals, Wnt inhibitors are expressed within the organizer and in the hypoblast/AVE and Wnt signaling is antagonistic to anterior development in a stage-dependent manner. Although Wnt signaling is critical for axis induction, other experiments show that activating Wnt/beta-catenin signaling during gastrulation causes anterior truncations (Christian and Moon 1993; Kelly et al. 1995b; Hoppler et al. 1996) whereas inhibiting late Wnt signaling results in hyperanteriorization (Hoppler et al. 1996; Leyns et al. 1997; Wang et al. 1997; Glinka et al. 1997, 1998). Additionally, inhibition of BMP and Wnt signaling together is needed to induce secondary axes with head structures, whereas induction with BMP antagonists alone generates only trunk structures (i.e., notochord; Glinka et al. 1997). Genetic studies in zebrafish *tcf3* (*headless*, *hdl*) mutants show a reduction in anterior structures and expanded ventrolateral derivatives (Kim et al. 2000), whereas the opposite occurs in *wnt8a* mutants (Kim et al. 2000; Lekven et al. 2001). Wnt antagonists are also required in vivo for anterior patterning. Interference with Dkk1 function in fish and frogs leads to anterior truncations (Hashimoto et al. 2000; Shinya et al. 2000), mimicking Wnt overexpression. Similarly, genetic deletion of *Dkk1* in the mouse epiblast led to loss of forebrain neural markers (Mukhopadhyay et al. 2001). Additionally, *Dkk1* is a direct target of *Otx2*, a key anterior specifying gene, in both the anterior mesendoderm (Ip et al. 2014) and anterior visceral endoderm (Kimura-Yoshida et al. 2005). And, either overexpression of *Dkk1* or additional deletion of one *Ctnnb1* allele is sufficient to partially rescue loss of Otx2 (Kimura-Yoshida et al. 2005), suggesting that controlling Wnt levels is a critical role of Otx2.

6.5.2.2 Self-Regulation of Nodal and Wnt Signaling During Anterior Patterning

As is the case for BMP signaling/antagonism and the organizer, Nodal and Wnt signaling are also precisely regulated through auto-regulation and negative feedback loops controlled by secreted antagonists. Nodal can directly auto-regulate its own expression in the epiblast and visceral endoderm through a Smad2/Foxh1-dependent enhancer in the first intron of the *Nodal* locus (Osada et al. 2000; Norris et al. 2002). Also, *Wnt3* is expressed in the proximal egg cylinder ectoderm and can regulate *Nodal* expression through a Wnt/beta-catenin-responsive proximal ectoderm enhancer (Brennan et al. 2001; Ben-Haim et al. 2006). *Wnt3* is in turn regulated by secreted Bmp4 signals emanating from a population of extra-embryonic ectoderm adjacent to the epiblast. This population is maintained by epiblast Nodal signaling, forming the second feedback loop (Ben-Haim et al. 2006). Nodal signals emanating primarily from the epiblast also induce the AVE at the distal end of the egg cylinder in a Smad2-dependent manner, regulating (both directly and indirectly) the expression of Nodal antagonists *Cerl* and *Lefty1*. These in turn feedback and inhibit Nodal signaling in the underlying epiblast.

Wnt3 also activates its own expression in the posterior epiblast and visceral endoderm directly through beta-catenin-dependent Wnt signaling (Tortelote et al. 2013) and Wnt signals (likely including Wnt3) directly activate *Dkk1* expression in a number of cell types (Chamorro et al. 2005). Interestingly, the autoregulation of *Wnt3* is evolutionarily ancient, occurring in sponges and in cnidarians (Holstein 2012). Both Wnt/Dkk1 and Nodal/Lefty1 interactions have been suggested to constitute reaction-diffusion systems in vivo, with regard to hair follicle spacing in the case of Wnt (Sick et al. 2006) and to left-right patterning in the case of Nodal (Nakamura et al. 2006). Whether or not these match the strict mathematical assumptions of Turing reaction-diffusion systems (the case for Wnt has been disputed, Meinhardt 2012), it is clear that interacting networks of self-propagating and self-limiting loops of Nodal, Wnt and BMP signaling underlie much of axis formation in vertebrates and indeed likely all animals.

In addition to reciprocal regulation of Nodal and Wnt, there is extensive interaction between Wnt/BMP and Nodal/BMP at the level of signaling integration during anteroposterior patterning. Wnt signaling can potentiate BMP signaling through several possible mechanisms. In the frog, BMP-activated Smad1 can be inhibited by Gsk3b phosphorylation of the linker region, and this in turn can be inhibited by Wnt leading to the perdurance of active Smad1 (Fuentealba et al. 2007). In zebrafish embryos, dorsoventral and anteroposterior patterning occur along a similar time course (Tucker et al. 2008). Correspondingly, manipulation of Wnt signaling can alter the anteroposterior character of Chrd-induced tissues at any time during gastrulation (Hashiguchi and Mullins 2013), suggesting that BMP and Wnt signaling mechanisms are active together, along with FGF and retinoid signaling (Hashiguchi and Mullins 2013). Additionally, in the chick epidermis Wnt signaling can block FGF inhibition of *Bmp4* transcription during neural plate induction (Wilson et al. 2001), although it is unclear whether this interaction is also critical for anteroposterior neural plate patterning.

Nodal and BMP signaling also interact at several different levels. Nodal and certain BMPs can form inactive heterodimeric ligands that fail to stimulate either signaling pathway and exhibit mutual antagonism (Yeo and Whitman 2001). Also, in the frog organizer Nodal and Admp (a BMP ligand) form a self-regulating network to control anteroposterior patterning (Inui et al. 2012). Nodal and Admp can compete for a shared receptor, Acvr2a, to oppositely regulate Nodal and Wnt antagonists in the anterior endoderm. High Nodal levels activate high expression of Nodal antagonists, which subsequently block Nodal-Acvr2a interaction and permit the occupancy by Admp. This alternate pathway limits the expression of Nodal antagonists, allowing Nodal signaling to reengage (Inui et al. 2012). Interestingly, Admp depletion results in the anterior endoderm gaining head-inducing activity in Einsteck transplantation assays, owing to overproduction of Nodal and Wnt antagonists (Inui et al. 2012). The high degree of interrelatedness of these signaling pathways thus can underlie many of the self-organizing properties of early axis formation.

6.5.3 Regulation of Posterior Development by Wnt Signaling

6.5.3.1 Default Specification of Anterior Neural Fate

In line with the activation-transformation model, experimental neural induction in *Xenopus* animal caps (ectoderm), either by Noggin treatment (Lamb et al. 1993) or by disaggregation (Grunz and Tacke 1989, 1990), results exclusively in anterior neural induction. Additionally, anteroposterior patterning is maintained in frog embryos lacking mesoderm and endoderm, generated by *vegt* depletion or CerS overexpression (Zhang et al. 1998; Wessely et al. 2001), and in those lacking both organizer and ventral BMP signals (Reversade and De Robertis 2005). Similarly in zebrafish, embryos lacking Nodal signaling and thus mesendoderm also retain anterior identity in the ectoderm (Gritsman et al. 2000). In the mouse, loss of Nodal signaling results in anterior neural specification in the epiblast in the absence of mesendoderm and the AVE (Conlon et al. 1994; Brennan et al. 2001; Camus et al. 2006). The molecules mediating this anterior default state are not known but are hypothesized to be present maternally in fish and amphibians and in the early epiblast of amniotes. Because embryonic stem cells can also acquire anterior neural fates by default (Tropepe et al. 2001; Chambers et al. 2009), it is possible that core pluripotency factors or proteins activated at the onset of epiblast differentiation would be good candidates for anterior specifiers.

6.5.3.2 Graded Wnt Signals and Posterior Specification

Although many different signaling molecules have been implicated in posterior neural fate specification, current evidence suggests that the graded activity of Wnt signaling is the critical proximate determinant of early posterior identity. Wnt

activity can be seen in *Xenopus* embryos as a gradient of nuclear beta-catenin in the prospective neural plate of the gastrula, with the highest levels posteriorly (Kiecker and Niehrs 2001). Similarly, in the mouse gastrula, posterior gradients of Wnt reporter construct activity have been observed (Maretto et al. 2003; Lewis et al. 2007, 2008; Fossat et al. 2011). Experiments in neuralized *Xenopus* animal caps showed that inducible expression of activated beta-catenin during gastrulation directly induces posterior neural markers (McGrew et al. 1997; Domingos et al. 2001). Additionally, cell–cell contact and FGF signaling are required in this case, suggesting the involvement of a "community-effect" response, as described for muscle formation (Standley and Gurdon 2004). Similar requirements for Wnt and FGF signaling were seen in chicken, where Wnt treatment directly and dose-dependently posteriorizes competent anterior neural tissue explants (Nordstrom et al. 2002). Correspondingly, Wnt inhibition in posterior neural explants causes anteriorization. FGF signaling is also required for posterior specification, but likely in a permissive role, acting in a non-dose-dependent fashion. And loss of FGF signaling does not anteriorize posterior neural fates (Nordstrom et al. 2002). Wnt signaling also has subsequent roles in specifying the posterior portion of major brain subdivisions later in development, in particular distinguishing diencephalon versus telencephalon (Heisenberg et al. 2001; Houart et al. 2002). It is therefore of great interest to determine how this signaling system is compartmentalized and reiterated during multiple aspects of individual tissue and organ development.

At the transcriptional level, Wnt/beta-catenin signaling during posterior specification is thought to proceed through the use of Tcf/Lef proteins as coactivators. This mechanism of gene activation is likely different from early, axis-inducing Wnt signals, which involve mainly derepression of Tcf3 (see Sect. 6.3.1). In mouse development, double homozygous genetic deletion of *Lef1* and *Tcf7*(*Tcf1*) results in posterior defects in addition to abnormalities in mesoderm specification (Galceran et al. 1999). Similarly in *Xenopus*, Lef1 and Tcf1 are required for the late/ventral response to Wnt (Roël et al. 2002; Liu et al. 2005), whereas Tcf3 is dispensable in this regard (Hamilton et al. 2001). Recall that Tcf3 is necessary for the repression of early Wnt target genes in vertebrates. Experiments in mouse and frog have suggested a "Tcf exchange" model for the changes in target gene responses to Wnt signaling prior to and following axis formation. *Lef1* can be transcriptionally activated by Wnt signaling (chick, Skromne and Stern 2001; mouse, Wu et al. 2012a), suggesting that cells experiencing Wnt signals undergo a qualitative change in their potential underlying responses to subsequent Wnt exposure during development. Similarly, early Tcf3 repressor function is inactivated by Wnt-activated kinases and replaced by activating Tcf1 function in both *Xenopus* and in mouse embryonic stem cells (Hikasa et al. 2010; Yi et al. 2011). Thus, the activation of genes during posterior specification is likely to involve both beta-catenin-dependent derepression of Tcf3, followed by replacement and activation by Lef1/Tcf1. In support of this idea, mouse knock-ins in which Tcf3 has been replaced by the deltaNTcf3 construct (non-derepressible) lack *Lef1* expression and show defective late Wnt responses (Wu et al. 2012a).

6.6 Axial Morphogenesis

The physical form of the vertebrate axis is brought about during gastrulation. During this process, the presumptive mesoderm and endoderm germ layers are internalized and undergo a set of morphogenetic events that elongate the axis in the anteroposterior direction and establish dorsoventral (mediolateral) organization in the mesoderm. Vertebrate embryos undergo gastrulation in one of two basic ways, depending on the architecture of the embryo. In spherical anamniote embryos, internalization occurs through the blastopore, which circumscribes the marginal zone, and forms the archenteron (primitive gut tube). In the flat blastoderm of amniote embryos, internalization generally occurs through the primitive streak in the posterior of the embryo, forming a trilaminar embryo with the endoderm remaining open to the yolk and forming a tube secondarily.

In recent years, the cellular mechanics of vertebrate gastrulation have been studied in great depth using live imaging approaches. Surprisingly, the underlying cell and tissue mechanics and their molecular regulation are extensively conserved, despite differing patterns and timing of gastrulation movements. Largely based on the seminal work of Trinkaus, Keller, Stern and others, a common set of vertebrate gastrulation movements and cell behaviors have been identified. These have been extensively reviewed in recent years (Keller 1986; Keller and Shook 2004; Stern 2004; Solnica-Krezel 2005; Tam et al. 2006; Shook and Keller 2008; Solnica-Krezel and Sepich 2012) and fall into three main categories, internalization, convergent extension and epiboly. Together, these processes form a "mosaic of regional processes" (Keller 1986) that are exquisitely integrated to accomplish the feat of gastrulation.

Mesendoderm internalization typically initiates by invagination (in-pocketing) in a tissue layer (the epiblast), followed either by involution as an in-rolling sheet of cells over a lip (*Einrollung*; Vogt 1929), or by ingression of individual cells. Invagination and ingression proceed by apical constriction of epiblast cells, and in the case of ingression, epithelial-to-mesenchymal transition and subsequent cell migration. Convergent extension is a thinning and narrowing of a tissue and occurs by mediolateral intercalation of closely packed cells or by migration of cells toward a midline. Cells in the mesendoderm undergoing convergent extension typically elongate along the mediolateral axis and exhibit bipolar protrusive behavior using lateral lamellopodia. The traction generated by this bipolar activity is thought to facilitate cell intercalation. Epiboly is a thinning and spreading/extension of a tissue and involves radial intercalation to form a thinner cell layer, as well as cell shape changes. In spherical embryos, epiboly is essential for the ectoderm to spread and cover the yolk mass during gastrulation. Uniquely in teleost embryos, epiboly encompasses all germ layers and the entire embryonic blastoderm covers the yolk cell.

6.6.1 Amphibian Gastrulation

Gastrulation in amphibians initiates at a latitudinal slit at the boundary of equatorial and vegetal regions, forming the dorsal lip of the incipient blastopore. Cell internalization begins with apical constriction of the so-called "bottle cells," creating the

initial invagination at the blastopore (Keller and Shook 2004). Extension of this cavity during gastrulation creates the archenteron. Internal vegetal rotation cell movements in the presumptive endoderm pull the marginal zone vegetally and initiate internalization by involution at the dorsal lip. Internalization spreads laterally from the dorsal lip eventually forming a circular blastopore, with axial mesendoderm entering at the early dorsal lip and more paraxial and lateral mesendoderm internalizing from the lateral and ventral blastopore (Keller and Shook 2004).

On the dorsal side, the anterior leading edge of the mesoderm (prechordal/head mesendoderm) migrates as a cellular stream along the blastocoel roof (Winklbauer and Nagel 1991; Winklbauer et al. 1992). This migration requires fibronectin fibrils deposited by the blastocoel roof cells (Boucaut et al. 1985; Winklbauer and Nagel 1991; Nagel and Winklbauer 1999). Internalizing mesendoderm cells maintain what is termed "tissue separation" with the ectoderm, and cannot intercalate back into the ectoderm (Wacker et al. 2000). This segregation is evident by the presence of Brachet's Cleft between the mesendoderm and ectoderm/neuroectoderm (Nieuwkoop and Florschütz 1950). An analogous cleft separating these tissue layers is found throughout the vertebrates.

In *Xenopus* and other anurans, the majority of the mesoderm arises from deep rather than superficial cells (Nieuwkoop and Florschütz 1950; Keller 1975). Involution of the mesendoderm is thus internal in these species and provides the main mechanical forces driving gastrulation. The mesendoderm and deep neural ectoderm undergo radial intercalation just prior to involution, occurring first in more anterior cells and later in posterior ones (Keller et al. 1985). Additionally, tissue extension is biased in the anterior–posterior direction, likely because some initial mediolateral intercalation occurs. After involution, cells become elongated perpendicular to the axis of extension and exhibit bipolar protrusive behavior and undergo mediolateral intercalation (Keller and Tibbetts 1989; Shih and Keller 1992b). This behavior drives convergent extension of the marginal zone and is the major biomechanical force behind internalization and elongation of the axis. Mediolateral intercalation behavior begins in more anterior tissue as it involutes and spreads to more posterior axial tissue as well as lateral mesoderm as the blastopore closes.

Closure of the blastopore at the ventral marginal zone is accomplished by convergent thickening, a little understood behavior in which prospective posterior somitic mesoderm converges into a pile of cells that do not extend, although they do so later in neurulation (Keller and Danilchik 1988). In general, the process of convergence can lead either to extension or thickening, or both, with the degree of each outcome depending on the constraints applied by the surrounding tissue. In either case, the progressive expansion of mediolateral intercalation behavior around the blastopore and the linking of forces around the marginal zone is thought to create "hoop stress" that pulls the blastopore lip over the yolk plug and internalizes the endoderm (Keller et al. 2000; Keller and Shook 2004).

Urodele and anuran amphibians undergo significant differences in development that have been long noted but perhaps not well appreciated. The process of gastrulation in salamanders and newts is outwardly similar to the situation described in

Xenopus, but has several important distinctions. Mesoderm is derived from the surface layer in urodeles (Vogt 1929). Axial mesoderm undergoes similar involution at the dorsal lip in urodeles, but internalization in the ventral and lateral regions occurs mostly by ingression of individual cells (Shook et al. 2002). This pattern of ingression has been referred to as a "bilateral primitive streak." In urodeles, lateral and ventral convergent forces are generated by the ingressing cells in these regions, as these cells undergo apical constriction and bring additional cells into the ingression zone (Keller and Shook 2004).

Interestingly, the urodele pattern of dorsal lip involution and lateral ingression is conserved in more primitive organisms including cyclostomes and lungfish (Shook and Keller 2008). It is likely that both the anuran and teleost mechanisms of primary internalization by involution evolved independently within those lineages. These changes in gastrulation may be correlated with the cellular organization of the ectodermal moiety, occurring as a multilayered ectodermal "deep layer" of non-polarized cells, covered by a superficial polarized epithelial layer in the anurans and teleosts, as opposed to the typical "epiblast" of pseudostratified interdigitating epithelial cells (Shook and Keller 2008).

6.6.2 Teleost Gastrulation

Similar morphogenetic events occur in the zebrafish/teleost gastrula, albeit in the context of a different embryonic architecture. One of the main features of fish gastrulation is prominent epiboly, in which all three germ layers (as opposed to just ectoderm) undergo extensive radial intercalation as the blastoderm expands to cover the yolk cell. Epiboly in teleost fish is somewhat unusual, and involves interconnections between the yolk cell and the superficial epithelial layer of the blastoderm. Epiboly begins just prior to gastrulation with YSL nuclei migrating vegetally in advance of the blastoderm margin. Cytoskeletal perturbations and in vivo observations suggest that the YSL "tows" the blastoderm margin vegetally (Betchaku and Trinkaus 1978; Solnica-Krezel and Driever 1994).

Internalization initiates by an involution-like "synchronized ingression" around the blastoderm margin, forming a bilayered structure, the germ ring (Adams and Kimmel 2004). Typical ingression is not thought to occur, as teleost gastrulation lacks a classical epithelial-to-mesenchymal transition (EMT) and cell migration characteristic of typical ingression movements (Shook and Keller 2008). Internalizing cells migrate beneath the outer layer toward the animal pole, forming an inner hypoblast (this is mesendoderm, and not homologous to the amniote hypoblast) and outer epiblast (ectoderm). Both layers are covered by a flattened superficial epithelium, the enveloping layer (EVL), which remains extraembryonic and may provide structural support for morphogenetic movements (Kimmel et al. 1990). On the dorsal side of the margin, involuting and single "delaminating" cells form a thicker layer, constituting the embryonic shield (Montero 2005). Also dorsally, a

teleost-specific population of epiblast cells, the dorsal forerunner cells (DFCs) detach and migrate vegetally ahead of the dorsal lip, in association with the margin of the EVL (Cooper and D'Amico 1996). Their function at this stage is unknown, but later these cells will form the Kuppfer's vesicle, which functions in left-right asymmetry. Also, similar to the process in anurans, convergent extension elongates the axial mesendoderm as a result of bipolar protrusive behavior and well as dorsally directed migration of more lateral cells (Solnica-Krezel 2005). Teleost thus appear to have independently evolved an amphibian-style method of gastrulation as a solution to concentrated yolk and discoidal cleavage in the egg.

6.6.3 Amniote Gastrulation

In the mammals and birds, gastrulation initiates at the primitive streak, a groove formed by internalizing cells along the posterior axial midline. Anterior–posterior patterning, and thus the positioning of the primitive streak towards the posterior, is controlled by the anterior migration of the hypoblast/AVE (Sect. 6.5.1). Mesoderm precursors are initially found throughout the posterior epiblast but migrate toward the posterior end prior to primitive streak formation. In the chick, this occurs through the double-vortex polonaise-like cell movements (see Sect. 6.5.1) and similar mechanisms have typically been thought to occur in mammals. However, mouse embryos appear to lack these large-scale cell movements in the epiblast (Williams et al. 2011), possibly owing to a smaller number of cells at the equivalent stage or to constraints of the cup-shaped architecture. Alternatively, the cell movements positioning the streak may occur in the visceral endoderm layer in mouse (Weber et al. 1999). Polonaise-like movements appear to occur in rabbit embryos, which have a flat morphology similar to chick embryos (Halacheva et al. 2011). Interestingly though, in this case, a more localized extreme intercalation cell movement event ("processional cell movements"), predominates (Halacheva et al. 2011). These observations indicate that overall primitive streak positioning in amniotes can result from many different patterns of cell movements depending on the species.

Internalization through the primitive streak involves massive epithelial-to-mesenchymal transition (EMT) and ingression of individual cells to form definitive mesoderm and endoderm (Stern 2004). Early ingressing mesoderm contributes mainly to pharyngeal and cardiac mesoderm anteriorly, as well as extraembryonic mesoderm posteriorly. Definitive endoderm ingresses by mid-streak stages and intercalates into and displaces the hypoblast/visceral endoderm. The primitive streak elongates and progresses anteriorly (distally in the mouse egg cylinder) until mid-gastrulation (late streak stage), approximately to the level of the future hindbrain, and begins to form the anatomically distinct Hensen's node at the anterior tip. Internalization at Hensen's node in many birds and mammals, including humans, resembles that of the amphibian dorsal lip, with invaginating bottle cells and involution of notochord and somitic mesoderm (Shook and Keller 2008). Chick and mouse

embryos however appear to have separately evolved ingression as the main mode of internalization, although dorsal lip involution may be evolutionarily ancestral.

Reptiles (non-avians) represent a useful but little-studied intermediate case. These embryos typically form a dorsal lip perpendicular to the axial midline in the posterior of the blastoderm, through which chordamesoderm involutes. Lateral and ventral cells ingress through a reptilian blastoporal plate, which resembles both the urodele blastopore and amniote primitive streak (Bertocchini et al. 2013).

After its formation, the Hensen's node begins to regress posteriorly and the primitive streak closes in an anterior-to-posterior progression. Notochord and somites form in the mesoderm behind the regressing node. At this point Hensen's node and surrounding cells likely comprise a pluripotent stem cell population that persists throughout regression and into tailbud formation (Wilson et al. 2009). It is often not discussed as such, but amniote primitive streak regression is likely an analogous process to blastopore closure, with mediolateral intercalation behavior driving convergent extension in the axial midline tissues and convergent behavior following ingression at the primitive streak. For the mouse, mediolateral intercalation of mesoderm has been established as a mechanism of axial elongation, and convergent-extension behavior has been demonstrated to initiate in pre-somitic mesoderm cells immediately after they exit the primitive streak (Yen et al. 2009).

In all vertebrates, the embryo lengthens and narrows along the AP axis at the culmination of gastrulation. The neural plate is visible in the dorsal ectoderm and the closed blastopore represents the posterior pole of the embryo. Patterning of the AP and DV axes is essentially accomplished during gastrulation. Although the left-right axis is thus specified topologically, the cellular and molecular mechanisms that regulate left-right asymmetry of the internal organs do not come into play until after gastrulation. These are primarily mediated by a distinct ciliated structure that forms during late gastrulation at the posterior end of the notochord along the gastrocoel roof plate, a part of the archenteron lining (Blum et al. 2014). This structure is derived from surface mesoderm in the late involuting dorsal lip and is homologous in many respects across vertebrates. Monocilia in the posterior notochord region are thought to create a leftward fluid flow, generating asymmetry in Nodal signaling in the left lateral plate mesoderm, leading to morphological left-right asymmetry and the generation of internal bilateral asymmetry in organs such as the heart, gut and lungs (also, see Blum et al. (2014) for review of caveats in chick and pig, which lack nodal cilia (Gros et al. 2009)).

6.6.4 Molecular Regulation of Axial Morphogenesis

The control of convergent extension has been extensively studied at the molecular level in *Xenopus* and zebrafish embryos. A host of cellular and genetic experiments have implicated components of the Wnt/PCP pathway as critical regulators of cell shape, cell polarity and activity during convergent extension. In *Xenopus*, overexpression of certain beta-catenin-independent Wnts and dominant-negative Dishevelled 2 (Xdd1; Sokol 1996) were sufficient to block axis elongation without

blocking mesoderm formation. Additionally, mutation or inhibition of *wnt11* function in fish and frogs indicated that Wnt/PCP signaling was indeed required for convergent extension behavior in vivo (Tada and Smith 2000; Heisenberg et al. 2000). Importantly, Wallingford et al. (2000) showed in detailed live imaging of explants that Dvl activity was needed for bipolar cell shape changes and for stabilizing mediolaterally oriented bilateral protrusive activity of lamellopodia in converging and extending mesodermal cells. And similar regions of the protein were required for controlling CE in *Xenopus* as for *Drosophila* PCP, namely the C-terminal DEP domain (Wallingford et al. 2000). Analogous results were found for zebrafish *wnt11* and *wnt5a* mutants, which showed unstable monopolar protrusive activity in dorsally migrating cells (Kilian et al. 2003; Ulrich et al. 2003). Other PCP proteins have also been shown to control cell intercalation by regulating mediolateral lamellopodia in fish and frogs, including Frizzleds, Vangl2, and Prickle (Djiane et al. 2000; Darken et al. 2002; Goto and Keller 2002; Jessen et al. 2002; Wallingford et al. 2002; Veeman et al. 2003b; Takeuchi et al. 2003), as well an extracellular modulator of Wnt, Glypican 4 (*knypek*, Topczewski et al. 2001).

More recent data from amniotes has shown that the regulation of cell shape and the formation of mediolateral lamellopodia are conserved in mouse (Ybot-Gonzalez et al. 2007; Yen et al. 2009; Williams et al. 2014) and chicken (Voiculescu et al. 2007). Additionally, mutations in core Wnt/PCP proteins in mice and humans display axis elongation and neural tube defects, both of which depend on convergent extension (Vangl1/2, Kibar et al. 2001; Murdoch et al. 2001; Montcouquiol et al. 2003; Kibar et al. 2007; Torban et al. 2008; Celsr1, Curtin et al. 2003; and Dvl1/2, Wang et al. 2006). Also, noncore vertebrate Wnt/PCP regulators Ptk7 and Scrib are also required for axis elongation and convergent extension, with Ptk7 likely directly controlling bipolar protrusive activity (Montcouquiol et al. 2003; Lu et al. 2004; Yen et al. 2009). The exact role of Ptk7 is unclear, although genetic experiments in zebrafish have suggested a role in antagonizing Wnt/beta-catenin signaling to allow Wnt/PCP signaling (Hayes et al. 2013). However, beta-catenin-activating roles have also been described suggesting temporal or context-dependent roles.

In addition to controlling cell intercalation during convergent extension, Wnt/PCP signaling is also implicated in cell intercalation prior to primitive streak formation. In the chicken, local cell intercalation in the presumptive primitive streak region prior to gastrulation is though to result from, and may facilitate the polonaise movements in the epiblast. A number of Wnt/PCP components are expressed in the midline prior to primitive streak formation and inhibition by electroporation of anti-PCP morpholino oligos can inhibit streak morphogenesis (Voiculescu et al. 2007). Additionally, and consistent with the role of the hypoblast in controlling polonaise movements, rotation of the hypoblast can alter Wnt/PCP gene expression, possibly through Fgf8 signaling (Voiculescu et al. 2007). Interestingly, it is likely that ingression itself can function locally to organize polonaise-like movements and pull additional cells into the streak, thus driving ingression in a feed-forward manner (Voiculescu et al. 2014).

The situation is less clear in mammals, as Wnt/PCP components have not been widely implicated in primitive streak formation in the mouse. However, ingression at the primitive streak in the mouse does require the down-regulation of Vangl2 protein,

mediated by Dact1 (Dishevelled-binding antagonist of beta-catenin 1; Suriben et al. 2009). Thus, whereas mice may exhibit a reduced role for cell movement-driven mediolateral intercalation during primitive streak formation (see above), a role for Wnt/PCP components may have been retained at the level of EMT in the forming primitive streak.

Wnt/PCP signaling exerts its effects on intercalating cells by coordinately regulating bipolar cell shape and polarization of actin dynamics to generate tensile forces along the cell's mediolateral axis. The mechanisms that orient this bilateral protrusive activity perpendicular to the elongating anteroposterior axis are not well understood. Experiments using dissociated *Xenopus* animal cap cells treated with graded Activin doses or the recombination of anterior versus posterior notochord explants showed that apposition of tissue from different axial levels was necessary for convergent extension along a perpendicular axis, whereas recombination of equivalent levels produced no elongation (Ninomiya et al. 2004). It is possible that cells could respond to this gradient of Tgfb signaling through changes in cadherin- and protocadherin-mediated cell adhesion during intercalation (Brieher and Gumbiner 1994; Kraft et al. 2012). Similar results have been obtained by opposing gradients of Nodal and BMP activity in zebrafish blastoderm explants (Xu et al. 2014), suggesting an important role for integration of the these pathways in controlling elongation. Temporal changes in Nodal signaling are also correlated with the onset of mediolateral intercalation behavior, further suggesting that the local activity of Wnt/PCP may be cued by differences in Tgfb activity.

Tgfb signaling, most likely through Nodal and Nodal-related proteins, is also involved in inducing ingression behavior and bottle cell formation by activating EMT. This can be shown in *Xenopus* by overexpression of Nodal in animal caps, which results in ectopic bottle cell formation (Lustig et al. 1996; Agius et al. 2000; Kurth and Hausen 2000). Also, loss of Nodal signaling dramatically reduces EMT-like cell ingression in zebrafish embryos (Keller et al. 2008). Movement of the hypoblast in amniotes, which expresses Nodal antagonists, is also highly correlated with primitive streak formation in the epiblast formed by those cells left behind. This Nodal-controlled ingression may also be self-regulating, as ingression may facilitate recruitment of additional cells to the streak (see above), which would expose more cells to Nodal signals, further triggering more EMT and ingression (Voiculescu et al. 2014).

Signaling by FGFs also plays a key role in regulating convergent extension and mediolateral intercalation. FGF signaling can regulate *brachyury* (*t*) expression, which in turn can activate *wnt11* (Tada and Smith 2000). In addition to its role in morphogenesis, FGF signaling also has a well-known role in mesoderm formation (Chap. 7). Experiments with cytoplasmic antagonists of FGF signaling, Sprouty/Spry and Spred, indicate that maternal Sprys antagonize convergent extension behavior by blocking PKC and calcium outputs of FGF signaling (Sivak et al. 2005). Later during gastrulation, zygotic Spred proteins accumulate and antagonize Mapk signaling, allowing the morphogenetic signals to predominate after medoderm specification has occurred (Sivak et al. 2005). Additionally, FGF regulation of convergent extension is controlled by the Nodal related 3.1 protein through an atypical and little characterized mechanism (Yokota et al. 2003).

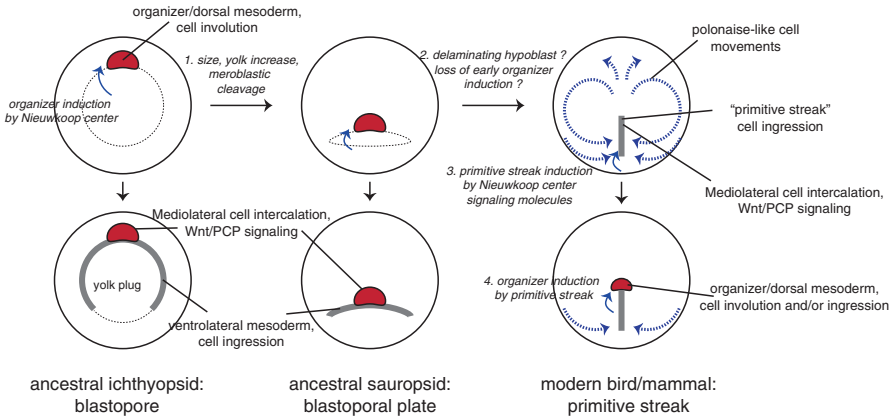

Fig. 6.14 Model for the evolution of axis formation in vertebrates. Highly generalized schematic diagrams of different vertebrate embryos during early (*upper panels*) and middle (*lower panels*) gastrulation. In the basal vertebrates (*left panels*; vegetal views), gastrulation initiates at the organizer with induction by the Nieuwkoop center (high/early Nodal signaling). The initial internalization movements are through involution. By mid-gastrulation, ingression of mesendoderm progresses around the vegetal cells (yolk plug) and forms the nascent blastopore. Involuted dorsal mesendoderm undergoes convergent extension via mediolateral cell intercalation under the control of Wnt/PCP signaling. During the evolution of amniotes (*middle panels*; top/dorsal views), eggs increased in size and yolk content and began to undergo meroblastic cleavage. Organizer induction by Nieuwkoop center molecules is retained in early gastrulation. Ingression proceeds through the horizontal slit of the blastoporal plate/blastopore and does not circumferentially envelop the non-cleaving yolk (not shown). In the evolution of modern birds and mammals, gastrulation initiates with ingression at the primitive streak, which would be homologous to the later ventrolateral blastopore in ancestral forms. The organizer (Hensen's node) is induced later by Nodal signaling from the middle primitive streak. The heterochrony in the pattern of gastrulation morphogenesis and organizer formation could result from several main events; the hypoblast/anterior endoderm segregating from the epiblast as opposed to forming from cleaving vegetal cells, the loss of early organizer induction, the apparent emergent behavior of polonaise-like movements leading to primitive streak formation and the relatively later induction of Hensen's node by the primitive steak

6.7 Concluding Remarks

The overall conservation of axial patterning mechanisms in vertebrates has long been recognized. However, detailed investigations at the cellular, molecular and genetic levels are only now revealing the true extent of this conservation and are also uncovering important differences. The broadest pattern of axis specification involves the generation of animal–vegetal polarity in the oocyte followed by the dorsal localization of determinants in the egg. This *ab ovo* specification mechanism operates throughout the primitive vertebrates, reptiles and possibly the egg-laying mammals. Based on various cell biological, genetic, and morphological observations, the basic pattern of axis generation is the involution of notochordal precursors at the early dorsal lip/organizer and the later ingression of ventrolateral precursors around the lateral blastopore, or "bilateral primitive streaks."

This pattern exists throughout the anamniotes (icthyopsids) and the reptiles and is initially established by cortical rotation in the egg, leading to Wnt/beta-catenin asymmetry, elevated dorsal Nodal signaling and organizer induction and dorsal mesoderm involution (Fig. 6.14). Interestingly, much of this sequence of events is likely specific to the vertebrates. In basal invertebrate chordates, such as *Branchiostoma*, cortical rotation and early dorsal enrichment of beta-catenin do not occur and gastrulation occurs symmetrically via cell ingression, with minimal involution (Zhang et al. 1997; Holland and Onai 2012). Early Nodal asymmetry is present and likely leads to the induction of organizer/BMP antagonist gene expression on the dorsal side of the blastopore (Yu et al. 2007; Holland and Onai 2012). Thus, a key series of events in early vertebrate evolution was likely the appearance of cortical rotation, leading to the restriction of gastrulation initiation dorsally, coupled with a transition to cell involution behavior in the organizer.

In contrast to this early asymmetry mechanism, therian mammals undergo apparent *ab embryo* axis specification, with the early phase of Wnt asymmetry being lost and evolving into new mechanisms of symmetry breaking in the blastocyst, based on cell position and migration in the visceral endoderm. In both *ab ovo* and *ab embryo* cases, the egg and the blastocyst exhibit marked metastability, rendering development with radial symmetry improbable under normal conditions. The evolutionary transitions between these mechanisms are not well understood. Increases in yolk and egg size and the shift to meroblastic cleavage have been suggested to drive the evolution towards primitive streak-based gastrulation (Arendt and Nübler-Jung 1999), although these cannot be the sole factors involved. Reptiles have large eggs and undergo meroblastic cleavage but still form the organizer first and develop a horizontal slit blastopore/blastoporal plate, maintaining the ancestral dorsal involution/lateral ingression pattern. Additionally, teleosts also evolved meroblastic cleavage but this change resulted in additional adaptations to keep a blastoporal gastrulation pattern, rather than evolving primitive streak-like movements. Furthermore, the "primitive steak" evolved independently in birds and in mammals, possibly multiple times, which could explain the differences in cellular mechanisms underlying formation of the primitive streak, as well as observations such as the presence of left-right asymmetries around Hensen's node and lack of nodal cilia in both chick and pig (Gros et al. 2009; Blum et al. 2014).

This convergent evolution toward the primitive streak could be correlated with changes in the development of the hypoblast/anterior endoderm. In amphibians and reptiles, these cells arise during cleavage from yolky vegetal cells, whereas in birds and mammals the hypoblast develops from cells delaminating or sorting out from the epiblast. Formation of the hypoblast in this manner could be a prerequisite for the emergent behavior of coordinated polonaise-like cell movements in the epiblast, which themselves are likely sufficient to position and initiate the primitive streak (Voiculescu et al. 2007). Additionally, owing to the more prominent role of hypoblast migration in birds and mammals, cell internalization initiates with the primitive streak, not with the organizer. Full organizer formation, in terms of gene expression and inducing activity, does not occur until mid-gastrulation. Thus, there is significant heterochrony in the main pattern of axis formation in birds and mam-

mals, with the appearance of the organizer and cell involution lagging behind that of ventrolateral cell ingression (Fig. 6.14). It is possible that the lessened importance of cortical rotation-like mechanisms led to subtle reorganization of the organizer gene regulatory networks, causing organizer genes such as Noggin to be expressed later relative to other dorsal genes.

Mammals exhibit the greatest changes in axis-forming mechanisms within the vertebrates. There is also wide variation in these mechanisms within the mammals themselves. Mammalian eggs have evolved to lose size and yolk content, as well as any notable animal–vegetal asymmetry and cortical rotation-related events in the process. The evolution of implantation also necessitated the early segregation of embryonic and extra-embryonic lineages and the maintenance of embryonic pluripotency. Furthermore, gastrulation in mammals initiates with far fewer cells than in other groups of organisms, ~250 cells in the mouse (compared with >10,000 in the frog and chicken), and is accompanied by rapid cell division. This rapid cell cycle of 2–6 h (Snow 1977) coincides with germ layer and axis patterning in the mammalian embryo, and has sparked evolutionary comparisons to the cleavage stage cell cycle in more primitive animals (O'Farrell et al. 2004). Whether this rapid division is indeed a conserved but temporally deferred event, as has been proposed, is unclear. Alternatively, there may exist a general correlation between pluripotency and shorter G1/G2 phases, with limited cell cycle checkpoints, as is seen in cultured stem cells (Becker et al. 2006).

What are some future prospects for the study of axis formation? After many decades of research, the ideas of symmetry breaking and self-organization in development remain compelling yet incompletely understood. Indeed, the ideas of self-organization and biological "regulation" predate the discovery of the organizer and have been developed into the concepts of the self-regulating embryonic "field" and pattern formation by positional information (Child 1915; Weiss 1926; Nieuwkoop 1967; Wolpert 1969, 1971; Green and Sharpe 2015). The ability of the organizer to undergo self-organization was noted by Spemann and others and clearly implies a complex network of interactions. Recent work showing inverse transcriptional regulation within the BMP regulatory circuitry has shed some light onto these processes. This observation is only the first step toward understanding self-organization at the gene regulatory network level, and additional general principles are likely to be identified. Although the conserved transcriptional control of genes like *Goosecoid* has long been noted, these mechanisms have not necessarily been fully characterized or compared. Remarkably, large and robust organizer gene regulatory networks have not yet been elucidated for any vertebrate organism. It should eventually be possible to generate comparative organizer gene regulatory networks across vertebrates and quantitatively compare their properties. Such analyses might shed light on possible changes in relative onset of organizer activity and also provide insight into the control of cell behaviors during gastrulation, as there have been several evolutionary transitions between involution and ingression in similar tissues.

Additionally, with regard to the complex interactions underlying symmetry breaking events in early vertebrate development, the molecular basis of microtubule orga-

nization during cortical rotation, the prime example of a symmetry-breaking process, remains largely unknown. The cell movements underlying asymmetry in the mammalian blastocyst are similarly mysterious. Both of these areas would likely benefit from advances in live imaging technology that are rendering the molecular activities of cells and tissues more visible and quantifiable. In particular, new methods for culturing and imaging different vertebrate embryos (e.g., Bedzhov and Zernicka-Goetz 2014; Keller et al. 2008) will likely provide novel insights into morphogenesis.

There have recently been rapid advances in whole genome analysis and genome editing technology that will likely transform the study of developmental biology and biology in general. Large-scale gene expression, chromatin analysis and proteomics are becoming commonplace and should yield abundant material for data science analysis. It is probably not a stretch to imagine that real-time whole transcriptome analyses will also become technologically feasible in living cells in the near future. TALEN and Cas9/CRISPR-mediated genome editing are also on the verge of becoming routine, extending mutational analysis to many areas previously intractable. Thus, in the foreseeable future, a variety of "-omics" data should be readily available for any organism, and investigators will have the capability to interrogate the function of any genomic region and immediately read out responses in gene expression and cell behavior.

The lack of detailed genomic and genetic information and methodologies has often been a barrier to work in many nontraditional model organisms. However, newer technologies should soon allow the genetic analysis of axis formation and other processes in organisms rationally chosen based on phylogenetic position or other criteria based on the biological question. Indeed multiple related species could be analyzed in parallel to capture extant variation in developmental mechanisms. Such ideas are not new (Tzika and Milinkovitch 2008) but are much closer to realization. Some interesting candidates for developmental analysis could include tree shrews, owing to their similarity to primates, and echidnas, as an egg-laying mammal model (Tzika and Milinkovitch 2008). The increased availability of human embryonic stem cells is also leading to a better understanding of human development. Gene regulatory networks can be analyzed in embryonic stem cells and induced-pluripotent stem cells and tissue "organoids" are increasingly being used to understand human organ development (e.g., McCracken et al. 2014). Certain ethical considerations would have to navigated however to study human axis formation in this way. Comparative studies of early development across all vertebrates could be facilitated by reviving the idea of a "standardized vertebrate normal table" (Witschi 1956; Hopwood 2007). Such a standard series could be based on conserved gene expression data, gene regulatory network organization and morphogenetic patterns. Potential in-roads to such a project have already been made with the construction of comparative drawings based on gene expression patterns, the "Molecular Haeckels" (Elinson and Kezmoh 2010).

It is perhaps ironic that just as long-awaited sequenced genomes and targeted genome editing technologies are becoming routinely available in organisms like fish and amphibians, interest in the basic science of development in these organisms is perceived to be waning in favor of human-centered translational research. It remains

to be seen whether this perception is borne out in the long term. An optimist might argue that the processes of asymmetry, axis formation, and self-organization have fascinated scientists and have generated fundamental biological insight for well over 100 years, and should continue to do so long into the future.

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Chapter 7
Establishment of the Vertebrate Germ Layers

Wei-Chia Tseng, Mumingjiang Munisha, Juan B. Gutierrez, and Scott T. Dougan

Abstract The process of germ layer formation is a universal feature of animal development. The germ layers separate the cells that produce the internal organs and tissues from those that produce the nervous system and outer tissues. Their discovery in the early nineteenth century transformed embryology from a purely descriptive field into a rigorous scientific discipline, in which hypotheses could be tested by observation and experimentation. By systematically addressing the questions of how the germ layers are formed and how they generate overall body plan, scientists have made fundamental contributions to the fields of evolution, cell signaling, morphogenesis, and stem cell biology. At each step, this work was advanced by the development of innovative methods of observing cell behavior in vivo and in culture. Here, we take an historical approach to describe our current understanding of vertebrate germ layer formation as it relates to the long-standing questions of developmental biology. By comparing how germ layers form in distantly related vertebrate species, we find that highly conserved molecular pathways can be adapted to perform the same function in dramatically different embryonic environments.

Keywords Pander • Ectoderm • Mesoderm • Endoderm • TGF-beta • Fgf • Nodal • Morphogen • Temporal gradient • Extraembryonic tissues • Teleost • Amniote • Amphibian

7.1 Introduction

The world is about to observe the 200th anniversary of the publication of two papers by Christian Heinrich Pander (1794–1865) in 1817 that mark the foundation of modern developmental biology (Pander 1817a, b). Pander invented the language

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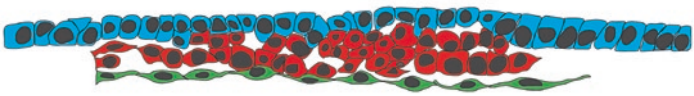
that we still use to describe embryos and raised scientific questions that we are still trying to answer today. In *Dissertatio Inauguralis*, published in January, Pander coined the term blastoderm (*blastoderma*) to define the membrane that "… *the Embryo chooses as its seat and its domicile, contributing much to its configuration out of its own substance*" (p. 21) (Pander 1817b). Subsequently, he showed that the blastoderm is divided into three "lamellae," called germ layers (*Keimhäute*) (Pander 1817a). Although he was not the first to observe embryonic membranes, Pander was the first to understand their significance:

> At the twelfth hour the blastoderm consists of two entirely separate layers, an inner one, thicker, granular and opaque, and an outer one, thinner, smooth and transparent. The latter, because of its development and for the sake of greater accuracy of description, we may call the serous layer and the former the mucous layer … There arises between the two layers of the blastoderm and third middle one in which the blood vessels are formed, which we therefore call the vessel-layer; from its origin events of the greatest importance subsequently occur … Actually there begins in each of these three layers a particular metamorphosis, and each one strives to achieve its goal; only each is not yet sufficiently independent by itself to produce that for which it is destined. Each one still needs the help of its companions; and therefore all three, until each has reached a specific level, work mutually together although destined for different ends (pp. 5–6) (Translated by Jane Oppenheimer (Oppenheimer 1940)).

Not only did Pander describe the three germ layers, but he realized that they originated from the blastoderm: "*For before incubation this membrane consists of a single layer, made up of granules which cohere to each other by their own viscosity. As incubation progresses, however, there originates from this another layer, more delicate but firmer in structure, so that at a specific time the blastoderm can be divided by a fairly long maceration into two layers*." (Pander 1817b; Oppenheimer 1940). Finally, he showed that each layer has distinct contributions to the embryo: the mucous layer, which we now call the endoderm, generates the gastrointestinal system; the vessel layer, or mesoderm, produces the blood and blood vessels and the serous layer, or ectoderm, gives rise to the epidermis and the central nervous system. We now understand the fates of the three germ layers in much greater detail (Fig. 7.1), but Pander's overall outline has withstood the test of time. Pander also recognized that mutual interactions between these three tissues were likely to be important for normal development, since they "*work mutually together*" to achieve their "*different ends*."

Pander's work was the culmination of an exhaustive series of observations on over 2000 live chicken embryos, conducted in collaboration with his doctoral advisor, the anatomist Ignaz Döllinger (1770–1841), and a skilled artist, Eduard d'Alton (1772–1840). They focused on the earliest stages of development and described only what they could see at a particular time without any preconceived notions about the fates of the tissues at later stages (Schmitt 2005). This unbiased approach was the intellectual breakthrough that allowed them to understand the biological significance of the embryonic membranes. Pander also used technical innovations that permitted him to see the embryo in greater detail than previously possible. He used a new dissection technique developed by Döllinger to isolate embryo and observe it on a black background for improved contrast (Schmitt 2005). Each of the three men viewed the embryos independently, and they consulted with each other to



Ectoderm	Mesoderm	Endoderm
Epidermis	Bones, cartilage, tendon	Digestive System
Central Nervous System	Muscle (smooth, striated)	Respiratory System
Neural Crest Derivatives: • Peripheral Nervous System • Melanocytes • Facial cartilage • Dentin of teeth • Skull bones	Circulatory System, including heart, vessels	Pancreas
	Lymphatic system	Liver
	Gonads	Thyroid Epithelium
	Kidney	Parathyroid Epithelium
	Adipose Tissue	Thymus
	Notochord	Bladder

Fig. 7.1 Diagram of the germ layers in a gastrulating rabbit embryo, after Kölliker (1882). *Blue* = ectoderm; *Red* = mesoderm; *Green* = endoderm. The table describes the derivatives of the three germ layers in vertebrates

verify their observations. d'Alton documented their findings with a series of drawings that stood out for their remarkable detail and three-dimensional complexity (Fig. 7.2A and B). To clarify the spatial relationship between the germ layers, remarkably, they examined the embryos edge-on and produced what may be the first optical cross sections of embryos (Fig. 7.2C, figs. 8 and 9). They complained that the images under the microscope were blurry, not surprising given the quality of lenses available to them. So to convince themselves that the folds in the germ layers were not merely optical illusions created by poor optics, they used needles and bristles to touch and physically move the tissue (Fig. 7.2C, figs. 6 and 7). Such caution was warranted because Pander and Döllinger realized the significance of their findings for a debate that had been raging for centuries.

7.2 Historical Context

The full impact of Pander's studies can only be understood in the broader context of the work of Albrecht von Haller (1708–1777) and Friedrich Caspar Wolff (1734–1794), two eighteenth century biologists who stood on opposing sides of the ancient question about "generation." von Haller was the main proponent of the theory of preformation of his time (Roe 1981). He differed from other preformationists, however, in that he emphasized the primacy of the egg, rather than the semen, as the residence of the preformed body. In his view, the egg contained a fully formed body that

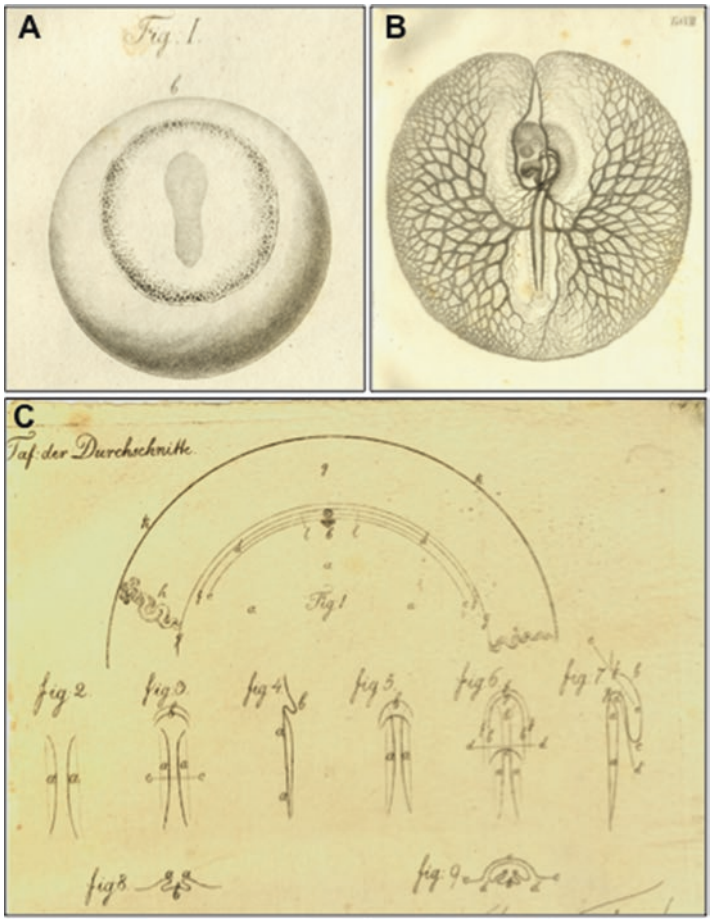


Fig. 7.2 Images of the germ layers from "Beiträge zur Entwickelungsgeschichte des Hühnchens im Eye" (1817). (**A**) d'Alton's drawing by of the chicken embryo showing the blood islands surrounding the area pellucida of the blastoderm on top of the yolk (Tab IV). (**B**) d'Alton's drawing showing a top view of the chicken embryo, including the entire circulatory system (Tab VIII). (**C**) Drawing of chicken eggs in cross section. Figure 1 shows the extent of the germ layer spreading over the yolk during development. (a) Yolk mass; (b) the site of entry into the egg shell; (arc c–c), endoderm, (d–d arc) mesoderm; (e) embryo, (f–f arc) ectoderm; (g) vitelline membrane; (h, i) Chalaza; (k) egg shell. Figures 2–9 show top views (Figs. 2, 3, 5, and 6), lateral (Figs. 4 and 7) and medial cross sections (Figs. 8 and 9) of the blastoderm at different stages. In all figures, (a) represents what Pander called the "primitive folds," which we call today the neural folds. (b) indicates the head folds. In Fig. 3, the line (c) indicates the point level of the cross section shown in Fig. 8. In Fig. 6, line (d) indicates the level of the cross section shown in Fig. 9. (c) and (g) indicate the upper and lower layers of the head fold, (f) indicates the anterior edge of the head fold. In Fig. 7, the lines (d) and (e) show the direction in which Pander and colleagues moved the embryonic folds with a bristle or needle (Tab der Durchschnitte). Images used with permission of Bayerische Staatsbibliothek, Münich. urn:nbn:de:bvb:12-bsb10315830=0. Shelfmark (2 Anat 49)

gradually revealed itself as the embryo grew in size and emerged from the yolk (Roe 1975). von Haller found evidence supporting his view in the earliest microscopic studies of developing vertebrates and invertebrates by Malpighi and Swammerdam, respectively. Malpighi and Swammerdam both observed embryonic versions of adult organs in their studies, but neither explicitly endorsed a theory of preformation (Cobb 2000). von Haller also examined chicken embryos himself, and found further support for his views on preformation. For example, he claimed that the membranes of the yolk sac were continuous with those of the intestine. Since the yolk membrane was clearly present in the unfertilized embryo, he concluded the intestinal membrane must also be present (Haller and Arnay 1758; Roe 1975). From this and other work, von Haller deduced the existence of an invisible embryo in untreated unfertilized eggs, complete with preformed versions of adult organs. Embryonic development was merely the process of organs growing in size after fertilization, becoming more opaque and visible to the eye over time. To explain why the different organs became visible at different times, he suggested that different parts of the embryo grow at different rates. He predicted that more powerful microscopes would eventually reveal the elusive preformed embryo (Keezer 1965).

Caspar Friedrich Wolff was a contemporary of von Haller's and a leading proponent of the theory of epigenesis (Roe 1981). The concept of epigenesis was originally formulated by Aristotle and held that organs and tissues are formed *de novo* with each generation (Aristotle and Peck 1943). Epigenesis was discredited in the Middle Ages along with Aristotelian physics, but was revived in a different form by William Harvey (1578–1657) in his 1651 treatise *Exercitationes de generatione animalium* (Harvey 1651). Harvey's epigenetic theory was not based on direct observation of development, since he never observed an egg or embryo under a microscope (Keezer 1965). The first experimental evidence supporting epigenesis came only in 1742, when Abraham Trembley (1710–1784) discovered the ability of freshwater polyps to regenerate after being cut in half (Roe 1975). This showed that some animals could regenerate structures *de novo*, but left open the question of how the animal was generated in the first place. To answer this question, Wolff performed a careful microscopic examination of early chicken embryos. He found that organs are generated through a process involving the folding of embryonic membranes in a precise and reproducible manner (Meyer 1932; Roe 1975). This folding could not be explained if embryonic development was merely the result of changes in size or opacity of preexisting organs. But Wolff did not fully appreciate the significance of the embryonic membranes. He thought they were an inorganic covering that moved passively in response to external forces (Schmitt 2005). Like all previous proponents of epigenesis, Wolff invoked the action of a mystical "vital force" to shape the embryo. To von Haller, this was the fatal flaw of epigenesis. He wrote that epigenesis invoked, "… *a force that seeks, that chooses, that has a purpose, that against the laws of blind combination always and infallibly casts the same throw … I do not find in all nature the force that would be sufficiently wise to join together the single parts of the millions and millions of vessels, nerves, fibers and bones of a body according to an eternal plan* …" (Roe 1981). Thus, epigenesis failed because no one could provide a satisfactory mechanism.

Pander's work addressed this criticism directly. He wrote:

> We must remind our readers that when folds of the membranes are mentioned, they should not imagine lifeless membranes, whose folds would necessarily extend over the entire surface if they were mechanically formed, and would not be restricted to a particular zone; because this view would inevitably lead to erroneous ideas. The folds that cause the metamorphosis of the membranes have a spontaneous organic origin, and form at the proper place, whether by expansion of already existing vesicles or by the emergence of new vesicles with no alteration in the remainder of the blastoderm. (p. 40) (Pander 1817a)

To scientists of the time, the ability to move was one of the defining characteristics of animal life. Pander realized that life itself was the driving force of morphogenesis, eliminating the mysticism of previous incarnations of epigenesis. After Pander, any lingering support for preformation evaporated. In a deeper sense, though, only now, we are only now beginning to get a satisfactory answer to von Haller's mechanistic critique of epigenesis by using hte modern molecular genetic and imaging techniques currently at our disposal.

7.2.1 The Germ Layer Theory

Pander quickly abandoned embryos to study fossils, but his work inspired others to study the germ layers in both vertebrate and invertebrate embryos, including his friend and fellow student of Döllinger's, Karl Ernst von Baer (1792–1876). von Baer is perhaps better known today for his discovery of the mammalian egg in 1826, but he was one of the first individuals to perform a comparative analysis of embryos from different species (Baer and Stieda 1828). von Baer extended Pander's observations of germ layers in the chicken embryo to all vertebrate embryos, while other scientists inspired by Pander found the germ layers in invertebrates (Oppenheimer 1940). By the middle of the nineteenth century it was recognized that germ layers are a universal feature of animal development. This was a major conceptual breakthrough because it suggested for the first time that all animals share a common mechanism of development. von Baer formalized this concept into the Germ Layer Theory, which stated that all gastrula stage embryos consist of three visible layers, each dedicated to play a specific role in the development of the organism (Baer and Stieda 1828). This theory provided an objective basis for comparing embryonic development in different species. Embryogenesis could be divided into three stages (at least) that are common to all animals. Before gastrulation, the embryo consists of an undifferentiated blastoderm. During gastrulation, the blastoderm divides into three germ layers, which make distinct contributions to the body. Finally, after gastrulation, the cells begin to differentiate into specific tissues.

The Germ Layer Theory also posited that homologous tissues in different species were derived from the same germ layers. For example, if the heart was derived from the mesoderm in chicken, it was also mesodermal tissue in amphibians and mammals. von Baer applied these fundamental insights into his systematic comparison of embryonic development in different species to develop his famous laws

of embryology, which Stephen J Gould paraphrased succinctly: "*Development is individualization*; *it proceeds from the general to the special; it is a true differentiation of something unique from something common to all*." (p. 55) (Gould 1977). In other words, embryos of the same phylum start out looking very similar to each other, displaying the general characteristics shared by all members of that phylum, and subsequently diversify as the more specific traits of each species develop (Baer and Stieda 1828). von Baer built a classification system of developmental history based on shared embryological characteristics. For example, animals containing an embryonic notochord were grouped together, and this group further divided into those with vertebrae and those without vertebrae. In his private notes, he expressed this classification system as a "tree of developmental history," which bears a striking resemblance to Darwin's evolutionary tree published 31 years later (Brauckmann 2012; Abzhanov 2013). The Germ Layer Theory was highly controversial for most of the nineteenth and early twentieth centuries, with much discussion focusing on which organs and tissues were true homologues in different species (Braem 1895). Modern methods of marking specific cell types have resolved this issue, and have shown that germ layers in different species do, in fact, produce homologous organs. von Baer left embryology in 1838 to study ecology, but his work inspired a new generation of scientists to use comparative embryology to understand the evolution of species (Brauckmann 2012).

7.2.2 The Developmental Hourglass

The stage in which all embryos of a phylum resemble each other was eventually called the phylotypic stage (Fig. 7.3b). After the phylotypic stage, the embryo morphologies increasingly diverge as the unique features of each species, such as hair, feathers, shells, beaks or teeth, begin to differentiate (Fig. 7.3c). Vertebrate embryos also display a wide variety of morphologies at the blastoderm stage, well before the phylotypic stage (Fig. 7.3a). This early morphological diversity may be surprising until one remembers that the egg is an adult cell type, produced by the mother for the purpose of reproduction in a particular environment. Therefore evolutionary pressures on the adult female shape the morphology of the egg and early embryo. The diverse vertebrate morphologies of the eggs converge on a common body plan at the phylotypic stage, in which diversity is constricted. After this stage, diversity increases as the specialized features characteristic of each species begin to develop. The constriction point has suggested an analogy with a sand-filled hourglass, which modern developmental biologists refer to as the Developmental Hourglass (Slack et al. 1993; Duboule 1994).

The phylotypic stage became a highly controversial concept over the years, in part because it was enthusiastically adopted by Ernst Haeckel to promote his erroneous views on evolution and development, which he encapsulated with the phrase "ontogeny recapitulates phylogeny" (Haeckel 1874; Sedgwick 1894). Some have even accused Haeckel of making fraudulent drawings to support his

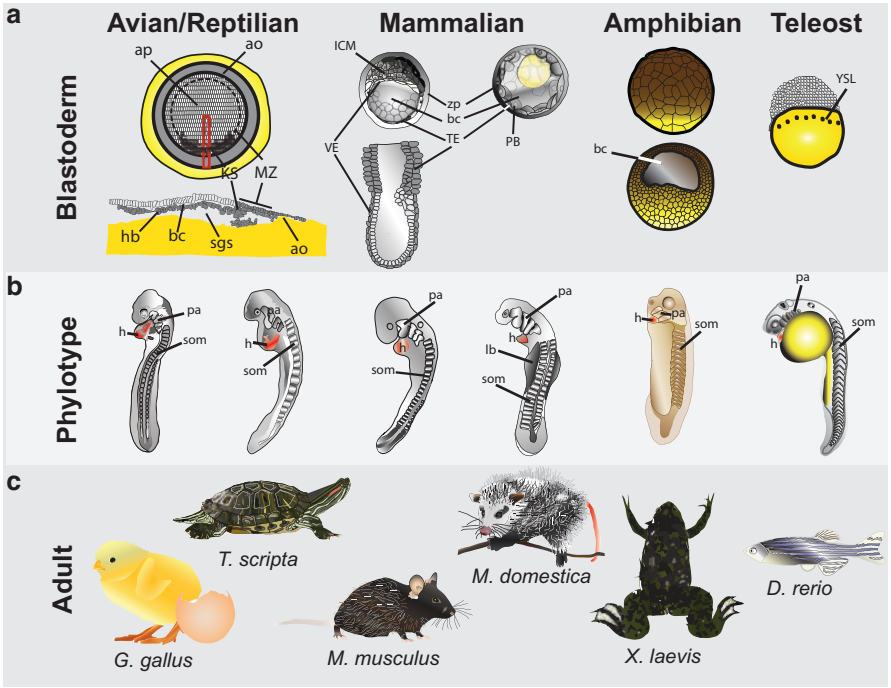


Fig. 7.3 Hourglass model of development. Diagrams showing morphologies of different classes of vertebrates at three different stages of development. (**a**) Comparison of a blastoderm stage chicken embryo (EGK stage XI), a mouse E3.5 blastocyst and E 5.5 stage embryo, a unilamellar blastocyst at day 5.5 (McCrady Stage 8–9) of a possum (*M. domestica*), an *X. laevis* blastula (7 h), and a zebrafish blastoderm stage embryo (3.5 h). (**b**) A comparison of a 2-day-old chick embryo, a 4-day-old turtle (*T. scripta*) embryo, an E8.75 mouse embryo, a day 10 (McCrady Stage 27) possum embryo, a 32 h (Stage 28) *Xenopus* tadpole, and a 28 h (prim-6) zebrafish embryo. To facilitate comparison, the orientation of the chick embryo has been flipped to match the other embryos. (**c**) A juvenile chicken, an adult turtle (*T. scripta*), possum (*M. domestica*), frog (*X. laevis*) and zebrafish (*D. rerio*). *ao* area opaca, *ap* area pellucida, *bc* blastocoel, *h* heart, *hb* hypoblast, *ICM* inner cell mass, *KS* Koller's sickle, *lb* limb bud, *MZ* marginal zone, *pa* pharyngeal arch, *PB* periblast, *sgs* subgerminal space, *som* somites, *TE* trophectoderm, *VE* visceral endoderm, *YSL* yolk syncytial layer, *zp* zona pellucida (Mate et al. 1994; Selwood et al. 1997; Keyte and Smith 2010; Kimmel et al. 1995; May 2013). *Note*: Drawings are not to scale

theory, but those accusations were convincingly refuted (Richardson et al. 1997; Richards 2009). The existence of the phylotypic stage in both vertebrates and invertebrates was recently confirmed by three independent studies using bioinformatics analysis of the genes expressed at different stages (Domazet-Loso and Tautz 2010; Kalinka et al. 2010; Prud'homme and Gompel 2010). These studies showed that the genes expressed at the phylotypic stage originated further back in evolutionary history than genes expressed at other stages, and evolved at slower rates. This indicates that the similarity in morphologies is indicative of a highly conserved developmental program.

In vertebrates, the phylotypic stage occurs around the pharyngula stage, just before the limb buds appear, when the pharyngeal arches are evident, the heart is beating, and 20 or more somites are present in the paraxial mesoderm (Fig. 7.3b). There are very few features at this stage that can be used to distinguish a fish embryo, from a frog or mammalian embryo. Some differences are only superficial, and mask an underlying similarity. The extraembryonic yolk, which can vary greatly in size from species to species, can superficially alter the appearance of the embryo. This is misleading, however, because the yolk does not contribute any tissue to the body (Richards 2009). Embryo size can also vary greatly from species to species at the phylotypic stage, but the overall body plan is constant. Some differences at the phylotypic stage, however, reveal important distinctions between the species. For instance, marsupial embryos can be readily distinguished by the precocious development of the forelimbs (Fig. 7.3) (Keyte et al. 2007). Marsupials spend a comparatively short time in the uterus in comparison with other mammals, and are born in a relatively immature state (Smith 2001). Their forelimbs are fully formed and functional, which enables the developing fetus to crawl out of the birth canal and into the pouch unassisted by the mother, where it completes the developmental program (Gemmell et al. 2002).

The conservation of morphology and gene expression at the phylotypic stage suggests that there are unknown evolutionary constraints on the morphology of the body plan that do not exist at early or late stages. Alternatively, the conservation of morphology may simply be due to the relative lack of evolutionary pressure driving morphological changes at this stage in comparison with the adult stages.

7.3 Blastoderm Formation and Gastrulation

7.3.1 Amniotes

7.3.1.1 Birds

Vertebrate embryos display enormous morphological diversity at the blastoderm stage, consistent with the Developmental Hourglass model. Avian and reptilian embryos are specialized for developing on land, outside the mother. They develop inside a shell that protects against evaporation while promoting gas exchange, and contain a large yolk that acts as a source of nutrition until hatching. The early cell divisions undergo meroblastic cleavages, which are characterized by incomplete cytokinesis that does not partition the yolk into the blastomeres, called. The earliest stages of embryonic development of birds occur *in utero*. Upon egg laying, the chicken blastoderm appears as a tiny flat disc composed of about 20,000 cells on top of the yolk, which expands to about 55,000 cells before gastrulation (Fig. 7.3a). The blastodisc is organized as a translucent circle of cells, called the area pellucida (ap) surrounded by an outer area, called the area opaca (ao) because it is not translucent. The outer ring of the area pellucida is called the Marginal Zone, and consists of cells that primarily contribute to extraembryonic tissues.

As first described by Pander, the chicken blastoderm originates as a single layer of cells called the epiblast. Initially, small clusters of cells delaminate from the epithelium, in a process of polyinvagination, to form islands of cells underneath the epiblast. These cells spread and proliferate to form a continuous layer of extraembryonic tissue called the hypoblast, or primitive endoderm (Fig. 7.3a) (Eyal-Giladi 1984). A small, fluid filled space, called the blastocoel (bc), separates the two layers. A subgerminal space separates the hypoblast from the yolk, which contains a layer of syncytial nuclei (YSL) (Nagai et al. 2015). Koller's sickle (KS), appears in the posterior marginal zone (PMZ) as a thickened crescent of extraembryonic cells (Izpisua-Belmonte et al. 1993; Callebaut and Van Nueten 1994).

Gastrulation begins in the posterior of the blastodisc, just anterior to the KS. During gastrulation, movements bring cells from the lateral edge of the blastodisc to the midline. These movements were named polonaise movements, after the polish dance in which "*couples walk down opposite sides of the hall in two columns, come together at the end of the hall, clasp hands and go forward in fours down the middle*," in a double vortex-like process (p. 391) (Gräper 1929). Cells converge at the midline on top of the KS, intercalate and extend toward the center of the epiblast (Voiculescu et al. 2007). Cells lateral to the KS move medially to replace the cells that leave the KS. These convergence and extension movements bring lateral cells within the blastodisc towards the posterior midline in long arcs (Fig. 7.4a, arrows). As cells accumulate, the midline becomes visible as a thickened epithelium, called the primitive streak (Fig. 7.4a). Within the streak, presumptive mesoderm and endodermal cells internalize by ingression. During ingression, individual epithelial cells lose their apical–basal polarity, detach from their neighbors and delaminate from the epithelial sheet as they transition to a mesenchymal state (Levayer and Lecuit 2008). As gastrulation proceeds, the primitive streak elongates towards the anterior as more cells converge at the posterior midline (Fig. 7.4a). The anterior limit of the primitive streak is marked by a thickened knot of epithelium called Hensen's node, which is derived from cells in the epiblast and deep cells of Koller's sickle (Fig. 7.4a) (Lawson and Schoenwolf 2001). After the streak reaches its full extent, the node retracts, migrating back to the posterior margin leaving condensing notochord in its path.

7.3.1.2 Reptiles

In most reptilian embryos, the early stages of embryonic development occur *in utero* and are difficult to observe. Therefore, the molecular and cellular events leading to gastrulation have not been characterized in as great detail as in other amniotes. Most of our understanding of early reptilian development comes from studies in certain turtle species, which initiate gastrulation soon after oviposition. At the blastoderm stage, turtle embryos have the same flat, bilamellar structure as avian embryos, complete with an area pellucida, area opaca and a marginal zone (Hubert 1970). These embryos contain a hypoblast formed by polyinvagination (Pasteels 1957). Some blastomeres on the edge of the blastodisc deposit their nuclei in the yolk through incomplete division, forming a yolk syncytial layer (YSL), similar to that observed in the chicken (Pasteels 1970).

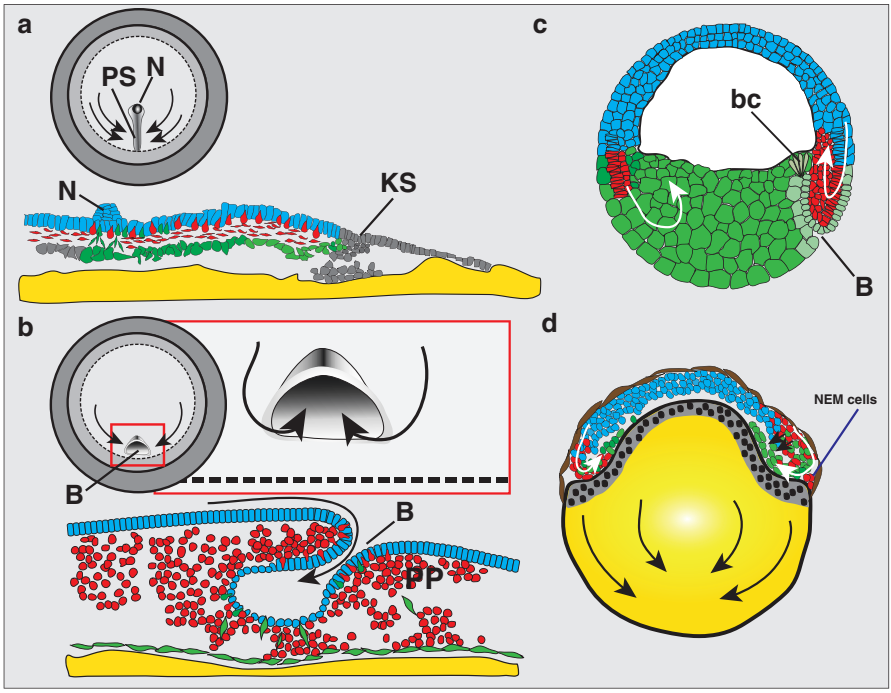


Fig. 7.4 Gastrulation in different vertebrate species. (**a**) Gastrulation in the chicken begins with the circular polonaise movements (*arrows*) of epiblast cells toward the posterior midline, where convergence and extension movements initiate formation of the primitive streak anterior to the Posterior Marginal Zone (PMZ). As the primitive streak extends, the Node forms as an epithelial thickening at the anterior end of the streak. Presumptive mesodermal and endodermal cells ingress from the epiblast. (**b**) In turtles, gastrulation begins with the flattening of the epithelium in the posterior margin, forming the Primitive Plate. An amphibian-like blastopore forms anterior to the primitive plate. Presumptive mesoderm and endoderm involute in the blastopore, and lose epithelial polarity (*black arrows*). Cells ingress from the epiblast after internalization. Mesoderm and endodermal cells ingress from the Primitive Plate to generate an uncharacterized tissue type. (**c**) Gastrulation begins in amphibians with the apical constriction of a small group of cells, called the bottle cells, in the subequatorial region of the dorsal margin. Constriction of the bottle cells draws the attached vegetal cells upward into the blastopore, and the bottle cells displace the involuting presumptive mesoderm inside the embryo and toward the animal pole (*white arrows*). Involution occurs all around the margin, but begins later on the lateral and ventral sides. (**d**) Gastrulation in teleosts begins when epiboly movements begin to spread the epiblast over the extraembryonic yolk (*black arrows*). A group of non-involuting, endocytic marginal cells (NEM) forms at the dorsal margin. At later stages, these cells become the dorsal forerunner cells. When cells reach the equator, presumptive mesoderm and endoderm internalizes around the entire margin by a polyingression mechanism (white arrows), which resembles the wheeling-in movement of involution. In the embryonic shield, cells also involute from the epiblast by ingression (*black*). *Blue* = epiblast, *Red* = presumptive mesoderm, *Green* = presumptive endoderm, *grey* = extraembryonic. *B* blastopore, *bc* bottle cell, *KS* Koller's sickle, *N* node, *NEM* non-involuting, endocytic marginal cells, *PP* primitive plate, *PS* primitive streak

Despite the obvious morphological similarity to avian embryos, reptilian embryos undergo a different method of gastrulation not observed in other amniotes. Instead of forming a Node and primitive streak, cells converge in the posterior epiblast and form an amphibian-like blastopore (see below) (Fig. 7.4b). Cells in the blastopore form a continuous rolling sheet of involuting cells, as in frogs, but then they lose their epithelial polarity and ingress to form mesoderm and definitive endoderm (Fig. 7.4b) (Coolen et al. 2008; Bertocchini et al. 2013; Stower et al. 2015). Prior to formation of the blastopore, cells in the PMZ flatten to form a structure called the primitive plate, which is found in all reptiles (Fig. 7.4b) (Coolen et al. 2008; Bertocchini et al. 2013; Stower et al. 2015). Cells in the primitive plate internalize by ingression, and contribute to the definitive endoderm and an uncharacterized type of mesoderm (Bertocchini et al. 2013). Thus, the mechanism that forms the germ layers in reptiles is a hybrid between the involution based mechanism of internalization found in amphibians, and amniote-like ingression.

7.3.1.3 Mammals—Monotremes

Mammalian embryos display a surprising amount of morphological diversity at the blastula stage. Monotremes are a family of egg-laying mammals that includes the platypus and the echidnas, making their embryonic development of particular evolutionary interest. Embryological studies of this group have been limited because they are protected species and difficult to breed in captivity. Platypus eggs have a large yolk and a disc-shaped blastoderm, like those in birds and reptiles, complete with an extraembryonic hypoblast (Wilson and Hill 1902; Selwood 1994; Hughes and Hall 1998; Selwood and Johnson 2006). The blastoderm forms by meroblastic cleavages, like those of reptiles and birds, and gastrulation occurs through a primitive streak located in the posterior of the blastodisc (Wilson and Hill 1902; Hughes and Hall 1998).

7.3.1.4 Mammals—Eutherians

Placental mammals, or eutherians, are a group that includes the animals we are most intimately familiar with, including the most commonly used mammalian developmental model organism, the mouse. The embryos in this group develop inside the mother and produce an extraembryonic yolk sac, but they lack yolk due to an accumulation of missense mutations in the vitellogenin genes (Brawand et al. 2008). Holoblastic cleavages divide the embryos into complete blastomeres. The early cleavages occur inside a membrane called the zona pellucida and produce a small spherical structure at the 8-cell stage, which is called the morula stage because it looks like a mulberry. After the 8- or 16-cell stages, the embryo undergoes compaction, a change in morphology driven by increased adhesion of cells on the outer surface of the embryo to each other. This change is accompanied by a lineage restriction such that the outer cells generate the trophoblast, an extraembryonic tissue specialized for invading the uterine wall and producing the elaborate tissues required for survival inside the womb (Pierce et al. 1988). The trophoblast express Na^+, K^+-ATPase pumps, which drive the influx of fluid that

forms the blastocoel in the middle of the embryo (Manejwala et al. 1989). The cells remaining inside the embryo after compaction form the Inner Cell Mass (ICM), a pluripotent group of cells that have the ability to generate any tissue in the embryo and some extraembryonic tissues (Beddington and Robertson 1989). The first cells from the ICM to differentiate form the visceral endoderm (VE), an extraembryonic tissue that separates the ICM from the blastocoel (Gardner 1983). The embryo at this stage is called a blastocyst (Fig. 7.3a). In most placental mammals, the epiblast forms a flat disc after implantation, similar to the disc observed in avian/reptilian blastoderms. Some rodents, however, like mice and rats, form an epiblast shaped like an inverted cup (Fig. 7.3a). In one strain of mice, the epiblast consists of only 660 cells just prior to the onset of gastrulation, but this expands rapidly to 14,500 cells by mid-gastrulation, due to a dramatic shortening of the cell cycle (Snow 1977). In the mouse, the primitive streak forms along the posterior side of the inverted cup, with endoderm and mesoderm egressing from the epiblast to surround the future ectoderm. A thickened tissue, called the node, marks the anterior of the streak.

7.3.1.5 Mammals—Metaeutherians

Marsupials are classified as non-placental mammals, or metatheria, because they undergo a significant portion of their development in a maternal pouch. Despite this categorization, marsupial embryos attach to the uterus and produce a yolk sac placenta, which has the same embryological origin as the yolk sac in eutherian mammals (Freyer et al. 2003). Marsupials do have active vitellogenin genes, and produce a yolk mass visible at the blastocyst stage (Fig. 7.3a) (Selwood et al. 1997). Marsupial blastocysts are also distinct because they do not form an ICM. Instead, they initially take on a unilaminar structure and resemble a hollow ball (Fig. 7.3a). This structure arises because the blastomeres strongly adhere to the zona pellucida during the early cleavages (Selwood 1986, 1992). Nonetheless, cells are allocated at this stage either to an extraembryonic trophoblast lineage or a pluripotent pluriblast lineage that generates the entire epiblast (Cruz et al. 1996). These cell populations can be distinguished based on morphological criteria, since the pluriblasts are large, round cells and the trophoblast cells are smaller and flattened (Selwood and Johnson 2006). The pluriblasts divide to generate a bilaminar blastocyst consisting of an extraembryonic hypoblast, or parietal endoderm, and an epiblast/pluriblast (Mate et al. 1994; Selwood et al. 1997; Kress and Selwood 2006). Growth of the embryo generates an a flat, pear-shaped epiblast visible on top of the trophoblast by the time of gastrulation (Cruz et al. 1996).

7.3.2 Amphibians

Amphibians are a large family of tetrapods that includes frogs, salamanders, and newts. They evolved to live on land as adults, but their embryonic and larval life occurs in an aquatic environment. The eggs are large and full of yolk platelets that become internalized into the blastomeres after fertilization, with the heavier

platelets settling in the large prospective endodermal cells at the vegetal pole (Fig. 7.3a) (Karasaki 1963). Amphibian embryos undergo holoblastic cleavage, similar to that observed in placental mammals. At the blastula stage, Na^+, K^+-ATPase pumps expressed in the cells at the animal pole are activated and control the influx of fluid to form a blastocoel underneath the prospective ectoderm (Gosner 1960; Morrill et al. 1974).

Gastrulation in *Xenopus* begins when a small group of presumptive endodermal cells, called the bottle cells, form in the sub-equatorial zone of the dorsal margin. These cells undergo a dramatic apical constriction, which generates the dorsal lip of the blastopore (Fig. 7.4c) (Hardin and Keller 1988). Invagination of the bottle cells internalizes the mesoderm and endoderm in two ways. First, the constriction of the apical surface results in an elongation of the basolateral surface, which displaces the presumptive mesoderm internally. Secondly, the vegetal cells attached to the bottle cells are drawn directly inwards. Presumptive mesoderm internalizes through the dorsal lip of the blastopore by the process of involution (Fig. 7.4c). According to John P. Trinkaus (1916–2003), "*Involution is the flowing of a sheet of cells over the edge of an inpocketing, where invagination has occurred, such as the blastopore during amphibian gastrulation.*" (p. 11) (Trinkaus 1984). Alexander Goette (1840–1922) first described this phenomenon in 1869, in embryos of the European aquatic toad, *Bombinator igneus* (Goette 1869). As involution proceeds, bottle cells subsequently form in more lateral and ventral marginal regions, drawing the entire circumference of the involuting marginal zone inside the embryo. Once internalized, the bottle cells change shape once again and spread to form the roof of the primitive gut, the archenteron (Hardin and Keller 1988). The extent of mesoderm internalized by involution varies greatly in different amphibian species. In the salamander, newt as well as in some frog species, the presumptive axial and paraxial mesoderm internalizes during neurulation by "subduction," a coordinated delamination from the epithelia (Smith and Malacinski 1983; Purcell and Keller 1993; Delarue et al. 1994; Shook et al. 2002). During gastrulation, convergence movements bring cells from the lateral regions to the dorsal midline. At the midline, they change their shape and align themselves with respect to the anterior–posterior axis (Solnica-Krezel 2005). These movements and cell shape changes transform the embryo from a sphere to a rod.

7.3.3 Teleosts

Teleost fish represent about half of all known vertebrate species, and they follow a common plan of embryonic development (Postlethwait et al. 2000). The eggs have a large amount of yolk, like avian/reptilian embryos, and the cells sit on top this yolk and undergo meroblastic cleavage. At the blastoderm stage, cells can be divided into the deep cells, which will produce the embryo, and an outer layer of extraembryonic cells called the enveloping layer (EVL), which produces a transient outer layer called the periderm (Kimmel and Law 1985b; Iwamatsu 1994). At the beginning of the blastoderm stage, the cells adjacent to the yolk fuse with each other and

release their contents into the underlying yolk cell, forming an extraembryonic YSL (Fig. 7.3a) (Betchaku and Trinkaus 1978; Ballard 1982; Kimmel and Law 1985a). In contrast to other vertebrates, the cells in the teleost blastoderm and early gastrula are loosely packed and "*constitute a population of individually motile cells*," according to Trinkaus (p. 357) (Trinkaus 1996). In zebrafish, for instance, cells move in random directions for distances up to 70 μm during the late blastula stages (Kane and Kimmel 1993; Concha and Adams 1998; Bensch et al. 2013). Movements are even more dramatic in the killifish blastoderm. Killifish blastoderm cells start blebbing at the end of the cleavage stages, and utilize an actin-based amoeboid-like mechanism to translocate in random directions up to 150 μm before gastrulation begins (Trinkaus 1973; Fink and Trinkaus 1988).

In teleosts, gastrulation begins when epiboly movements start and cells migrate toward the vegetal pole to cover the extraembryonic yolk (Fig. 7.4d, black arrows) (Trinkaus 1996). During gastrulation, cells converge toward the future dorsal side of the embryo, resulting in a thickened region called the embryonic shield (Oppenheimer 1959). As in amphibians, cells at the dorsal midline extend to change the shape of the embryo from a sphere to a rod (Solnica-Krezel 2005). The mechanism by which cells internalize during gastrulation in teleosts has been controversial. Teleosts lack bottle cells and do not forma classic, amphibian-like blastopore. Instead, the function of the bottle cells may be performed by the YSL and a group of *N*on-internalizing, highly *E*ndocytic *M*arginal cells (NEM), also called the dorsal forerunner cells (D'Amico and Cooper 2001; Feldman et al. 2002). The NEM cells may physically constrain the possible movements of internalizing cells. Early studies were confounded by the fact that the easiest cells to label and visualize, the EVL, never internalize. Some of the first investigations of teleost gastrulation reported that deep cells undergo involution in sea bass, trout, and salmon (Goette 1873; His 1878; Wilson 1891). At the same time, other studies in trout and cod concluded that deep cells internalized by delaminating from the blastodisc (Stricker 1865; Reineck 1869; Ryder 1884). The first cell labeling experiments in teleosts found no evidence of involution, invagination or ingression in trout (Ballard 1966a). Instead, the evidence supported a novel model, in which cells deep in the center of the blastoderm migrate outward during gastrulation and form a hypoblast due to convergence movements during epiboly (Ballard 1966b).

The ability to record the behavior of individual cells in live embryos provided the most detailed description of how teleost cells are internalized during gastrulation. The large nuclei of cells in the cyprinid fish, *Barbus conchonius* (Rosy Barb) make it very easy to follow the behavior of single cells under a microscope using DIC optics (Wood and Timmermans 1988). In this species, the presumptive mesoderm and endoderm clearly involute. Similar results were obtained in the Atlantic herring (Hill and Johnston 1997). By contrast, live imaging of the early stages of gastrulation in the killifish revealed that cells internalize by ingression, not involution (Trinkaus 1996). The behavior of individual cells during gastrulation has been analyzed in the greatest detail in zebrafish (Kimmel and Law 1985b; Warga and Kimmel 1990; Shih and Fraser 1995). When epiboly movements bring cells to the equator, precursors of mesoderm and endoderm involute and migrate towards the animal

pole along the inside of the embryo (Fig. 7.4d, white arrows) (Warga and Kimmel 1990). Imaging of zebrafish embryos by light sheet microscopy confirmed that cells around the margin internalize by the "wheeling-in" type of movement, as if they were moving around a blastopore lip (Keller et al. 2008). The movements in zebrafish are often referred to as "involution" in the literature because they are reminiscent of the movements during amphibian gastrulation. A significant difference, however, is that cells internalize individually in teleosts rather than as an epithelial sheet, as in amphibians. Therefore, a more accurate term to describe this type of internalization movement is "synchronized ingression" (Kane and Adams 2002; Adams and Kimmel 2004; Keller et al. 2008). In synchronized ingression, cells in the blastoderm undergo a coordinated epithelial-to-mesenchymal transition as they internalize at the margin, similar to the process of subduction described for axial and paraxial mesoderm in some species of salamander (Purcell and Keller 1993; Shook et al. 2002). Imaging studies revealed that cells within the shield also internalize by a more traditional form of ingression (Keller et al. 2008). Thus, zebrafish utilize two distinct methods of internalization in different regions of the embryo: Cells around the margin internalize as a group by synchronized ingression, while some cells in the shield internalize individually by ingression. The seemingly contradictory observations from different teleost species could reflect true species-specific differences in the mechanisms of internalizing mesoderm and endoderm. Alternatively, the apparent contradictions could simply be an artifact of investigators focusing on different regions of the embryo that use different methods of internalization. Detailed imaging studies of other teleosts are necessary to determine if the results in zebrafish are representative of all teleosts.

7.3.4 Non-teleost Fish

Lampreys (*Lampetra*) are jawless fish that sit at the base of the vertebrate tree, well before the origin of the hinged jaws observed in all cartilaginous and bony fish (Kuratani et al. 2001). Sturgeon (*Acipenser*) and Bichers (*Polypterus*) are two families of ray-finned fish that evolved soon after they branched off from the Sarcopterygian lineage, which produced the tetrapods (Hurley et al. 2007). Embryos from these three groups undergo holoblastic cleavage, and form a blastocoel (Bolker 1993a; Bartsch et al. 1997; Takeuchi et al. 2009b). The vegetal blastomeres in lampreys and bicher are extraembryonic cells specialized for the purpose of providing nutrients during embryogenesis and do not contribute to the embryo (Takeuchi et al. 2009b).

Gastrulation in the sturgeon has been very well described (Ballard and Ginsburg 1980; Bolker 1993a). The mesoderm and endoderm internalizes during gastrulation by involution, which begins with the formation of a blastopore in the dorsal margin. The lip of the blastopore is formed by constriction of bottle cells, which drive the involution of mesoderm and draw the endoderm directly inside the embryo. This process strongly resembles that described for *Xenopus* and other amphibians, but there are some slight differences. First, in sturgeon the notochord is formed after gastrulation by the ingres-

sion of axial mesoderm from the archenteron roof (Bolker 1993a). Secondly, the blastopore forms at the equator in sturgeon, instead of subequatorially as in amphibians. The embryo then undergoes a dramatic thinning of the marginal zone and blastocoel roof, likely by radial intercalation (Bolker 1993b). This thinning process resembles the epiboly movements in teleost embryos, and acts to push the bottle cells into a more vegetal location. The process of involution is very similar in bichir and lamprey, although gastrulation in the lamprey has not been described in extensive detail (Piavis 1961; Takeuchi et al. 2009a). Bottle cells have not been described in the lamprey, but the presumptive mesoderm and endodermal appear to internalize by a process of involution similar to that in amphibians. There is some controversy about the origins of the definitive endoderm in these species (Richardson et al. 2010).

7.3.5 Conclusion

The comparison of blastoderm structure and gastrulation in different vertebrate phyla is instructive to understand how gastrulation evolved. The origin of the vertebrate lineage coincided with the acquisition of a large yolky mass, which influenced the mode of early cleavages and gastrulation (Arendt and Nubler-Jung 1999). The basal vertebrates lamprey and bichir (*Polypterus*) have large extraembryonic yolk cells and undergo holoblastic cleavage. The presence of yolk in the vegetal pole of lamprey and *Polypterus* places constraints on cell movements that influence the mechanics of gastrulation. Yolk cells also influence the timing and mechanism of gastrulation in vertebrates with internalized yolk. In *Xenopus*, for instance, the presence of the yolky endodermal cells in the vegetal pole directs the constriction of the bottle cells toward the equator, which in turn controls the involution movements of mesoderm during gastrulation (Hardin and Keller 1988). The subequatorial location of the bottle cells is important for *Xenopus* morphogenesis, and a more equatorial position was predicted to cause a high frequency of exogastrulation. Consistent with this prediction, in the bottle cells in sturgeon embryos are formed at the equator, but gastrulation is delayed until radial intercalation movements extend the embryo and bring the bottle cells to a more vegetal location (Bolker 1993a).

Embryos with a larger extraembryonic yolk mass, like teleosts, reptiles, and birds undergo meroblastic cleavage (Sheng 2015). If the yolk is large enough, the blastoderm forms a disc instead of a sphere. The reptile blastoderm is a flat disc on a large yolk. These embryos undergo meroblastic cleavage, but form a blastopore and involute rather than forming a primitive streak and node. Thus gastrulation in reptiles occurs by an intermediate mechanism, between that of amphibians and of other amniotes. Monotremes are a family that includes egg-laying mammals like the platypus and echidnas. They occupy a similarly intermediate position in the vertebrate phylogeny and their embryos follow a bird-like pattern of meroblastic cleavages followed by formation of a node and primitive streak (Wilson and Hill 1915; Hughes and Hall 1998; Werneburg and Sánchez-Villagra 2011). As mammals lost their yolk genes, they retained their disc-like morphology, and elaborated extraembryonic structures to facilitate survival in the womb.

The evolutionary origin of the YSL is unclear. Reptile and avian embryos have a YSL, but it is not likely that these are homologous to the teleost YSL, as has been suggested (Nagai et al. 2015). In reptile and bird embryos, the YSL forms during the incomplete meroblastic divisions of the cleavage stage embryo, whereas the teleost YSL forms at a later stage by the fusion of blastoderm cells. Although lamprey and bicher embryos have large extraembryonic yolk cells, the garpike is the most basal vertebrate with a true YSL (Long and Ballard 2001). The garpike (*Lepisosteus*) YSL may be homologous to the teleost YSL in structure and function, but it has also been proposed that the teleost YSL is evolved from the primary hypoblast in bowfin (*Amia*) embryos, an intermediate species between *Lepisosteus* and teleosts (Long and Ballard 2001; Cooper and Virta 2007; Comabella et al. 2014). The presence of extraembryonic yolk cells in basal vertebrates, teleosts, gar, reptiles, and birds, in addition to the formation of the trophoblast in mammals indicates that a common strategy in vertebrate development is to segregate extraembryonic and embryonic lineages before gastrulation. A major, and potentially interesting exception is the amphibians, in which the yolk is internal to the cells.

7.4 The Fate Map of the Vertebrate Blastoderm

7.4.1 The Amphibian Fate Map

Pander and von Baer understood that the three germ layers generate different organs in embryo, but it was not until the early twentieth century that the first fate maps of vertebrate embryos were made. In 1925, the German embryologist Walther Vogt (1888–1941) developed the first technique to heritably label embryonic cells and their descendants (Vogt 1925). He used dried chips of agar stained with Neutral Red or Nile Blue to physically mark the surface of a particular region of the blastula or gastrula stage embryo. The residue of the agar chip would stick to the surface of the cells, and be inherited by its daughters at each subsequent cell division. He could then determine which regions of a blastula stage embryo produced a specific tissue or organ. Amphibians were ideally suited for this analysis, because there is very little cell mixing at the early stages and the future dorsal axis is apparent during the first cell cycle with the formation of the grey crescent. Vogt constructed detailed fate maps of a wide variety of urodele and anuran embryos, including axolotls, newts, and toads (Vogt 1929). Osamu Nakamura (1911–) revised this fate map in 1938 and again in 1942 with improved vital dye staining techniques (Nakamura 1938, 1942). Other scientists used similar surface labeling techniques to fate map the South African Clawed Frog, *Xenopus laevis* and other amphibian embryos (Pasteels 1942; Keller 1975). There are, however, several limitations of the technique. The mark is diluted over successive generations of cell divisions, and can be transferred to neighboring cells by contact. Deep cells of the embryo cannot be lableled in this manner, making it impossible to map the origin of internal organs and tissues with this technique. These weaknesses were addressed by the development of techniques to inject embryos with Horse Radish Peroxidase (HRP), an enzyme that can be detected even

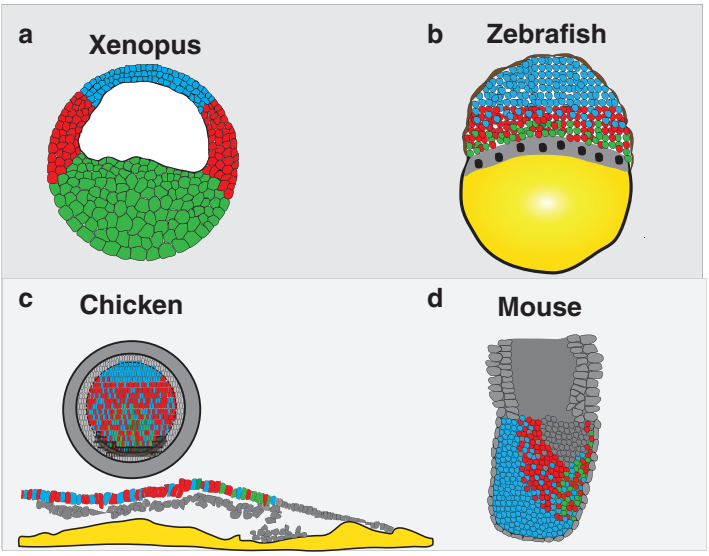


Fig. 7.5 Blastoderm stage fate maps. (**a**) Diagram of a *Xenopus* blastoderm fate map, after those constructed by injecting cells with a fluorescent lineage tracing dye at the 32-cell stage (Cooke and Webber 1985; Dale and Slack 1987a; Moody 1987b). (**b**) Diagram of a zebrafish blastoderm fate map, after those constructed by injecting single blastomeres with lineage tracing dye at the blastoderm stage (Kimmel et al. 1990; Shih and Fraser 1995; Melby et al. 1996; Warga and Nusslein-Volhard 1999). (**c**) Diagram of a chicken blastoderm fate map, after those constructed by labeling cells with DiI or by homotypic transplantation of quail tissue into chick embryos (Hatada and Stern 1994; Callebaut et al. 1996). (**d**) Diagram of a fate map of a mouse pre-gastrula stage epiblast, after experiments injecting single blastomeres with an HRP-lineage tracer followed by ex vivo culturing of the embryo (Lawson et al. 1991). *Blue* = ectoderm, *red* = mesoderm, *green* = endoderm, *grey* = extraembryonic structures

in fixed tissue by a chemical reaction that produces a dark colored precipitate (Weisblat et al. 1978). This method also has some weaknesses. It is labor intensive, since the embryos have to be sectioned to visualize the internal cells carrying the label (Jacobson and Hirose 1978, 1981; Hirose and Jacobson 1979). Since HRP is a protein, it is degraded over time in live cells and therefore cannot permanently mark a cell and its descendants. Modern fate mapping techniques in amphibians utilize nontoxic, nondegradable fluorescent dyes conjugated to sephadex beads, injected into cleavage stage embryos (Cooke and Webber 1985; Dale and Slack 1987a; Moody 1987a, b). These are stable, nondiffusible compounds that permit visualization of labeled cells in live embryos. The fate maps produced by these methods revealed that the conserved morphology of amphibian blastulae reflects a deeper homology in the organization of cell fates, which is exemplified by the *Xenopus* fate map (Fig. 7.5a). Endodermal tissues, such as the gut, pancreas and liver, are derived from the large, yolky cells at the vegetal pole of the blastula, while the ectodermal tissues like the neural tube and epidermis are derived from cells at the animal pole, above the blastocoel (Fig. 7.5a). Mesodermal tissues, like the skeletal muscle, heart, and bone are derived from the cells around the equator, or the marginal zone.

7.4.2 The Teleost Fate Map

In the 1930s, Jane Oppenheimer (1911–1996) and Jean J. Pasteels (1906–1991) used Vogt's technique of labeling cells to produce the first fate maps of killifish and trout embryos, respectively (Oppenheimer 1935; Pasteels 1936). These fate maps were limited by the extensive cell mixing that occurs in early teleost embryos and by the rapid fading of the dye, which occurred more quickly on teleost embryos than on amphibians (Oppenheimer 1947). Later studies produced a more accurate fate map of trout by using a different method to mark cell surfaces (Ballard 1973). The zebrafish fate map was made by injection of fluorescent lineage tracing dyes into individual cells at the late blastula or early gastrula stage (Fig. 7.4) (Kimmel et al. 1990; Shih and Fraser 1995; Melby et al. 1996). Extensive cell movements preclude making a detailed fate map at earlier stages (Warga and Kimmel 1990). In teleost embryos, cell fates are distributed with the same basic organization with respect to the yolk cell. Broadly, endodermal tissues arise from blastomeres closest to the yolk cell, ectodermal tissues arise from blastomeres at the animal pole and mesodermal tissues arise from the cells in between the yolk and the animal pole (Fig. 7.5b). A more detailed examination of the data for zebrafish, however, shows that the territories of the three germ layers overlap significantly. For example, endoderm progenitors are juxtaposed to mesoderm precursors in the region closest to the yolk (Kimmel et al. 1990; Warga and Nusslein-Volhard 1999). Because the precursors for the two germ layers are intermixed, this region is called the mesendoderm. Farther from the yolk, mesodermal precursors are juxtaposed to presumptive ectodermal cells. The extensive intermingling of precursors distinguishes the teleost fate map from that of amphibians. Like the frog fate map, the zebrafish fate map is at single cell resolution. But the technical limitations of injecting embryos at the blastula stage mean that the fate map is restricted to cells close to the embryo surface. The fates of more internal cells have not been determined, although that may change with advances in fluorescent microscopy that permit the live tracking of every cell (Keller et al. 2008).

7.4.3 The Avian Fate Map

Because of the relatively small size of the blastoderm, avian and reptilian embryos presented a difficult challenge for fate mapping studies. Some early, inconclusive attempts were made to label reptile blastoderm cells using Vogt's technique with vital dyes (Pasteels 1937; Chandrasekharan 1966). The first maps of the chicken blastoderm were made by explanting parts of the blastodisc and determining which tissues formed when they were grown in isolation (Rudnick 1935). Today two main methods are used to fate map chicken embryos. Quail cells are histologically distinguishable from chicken cells because they have large nucleoli that act as heritable lineage tracers when transplanted into chicken embryo hosts (Le Douarin 1969). Quail cells develop normally in chick hosts, and the morphological differences

between quail and chick cells can be used to determine the origin of various cell types in orthotopic xenografts (Le Douarin 1973). Cells in the chick blastodisc are too small to reliably inject lineage tracing dyes, but the lipophilic dyes DiI and DiO have been used to heritably label the cell membranes in particular embryonic regions (Hatada and Stern 1994). Both methods have been applied to fate map the chicken blastoderm (Hatada and Stern 1994; Callebaut et al. 1996). For the purposes of building a fate map, the pre-gastrula stage blastodisc (EGK Stage XII), is organized in regions defined by their distance from the PMZ, using Koller's sickle as a reference point. The studies agree that the germ layers arise from three distinct regions of the blastodisc. Cells that form the gut and other endodermal organs are restricted to a pie-shaped sector next to the Koller's sickle, cells at the opposite end of the disc generate ectodermal tissues, and mesodermal progenitors arise from the middle region (Fig. 7.5c). A detailed view of the fate maps shows considerable overlap between the territories. Ectodermal precursors can be found throughout the entire blastodisc, whereas mesodermal progenitors are excluded from the anterior 20 % epiblast. Precursors of all three germ layers can be found juxtaposed in the posterior region.

7.4.4 The Mammalian Fate Map

Fate mapping mammalian embryos presented a greater technical challenge, since they develop internally. The first fate maps of mouse embryos were made by injecting blastomeres from the ICM of one blastocyst into the ICM of another, genetically distinct embryo (Gardner and Rossant 1979). The resulting chimeras showed that cells in the ICM contribute to all three germ layers of the fetus, as well as some extraembryonic tissues. Fate maps at later stages used a strategy conceptually identical to the quail-chick chimeras used to map the avian blastodisc. In these experiments, grafted mouse epiblast tissue was labeled either by incorporation of radioactive thymidine or by treatment with a Wheat Germ Agglutinin-gold conjugate, which binds to cell surfaces (Beddington 1982; Copp et al. 1986; Tam and Beddington 1987; Tam 1989). Labeled explants were orthotopically transplanted into unlabeled host embryos and grown in culture. Fate maps drawn from these experiments showed that all regions of the epiblast contribute to the three germ layers until late in gastrulation, when regions become specialized to contribute to different tissues. A fate map of the pre-gastrulation stage epiblast was made at single resolution by injecting single cells of epiblast with an HRP lineage tracer, and culturing the embryos until mid-gastrulation (Lawson et al. 1991). Precursors of the three germ layers are organized in broad territories, with progenitors of the definitive endoderm restricted to the posterior most region of the cup, while the anterior is populated only by ectodermal precursors (Fig. 7.5d). Precursors of all three germ layers are intermingled in the posterior, while ectodermal and mesodermal precursors are juxtaposed in the central region of the epiblast. A significant portion of the epiblast also generates extraembryonic mesodermal tissues, such as the amnion and chorion.

7.4.5 Conclusion

A comparison of the fate maps of the frog, fish, bird and mammalian blastoderm reveals a striking conservation of organization, despite the dramatic differences in morphology. In all species, ectodermal tissues arise from cells in the future anterior of the embryo, while endoderm arises from the future posterior, where gastrulation initiates. Mesodermal precursors are distributed in between. The deep conservation of the vertebrate pre-gastrula fate map suggests that the conserved body plan observed at the phylotypic stage originates with a common mechanism of germ layer specification.

The intermingling of germ layer territories observed in the fish, avian and mammalian fate maps has largely been overlooked due to the prevalence of oversimplified fate map cartoons in textbooks and review articles. Precursors of the germ layers are highly segregated in the fate map of the *Xenopus* blastula, but there is considerable intermixing of germ layer precursors in other amphibian species (Smith and Malacinski 1983; Purcell and Keller 1993; Delarue et al. 1994). The intermingling of germ layer precursors reflects the fact that cells are constantly changing their positions, due to both passive movements in response to cell division and division and by active, but random movements apparent in some species. The directed movements of gastrulation result in even more dramatic changes in cell position. Cells move between 20–70 μm in random directions in the zebrafish blastoderm, which is approximately 500 μm in diameter (Trinkaus 1973; Bensch et al. 2013). Cells migrate up to 150 μm in the killifish blastoderm, which is about 1.8 mm in diameter (Trinkaus 1973; Bensch et al. 2013). In the amniote epibilast, cells also move great distances before the primitive streak forms, and cell proliferation in the mouse is coupled to a dramatic increase in embryonic volume (Snow and Bennett 1978; Gardner and Cockroft 1998; Chuai et al. 2006). These movements have profound consequences for the molecular mechanisms that induce the germ layers.

7.5 Germ Layer Induction

Although Pander understood that the three germ layers "*work mutually together although destined for different ends*," von Baer's Germ Layer Theory took a more rigid deterministic view of the germ layers as it developed over the century. At the turn of the twentieth century, tissue grafting and explant culturing methods were developed that finally permitted scientists to functionally test the roles of different embryonic tissues (Oppenheimer 1940). Vogt's fate map made it possible to correctly interpret these experiments, since it is necessary to understand how tissues behave in their normal locations before trying to understand how they behave when placed in an abnormal environment. These embryological experiments demonstrated the source of signals that induce the germ layers, and showed that these signals act before gastrulation.

7.5.1 Amphibians

7.5.1.1 The Organizer

As a part of her doctoral thesis under Hans Spemann (1869–1941), Hilde Proescholdt (later Mangold) (1898–1924) worked with embryos from two differently pigmented species of salamander. She transplanted the dorsal lip of the blastopore from one embryo onto the ventral side of a host embryo. The dorsal lip is the site where gastrulation initiates in amphibians, and is visible as a small region of darkly pigmented cells at the margin. These experiments were particularly challenging because she could only obtain embryos from natural mating of a species that had only one mating season a year, and in the pre-antibiotic era, the operated embryos did not survive very well in the pond water she used to incubate her embryos (Hamburger 1984). She repeated the experiment several hundred times over the course of 2 years in order to obtain the six survivors that formed the basis of her Nobel prize winning paper (Spemann and Mangold 1924). The grafted tissue induced an entire secondary body axis. The transplant differentiated into notochord, muscle and some gut, and the overlying host cells adopted neural fates (Spemann and Mangold 1924). The mesoderm of the dorsal lip is sufficient to induce the overlying ectoderm to form neural tissue, and to organize an entire secondary body axis. Spemann called this tissue the Organizer, and laid out three characteristics that functionally define organizer tissue. The Organizer tissue should induce neural tissue from naïve ectoderm, self-differentiate into notochord and prechordal plate, and reprogram surrounding tissue within the mesoderm.

7.5.1.2 Mesoderm Induction

Johannes Holtfreter (1901–1992) used a different approach to determine how the germ layers interact to produce the embryo. He developed culturing conditions that decreased the incidence of bacterial infection of operated embryos (Holtfreter 1929). He found that when raised in particular salt conditions, amphibian embryos gastrulate abnormally, such that the mesoderm and endoderm egress from the embryo instead of internalizing and migrating underneath the prospective ectoderm (Holtfreter 1933). In these exogastrulae, the mesoderm never underlies the ectoderm, and the neural plate never forms. He also performed explant studies, in which he removed cells of the animal cap from the blastula, and cultured them in his solution. The caps quickly healed into round spheres, but only differentiated into “atypical ectoderm” (Fig. 7.6a) (Holtfreter 1938a, 1938b). This was surprising because, according to Vogt’s fate map, some of the cells he explanted would become neural tissue if left in its original location. The Organizer experiment, Holtfreter’s exogastrulae and the animal cap explants all showed that mesodermal tissue interacts with the ectoderm to generate the neural plate. This interaction was termed “induction” because it involved communication between the germ layers and was presumably

mediated by the transfer of an unknown molecule. More broadly, these experiments, along with others, showed that germ layer identity is not fixed before gastrulation, and they confirmed Pander's belief that interactions between the germ layers are essential for normal embryonic development.

The first indication that ectodermal cells could be induced to form mesodermal and endodermal tissue, came from experiments in which differentiated tissues from adult guinea pigs were implanted into newt gastrulae, or cultured in sandwiches between two ectodermal explants (Toivonen 1953; Takata and Yamada 1960; Saxen et al. 1964). Two important conclusions were drawn from these results. First, they showed that in the proper environment, presumptive ectoderm could be converted to the other germ layers. Secondly, they showed that some tissues produced instructive signals that could change the fate of ectoderm. This raised the possibility that such interactions occur during normal development to form the germ layers. Pieter Nieuwkoop (1917–1996) devised an experiment to identify the source of the endogenous mesoderm inducing signals. In a variation on Holtfreter's explant experiments, he explanted animal caps and vegetal pieces from salamander embryos and grew them either separately or in combination (Nieuwkoop 1969). He found that neither the animal caps nor the vegetal pieces were able to form mesoderm when grown separately, but mesoderm was present when the vegetal pieces were grown in combination with the animal caps (Fig. 7.6a). In salamanders, mesodermal tissue was derived exclusively from cells in the pigmented animal cap ectoderm (Nieuwkoop 1969). Similar results were obtained in *Xenopus laevis* (Nakamura et al. 1970). These experiments identified the vegetal pole as the likely source of the endogenous mesoderm inducing signals, although Nakamura cautioned that "'*mesodermal induction*', *meaning the conversion of presumptive ectoderm into mesoderm, is an abnormal phenomenon*" which is only suggestive of the process that occurs in normal development (p. 318) (Nakamura et al. 1970).

7.5.1.3 The Nieuwkoop Center

A series of more spatially and temporally refined recombination experiments showed that the vegetal cells contained at least two types of inductive signals, and that these signals act before gastrulation. Dorsally located vegetal cells were able to induce ectodermal cells to become notochord, a dorsal mesodermal structure, with high frequency, but could only rarely induce ectoderm to form blood, which is derived from the ventral mesoderm. Ventral vegetal cells were able to induce ventral and intermediate mesodermal cell types easily, but could only rarely induce notochord (Dale et al. 1985; Dale and Slack 1987b). A series of heterochronic recombinants showed that vegetal pieces from young embryos could not act on gastrula-stage explants, but that gastrula stage vegetal pieces could act on younger ectoderm (Dale et al. 1985). This showed that the vegetal pieces continue to produce the mesoderm-inducing signal after gastrulation, but that the ectoderm loses the competence to respond to these signals. The dorsal mesoderm inducing activity was further localized to the dorsalmost vegetal cells of a 64-cell stage embryo, and its descendants (Gimlich and Gerhart 1984). When transplanted to the ventral side of a host, these cells could

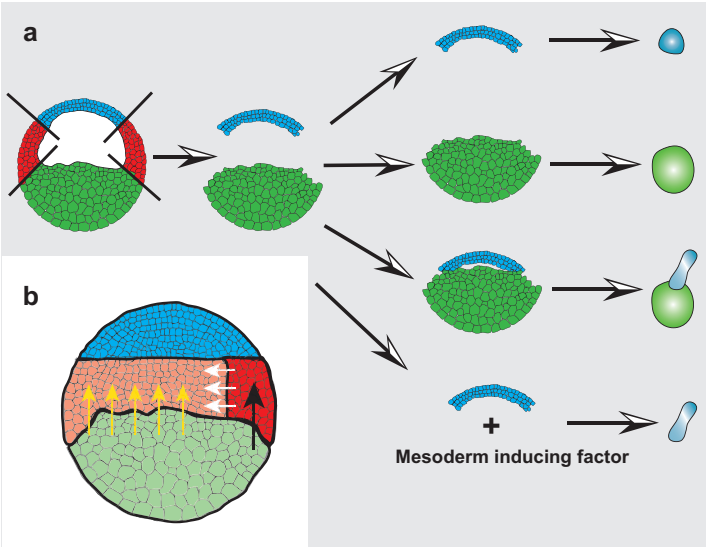


Fig. 7.6 Experiments to find the mesoderm inducing signals in *Xenopus*. (**a**) Animal cap explants differentiate into atypical epidermis when cultured alone, but form mesoderm in combination with vegetal cells, or with mesoderm-inducing factors. (**b**) The Three Signal Model hypothesizes that there are three mesoderm-inducing activities in frogs: The first signal induces dorsal mesoderm (*black arrow*) in animal caps, the second signal induces ventral and ventrolateral mesoderm (*yellow arrows*) in animal caps, while the third signal dorsalizes ventral mesoderm in Ventral Marginal Zone (VMZ) explants (*white arrows*) (Dale and Slack 1987b)

induce a full body axis, like the Organizer. Unlike the organizer, however, these cells did not contribute any cells to the axis itself and differentiated into gut endoderm. This region of the embryo was named the Nieuwkoop Center, in honor of the man who discovered the mesoderm-inducing capacity of cells in the vegetal pole (Gerhart et al. 1989). The Nieuwkoop center does not form in embryos treated with UV light during the first cell cycle, and all cells in resulting embryos adopt ventral fates (Gimlich and Gerhart 1984). Thus, the Nieuwkoop center is both necessary and sufficient to induce overlying cells in the embryo margin to become the Organizer.

7.5.1.4 The Three-Signal Model

A third inducing activity was uncovered by experiments that recombined the dorsal marginal zone (DMZ) with the ventral marginal zone (VMZ). In these experiments, the tissue was explanted after mesoderm had been specified, just before the onset of gastrulation. When explanted at this stage and grown in isolation, the VMZ generates ventral mesodermal cell types, such as blood and mesothelium, but does not produce notochord and generates only a little muscle. By contrast, when combined with the DMZ, cells from the VMZ differentiate into muscle at a high frequency and

no longer produce blood or mesothelium (Slack and Forman 1980; Smith and Slack 1983; Dale and Slack 1987b). The DMZ is the site of the Organizer, and was already known to induce neural tissue from ectoderm. These experiments showed that the DMZ contains another activity that acts to "dorsalize" presumptive ventral mesoderm so that it produces a more dorsal mesodermal cell type than it would in isolation. These results suggested the Three Signal Model for mesoderm formation (Fig. 7.6b) (Dale and Slack 1987b). According to this view, a signal from the dorsal vegetal cells induces the overlying marginal cells to adopt dorsal mesodermal fates (Fig. 7.6b, black arrow), including the notochord, while a second signal from the ventral vegetal cells induces overlying cells to adopt ventral mesodermal fates, including blood and pronephros (Fig. 7.6b, yellow arrows). At a subsequent stage, signals from the DMZ dorsalize other marginal cells to adopt dorsolateral mesodermal cell fates, like muscle (Fig. 7.6b, white arrows). These experiments only tested the functional activity of the signals produced by vegetal cells and did not clarify the molecular nature of the signals. The three activities could be explained by one molecule acting in a gradient, with different effects at different concentrations, or by multiple molecules acting synergistically to induce different fates.

7.5.2 Teleosts

7.5.2.1 The Embryonic Shield

It quickly became apparent that embryonic induction was not restricted to amphibian embryos. Several lines of evidence indicated that the teleost embryonic shield is the functional equivalent of Spemann's Organizer. Working in the yellow perch, Oppenheimer transplanted the embryonic shield from one embryo into ectopic locations in the blastoderm or yolk of a host embryo (Oppenheimer 1934a). She stained the host and graft with different color vital dyes, and found that the grafted shield self-differentiated into notochord and somites, and induced neighboring host cells to form a secondary neural axis, like Spemann and Mangold had observed in salamanders a decade earlier. Similar results were obtained in subsequent transplant experiments with shields from killifish (*Fundulus heteroclitus*), trout, zebrafish, and medaka embryos (Oppenheimer 1934b, 1936c; Luther 1935; Shih and Fraser 1996; Inohaya et al. 1999). Conversely, neural tissue failed to form when the prospective neural ectoderm of a trout embryo was extirpated and grafted onto the yolk, far from any inductive signals of the shield (Luther 1936). The shield is necessary and sufficient to induce neural tissue from naïve ectoderm and the shield fulfills Spemann's definition of an organizer tissue.

Oppenheimer went one step further and showed that amphibian cells could respond to signals from the shield (Oppenheimer 1936b). She implanted the shield of a zebrafish embryo into the blastocoel of a newt embryo. In the resulting chimeras, the zebrafish tissue differentiated into a notochord and somites, as expected, and the amphibian host cells surrounding this graft formed ectopic neural tissue. This striking result showed that the inductive signals themselves were likely to be conserved between amphibians and teleosts.

7.5.2.2 The YSL

Early experiments to determine the role of the yolk during teleost development yielded ambiguous results. The great geneticist Thomas Hunt Morgan (1866–1945) was the first person to perform a systematic experimental analysis of teleost development, working with wrasse, sea bass, and killifish embryos at the Marine Laboratory in Woods Hole, Massachusetts (Morgan 1893, 1895). He used a needle to poke a hole in the vegetal pole of the embryo, opposite the blastodisc, gently squeezed out the yolk, and permitted the sac to backfill with seawater. He concluded that, "*The yolk may be removed from the egg of Fundulus at almost any stage of development and the embryo still forms*." (p. 809) (Morgan 1893). The caveat to this conclusion was that embryonic development ceased if he removed more than two-thirds of the yolk content. Morgan viewed the yolk as a passive structure, important only for the mechanics of embryonic cleavages and epiboly. Oppenheimer reinvestigated the role of the yolk in killifish, with a full understanding of embryonic induction (Oppenheimer 1936a). Her approach was to remove the entire blastoderm from the yolk at different stages and to grow them as isolates in culture. Blastoderms isolated after the 32-cell stage produced embryos with structures from all three germ layers, although they typically lacked posterior structures. Blastoderms isolated before this stage formed a hollow ball of epidermis reminiscent of the atypical ectoderm formed in Holtfreter's animal cap explants. Oppenheimer concluded that the yolk syncytial layer (YSL), which she called the periblast, contains a substance that is transferred to the blastoderm at or before the 32-cell stage, and is necessary for formation of the mesoderm and endoderm. Similar results were obtained from cultures of isolated goldfish, trout, medaka and zebrafish blastoderms (Fig. 7.6a) (Tung et al. 1954; Devillers 1961; Hyodo et al. 1996; Sagerstrom et al. 1996; Xu et al. 2014b). These transplant experiments were often difficult to interpret, given differences in staging, and whether or not a bit of the YSL had been included in the blastoderm isolates. Conclusive proof that molecules produced in the yolk are essential for embryonic development came from experiments in which RNase was injected into the YSL of zebrafish embryos (Fig. 7.7a) (Chen and Kimelman 2000). This treatment had dramatic consequences, and the embryos died before gastrulation, failing to express the earliest markers for mesoderm and endoderm. Since zebrafish embryos lacking mesoderm and endoderm survive perfectly well for days, it is likely that the RNase treatment affected a number of different processes that are vital for survival past early embryonic stages.

A complementary set of experiments demonstrated that signals from the yolk can induce mesoderm and endoderm. In a series of heterochronic transplants, old blastoderms with visible shields were replaced with younger blastoderms, lacking shields. As development proceeded, the transplanted blastoderms always developed shields in the same location as the original blastoderm (Long 1983). In zebrafish, animal cap cells expressed markers of mesendoderm, including the shield, when they were directly exposed to the YSL in grafting experiments (Fig. 7.6a) (Mizuno et al. 1996; Ober and Schulte-Merker 1999; Rodaway et al. 1999). Because the yolk cell cannot be further physically subdivided, specific regional capabilities of the YSL have not been addressed by grafting experiments. Targeted knockdown of

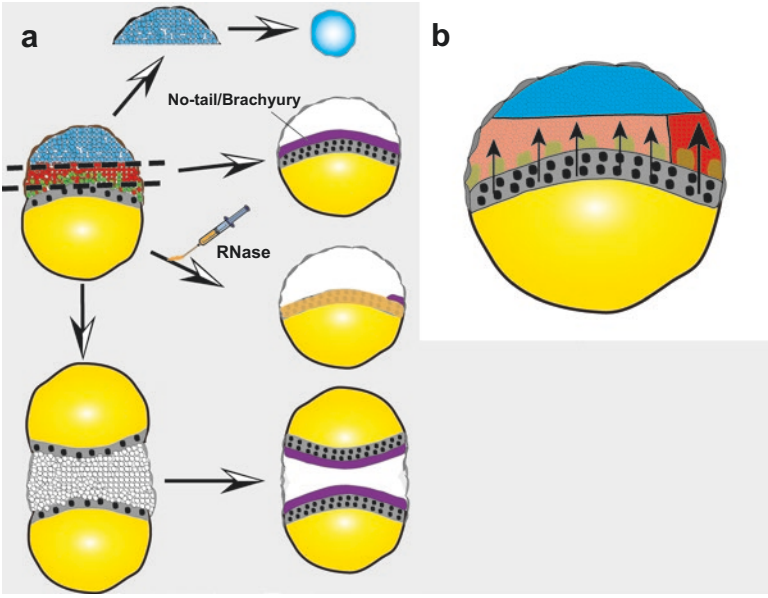


Fig. 7.7 Experiments to find the mesoderm inducing signals in zebrafish. (**a**) Zebrafish animal cap explants differentiate into epidermis when cultured alone, but express mesodermal markers when cultured on a free yolk cell (Ober and Schulte-Merker 1999). In untreated embryos, cells near the margin express the pan-mesodermal marker, No-tail/Xbra. No-tail/Xbra expression is greatly reduced when RNase is injected into the YSL, and an ectopic *no-tail/Xbra* appears when an ectopic yolk cell is grafted on the animal pole of a host embryo. (**b**) Signals from the YSL induce mesoderm and endoderm (*small black arrows*), and signals from the dorsal YSL induce dorsal mesoderm and endoderm (*large black arrow*)

specific genes in the YSL, however, indicates that it expresses an essential ventralizing signal in addition to its ability to induce dorsal mesoderm (Fan et al. 2007; Sun et al. 2014). Thus in teleosts, the extraembryonic YSL performs the same functions during development that are fulfilled by the vegetal blastomeres in amphibians (Fig. 7.7b). The YSL fits all the criteria for Nieuwkoop Center. Signals from the YSL are both necessary and sufficient to induce the shield, and the YSL does not contribute to the embryonic axis, since it is an extraembryonic tissue.

7.5.3 Amniotes

7.5.3.1 The Node

The functional equivalent of Spemann's Organizer in amniotes is called the node. In 1875, Viktor Hensen (1835–1924) was studying the embryonic development of the rabbit when he noticed an epithelial thickening at the anterior end of the primitive streak in gastrulating embryo (Hensen 1876). Because of its appearance, he called this structure the node, although in some texts it is referred to as the primitive knot.

This structure, now called Hensen's node, was subsequently discovered in all gastrulating mammalian and avian embryos. Hensen observed that a bridge of invaginating mesoderm at the node connects the hypoblast to the epiblast. This unusual feature suggested to him that the node plays an important role in germ layer formation, but it would take nearly 60 years before experimental embryologists began to decipher its functions. After its discovery, biologists presumed the node was an embryonic growth zone (Leikola 1976). This changed when Vogt's vital dye staining technique was used to trace the cell movements in the chicken epiblast (Wetzel 1925, 1929; Gräper 1929). These studies revealed that the cells of the epiblast migrate in an arc-like pattern toward the midline where they converge and invaginate in the streak and node. Later fate maps, using the more reliable lipophilic lineage tracers DiI and DiO, confirmed these observations (Hatada and Stern 1994). The node and primitive streak, therefore, are the sites where the germ layers form as the presumptive mesoderm and endoderm internalize. Rather than being a static structure, the node is composed of a highly dynamic cell population of endoderm and mesoderm precursors, equivalent to the teleost shield and the amphibian dorsal lip.

Early grafting experiments showed that the node and anterior primitive streak have the ability to self-differentiate when transplanted to the chorioallantoic membrane (Hunt 1931; Willier and Rawles 1931). Conrad H. Waddington (1905–1975) developed a technique for culturing chicken and duck blastoderms on a blood clot in a watch glass, with all the yolk removed (Waddington 1932). With this method, he could visualize development continuously for a period of days after various experimental manipulations. His first report describes 650 different operations. In one set of experiments, the epiblast was divided into pieces that were cultured separately. Only sections containing the node and anterior streak could produce embryonic organs such as the neural plate, notochord, heart and liver. This suggested that the node was essential for axis formation. Waddington next inserted pieces of the primitive streak underneath the epiblast of a cultured blastoderm. Anterior streak and node, but not the posterior streak, could induce the overlying epiblast to form a secondary body axis. Furthermore, nodal tissue from a duck could induce a secondary axis in a chicken epiblast, and vice versa (Waddington and Schmidt 1933). A limitation of these experiments is that they relied exclusively on morphological criteria to distinguish host tissue from graft because the vital dyes did not label tissue with sufficient intensity to be visible in sections, and because duck tissue was indistinguishable from chicken tissue under the microscope (Waddington 1932). Waddington was cautious in his conclusions, but his results were replicated much later using modern tissue labeling methods and quail-chick xenografts (Storey et al. 1992; Psychoyos and Stern 1996). These experiments show that the amniote node meets Spemann's definition of an organizer tissue. The mouse node can also induce a secondary neural axis in grafting experiments, indicating that organizer activity is a property common to the node in all amniote embryos (Beddington 1994).

A series of xenograft experiments showed that zebrafish and *Xenopus* blastulae respond to signals from the chicken and mouse nodes (Kintner and Dodd 1991; Blum et al. 1992; Hatta and Takahashi 1996). Similarly, the rabbit node can induce neural ectoderm in chicken embryo (Waddington 1937). The conserved activities of these tissues suggested that the signals themselves were likely to be conserved

among all vertebrates. This was confirmed when Chordin and Noggin were identified as the endogenous, neural inducing, and dorsalizing signals expressed in the amphibian Organizer, teleost shield, and amniote node (Smith and Harland 1992; Sasai et al. 1994; Connolly et al. 1997; Fisher et al. 1997; Schulte-Merker et al. 1997; Streit et al. 1998; Furthauer et al. 1999; Bachiller et al. 2000).

7.5.3.2 Koller's Sickle/PMZ

It has been more challenging to identify the equivalent of the Nieuwkoop center in amniotes than in other phyla, mostly due to the more complicated anatomy of the blastoderm. Attention has focused on three extraembryonic tissues: the hypoblast, Koller's sickle and the posterior marginal zone (PMZ). The location of the hypoblast underneath the epiblast is reminiscent of the location of the vegetal blastomeres under the marginal zone of amphibians. Waddington was the first to test the capacity of the hypoblast to induce mesoderm, working in chicken and duck embryos (Waddington 1932, 1937). He separated the hypoblast from the epiblast, rotated the it 90° or 180° along the anteroposterior axis, recombined the pieces and grew them in culture. In all cases, the primitive streak developed abnormally, and sometimes secondary primitive streak and body axis formed with its polarity coordinated with that of the rotated endoderm. Although this result was suggestive, the lack of adequate lineage-tracing techniques meant that Waddington could not definitively conclude that the formation of a new node and streak was due to an inductive event. More recent experiments used lineage labels to unambiguously demonstrate that the node is induced, but have variously identified Koller's sickle, the PMZ, and the hypoblast as the inductive tissue (Azar and Eyal-Giladi 1979; Eyal-Giladi et al. 1992; Callebaut and Van Nueten 1994; Khaner 1998; Callebaut et al. 2003). For instance, a secondary body axis forms when a square piece of tissue from the PMZ including Koller's sickle, is grafted orthogonally to the endogenous PMZ (Fig. 7.8a) (Khaner and Eyal-Giladi 1989; Eyal-Giladi et al. 1992). Subsequent experiments found that Koller's sickle alone could not induce a primitive streak (Khaner 1998). Furthermore, detailed fate mapping showed that cells in Koller's sickle contribute to the axial mesoderm (Bachvarova et al. 1998). These results eliminated the sickle from consideration as the Nieuwkoop Center. PMZ dissected from a chicken or duck embryo in the absence of Koller's sickle can induce a primitive streak and body axis when grafted to an epiblast (Fig. 7.8a) (Bachvarova et al. 1998; Khaner 1998). Importantly, labeled cells in the graft did not contribute to the new body axis, indicating that the avian PMZ expresses signals that induce formation of the primitive streak near the marginal zone, which is comprised of future mesoderm and endodermal cells (Fig. 7.8b, large arrows). Similar grafting experiments have not been performed in mammals, but genetic studies in the mouse demonstrated that signals from the extraembryonic visceral endoderm (VE) are required to induce the node and primitive streak (Varlet et al. 1997). Thus, the avian PMZ and the mammalian VE are functionally equivalent and meet the criteria of the Nieuwkoop center. As the primitive streak extends and the node migrates toward the anterior, signals from the posterior streak induce surrounding epiblast cells to form lateral

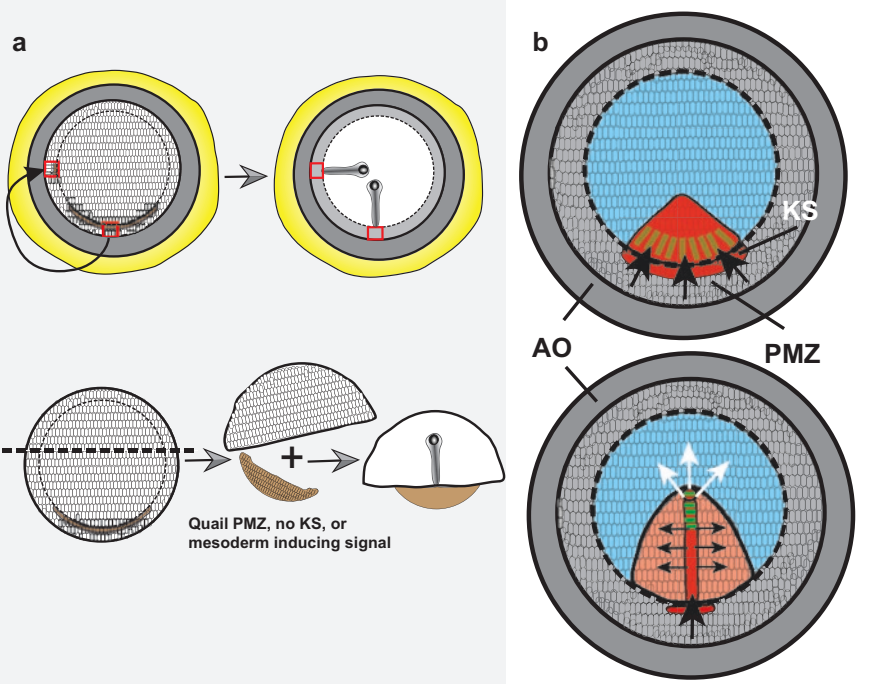


Fig. 7.8 Experiments to find the mesoderm inducing signals in chicken. (**a**) The PMZ can induce an ectopic primitive streak and Hensen's Node when grafted at a 90° angle with respect to the original axis. The PMZ from a quail embryo can induce a primitive streak to form when grafted to the anterior portion of a chicken epiblast. (**b**) Signals from the PMZ induce formation of the primitive streak in the posterior area pellucida (top embryo, *black arrows*). At a later stage, the primitive streak and Hensen's Node express signals that induce mesoderm and endoderm, and signals from Hensen's Node pattern the neural tissue (*white arrows*)

mesoderm (Fig. 7.8b, small arrows). Cells only become committed to these fates, however, after they invaginate through the primitive streak (Kimura et al. 2006). The node gains the ability to induce neural tissue when the streak has reached its full extension (Fig. 7.8b, white arrows) (Storey et al. 1992).

7.5.4 Conclusion

The discovery of the germ layers by Pander and von Baer raised the question of how the three layers cooperate to ensure the proper placement of organs relative to each other and with respect to the body axis. Some mechanism must act to guarantee that the foregut, for instance, always forms in the head rather than in some other location. The extent of communication between the germ layers only became apparent in the early twentieth century, when experimental embryologists began dissecting embryos and transplanting tissues to different locations, or developing them in

culture. Comparisons of the effects of these manipulations to the normal fate maps demonstrated that cell fates depend on their environmental context. Spemann and Mangold found that in tissue transplants, the dorsal lip of the blastopore self-differentiates as axial mesoderm and induces the overlying ectoderm to form neural tissue. Because of these properties, they named this tissue the Organizer. Subsequently, tissues with Organizer properties were discovered at the onset of gastrulation in all vertebrate embryos. The fact that Organizer tissues function across phyla boundaries indicated that a common mechanism acts in all vertebrates to induce neural tissue, despite the obvious morphological differences between the embryos at these stages. The discovery that Chordin and Noggin are expressed in the amphibian Organizer, the teleost shield and the amniote node and streak confirmed this prediction and transformed our understanding of how the vertebrate body plan is constructed. Chordin and Noggin are both secreted antagonists of the Bone Morphogenetic Proteins (BMP), a subclass of the TGF-β superfamily that ventralizes the embryo (Piccolo et al. 1996; Zimmerman et al. 1996). Thus, instead of providing a signal that induces dorsal fates, signals from the organizer the act to block ventralizing signals.

Experimental embryologists also identified the tissues that induce the Organizer, called the Nieuwkoop Center, as discussed above. In amphibians, the Nieuwkoop Center is located in the dorsal vegetal blastomeres, which are fated to become gut endoderm, and other vegetal cells induce ventral mesodermal cell types. In teleosts and amniotes, the Nieuwkoop Center is located in extraembryonic tissues, including the YSL, PMZ and the mammalian VE. Signals from these tissues induce all mesodermal and endodermal cell types in the overlying blastoderm, including the shield and node. The combined action of the Nieuwkoop Center and the Organizer explains how development of the germ layers is coordinated. Therefore, the reason the foregut always forms in the head is because prior to gastrulation, precursors of the foregut induce organizer tissue, which induces and patterns the brain and head. In teleosts and amniotes, the foregut precursors and the organizer tissues are both induced by signals emanating from extraembryonic tissues, but the result is the same: coordinated development of organs within the three germ layers.

7.6 Identification of Mesoderm Inducing Signals

The fact that extraembryonic tissues of teleosts, birds, and mammals have the same mesoderm inducing capacity as the vegetal pole cells in amphibians suggests that a common mechanism is responsible for inducing the germ layers in all vertebrates. The embryological studies that identified the sources of the signals that induce the germ layers provided the experimental framework for searching for the endogenous mesoderm-inducing factors. Other mesoderm inducing factors were identified through genetic studies in the mouse and zebrafish. The results from this work demonstrated that a common mechanism acts in all vertebrates to specify the germ layers.

7.6.1 Transforming Growth Factor-β

The first mesoderm-inducing factor, called Vegetalizing Factor, was purified from 9–13 day old chicken embryos (Tiedemann and Tiedemann 1959). This 30 kDa protein was purified based on its ability to induce muscle, pronephros, and notochord when applied as an insoluble pellet to salamander gastrulae (Born et al. 1972). Interestingly, the type of mesoderm induced depended upon the concentration or duration of exposure to Vegetalizing Factor (Asashima and Grunz 1983; Grunz 1983). Treatment of *Xenopus* animal caps with low doses or short exposures of Vegetalizing Factor induce ventral mesodermal cell types, like blood or heart, and higher doses or longer exposures induced dorsal mesodermal tissues, like notochord. The highest doses or the longest exposures induce ectoderm to form endoderm. A similar activity was secreted into the media by XTC cells, a line of transformed fibroblasts from tadpoles, and by the mouse leukemia cell line, WEHI-3 (Smith 1987; Godsave et al. 1988).

7.6.1.1 Activin

The first indication of the molecular identities of these mesoderm-inducing factors (MIF) came from the observation that TGF-β2 could mimic their activities in a variety of assays, including mesoderm induction from *Xenopus* animal caps (Rosa et al. 1988; Smith et al. 1988). Biochemical purification and peptide sequencing of the Vegetalizing Factor, XTC-MIF, and the WEHI-3 MIF revealed that Activin-A was responsible for all three activities (Albano et al. 1990; Huylebroeck et al. 1990; Smith et al. 1990; Tiedemann et al. 1992). Analysis of the expression patterns of *activin* genes in *Xenopus* showed that transcripts encoding Activin-B, but not those for Activin-A, were expressed at the correct time and place to induce mesoderm (Thomsen et al. 1990; Dohrmann et al. 1993). Since Activin-B had the same ability as Activin-A to induce mesoderm from animal cap ectoderm, Activin-B became the first candidate molecule for the endogenous mesoderm-inducing factor (Thomsen et al. 1990). Furthermore, Activin induces mesoderm in a dosage dependent manner, as predicted by the transplant experiments (Green and Smith 1990; Green et al. 1992). Activin is also expressed in fish oocytes, and can induce the mesoderm when overexpressed in zebrafish and medaka embryos (Schulte-Merker et al. 1992; Ge et al. 1993; Wittbrodt and Rosa 1994). Activin is expressed in the chicken hypoblast and in the primitive streak, when mesoderm is being specified (Mitrani et al. 1990b; Stern et al. 1995). Exposure to Activin can induce an entire body axis, including a notochord and somites, to form in a cultured chick epiblast (Albano et al. 1993). Activin can also induce an ectopic primitive streak or when locally misexpressed in a whole embryo (Ziv et al. 1992; Cooke et al. 1994). This indicates that the Activin pathway has a highly conserved function in vertebrates and can induce mesoderm when activated at the proper place and time of development. In mice, Activin is expressed in the oocytes and early blastocyst, but not in the embryo during

gastrulation (Albano et al. 1993, 1994). This suggested that either Activin was not the endogenous mesoderm-inducing factor, or that the role of the Activin pathway was not conserved in mammals. This question was only resolved by the identification of additional mesoderm inducing molecules.

7.6.1.2 Vg1/Gdf1

A differential screen for maternally expressed genes preferentially localized to the vegetal pole of the oocyte revealed a second candidate, called *vg1* or *growth differentiation factor-1 (gdf1)* (Rebagliati et al. 1985). *vg1* encodes a TGF-β molecule with mesoderm-inducing activity (Weeks et al. 1985; Dohrmann et al. 1993; Thomsen and Melton 1993). Because of their dorsal mesoderm-inducing activity and their expression patterns in *Xenopus*, Activin-B and Vg1/Gdf-1 were good candidates for the dorsal mesoderm-inducing signal predicted by the Three Signal Model (Fig. 7.6b, black arrow) (Dale and Slack 1987b). Vg1/Gdf-1 is also maternally expressed in zebrafish and can induce axial mesoderm when overexpressed (Helde and Grunwald 1993; Dohrmann et al. 1996). The chicken orthologue of Vg1/Gdf-1 is expressed in the PMZ, and it can induce a new primitive streak when expressed in the lateral marginal zone (Seleiro et al. 1996; Shah et al. 1997). In the mouse, Gdf-1 is expressed ubiquitously in the epiblast before gastrulation, consistent with a role in mesoderm induction (Rankin et al. 2000; Wall et al. 2000).

7.6.1.3 Derriére/Gdf3

A screen for secreted factors expressed in *Xenopus* embryos uncovered a zygotically expressed TGF-β molecule closely related to Vg1, called Derriére or Gdf-3 (Sun et al. 1999a). Derriére/Gdf-3 can induce mesoderm and endoderm in animal caps, and inhibits head formation when overexpressed in the dorsal blastomeres. When injected ventrally, Derriére/Gdf-3 induces a secondary body axis containing posterior trunk mesoderm, including muscle. This indicates that Derriére/Gdf-3 has the ability to dorsalize ventral mesoderm, as expected for the dorsalizing signal in the Three-signal model (Fig. 7.6b, white arrows). Mouse Gdf-3 is ubiquitously expressed prior to gastrulation (Chen et al. 2006).

7.6.1.4 Nodal

In 1993, a retroviral insertion was identified that disrupted a new member of the TGF-β superfamily in the mouse (Zhou et al. 1993). The affected gene was named *nodal* because of its expression pattern in the node. It is also expressed in the primitive streak and in the extraembryonic visceral endoderm (Conlon et al. 1994). The chicken *nodal* orthologue, *cNR-1*, is expressed in the middle two-thirds of the primitive streak until it is fully extended (Levin et al. 1995). Nodal can induce formation

of a secondary primitive streak in a chick embryo when it is expressed in the lateral region of an epiblast that lacks the hypoblast, which secretes Nodal antagonists (Bertocchini and Stern 2002). Of the three teleost *nodal-related* genes, two, *nodal-related-1* (*ndr-1*)/*squint* (*sqt*), *nodal-related-2* (*ndr-2*)/*cyclops* (*cyc*), are expressed in the presumptive mesendodermal cells (Rebagliati et al. 1998a; Fan and Dougan 2007). *ndr-1/sqt* expression begins in dorsal marginal cells and the dorsal YSL, followed by transient expression of both *ndr1/sqt* and *ndr2/cyc* around the entire margin, including the YSL (Erter et al. 1998; Feldman et al. 1998; Fan et al. 2007). Subsequently, *ndr1/sqt* expression is lost from the margin, persisting only in the NEM/Dorsal Forerunner Cells, while *ndr2/cyc* continues to be expressed in the involuting axial mesoderm (Rebagliati et al. 1985, 1998a). Thus, dorsal marginal cells are continuously exposed to Nodal signals, while ventrolateral cells are only transiently exposed to Nodal. *ndr1/sqt* is also expressed maternally (Feldman et al. 1998). The third *nodal-related* gene, *nodal-related-3* (*ndr-3*)/*southpaw* (*spaw*), is expressed after gastrulation and is not involved in mesoderm formation (Long et al. 2003). In *Xenopus*, six genes, *xnr1-6*, were cloned based on homology to *nodal* (Jones et al. 1995; Joseph and Melton 1997; Takahashi et al. 2000). All of the *xnr* genes are expressed in the presumptive mesoderm in a graded fashion, with highest levels in the Organizer, and can induce mesoderm in a dosage dependent manner. Mouse Nodal induces mesoderm and endoderm in a dosage-dependent manner in both frog and fish embryos (Jones et al. 1995; Toyama et al. 1995). Ndr-1/Sqt, Ndr-2/Cyc, and each of the *Xenopus nodal* genes have similar activities when overexpressed (Jones et al. 1995; Joseph and Melton 1997; Erter et al. 1998; Feldman et al. 1998; Rebagliati et al. 1998b; Takahashi et al. 2000; Aoki et al. 2002). The exception is Xnr-3, which lacks a conserved cysteine in the mature ligand domain, and acts as a neural inducing signal rather than a mesoderm-inducing signal in animal cap assays (Smith et al. 1995). The ability of Ndr-3/Spaw to induce mesoderm has not been tested, but its strong homology to Ndr-1/Sqt suggests it should have a similar activity. The late expression pattern of Ndr-3/Spaw, however, eliminates it as a possible endogenous mesoderm-inducing signal.

7.6.2 TGF-β Signal Transduction Pathway

Activin, Vg1, Derriére and Nodal are all members of the TGF-β superfamily, named after its founding member, Transforming Growth Factor-β. TGF-β proteins are synthesized as precursor proteins that undergo extensive post-translational modifications and covalent dimerization before the final cleavage event that releases the mature ligand from the C-terminus. These ligands bind to transmembrane receptors on the surface of a responding cell and activate their serine/threonine kinase activity. This initiates a signaling cascade that results in activation of specific target genes. There are two types of TGF-β receptors: Type I receptor and Type II receptor (Massague 1992). When a TGF-β ligand binds to the type II receptor, the ligand-bound type II receptor has higher affinity to the type I receptor and forms a complex

in which the type II receptor phosphorylates the type I receptor for activation. Nodal and Vg1 require an EGF-CFC co-receptor, called One-eyed Pinhead (Oep)/Cripto/FRL, for binding and activation of the receptor complex (Ding et al. 1998; Gritsman et al. 1999; Yeo and Whitman 2001; Cheng et al. 2003). The activated type I receptor phosphorylates cytoplasmic effector proteins called the Smad proteins. The name Smad is a historical relic of how the genes were discovered. Genetic screens identified the *mothers against decapentaplegic* (*mad*) genes and the *sma-2*, *sma-3* genes as components of the TGF-β signaling pathway in flies and worms, respectively (Sekelsky et al. 1995; Savage et al. 1996). For simplicity, the nomenclature of the gene family was revised, combining *sma* and *mad* to produce gene family name, *smad*. The Smad proteins fall into two categories: Receptor-regulated smads (R-smads) are phosphorylated by the receptors and coverted to an active state. There are four main R-smads, called Smad1, Smad2, Smad3, and Smad5 (Massague et al. 2005). The TGF-β/Activin/Vg1 class of ligands binds to the Type I Receptor, ActR1b/ACVR1b and the Type II Receptor ActR2a/ACVR2a or ActR2b/ACVR2b, and activates Smad2 and Smad3. The BMPs bind to different Type I and Type II Receptors and activate Smad1 and Smad5. Smad4 is common (co-Smad) to all TGF-β signal transduction pathways. Smad4 binds to phosphorylated R-Smad proteins, forming a complex that translocates to the nucleus and activates transcription of target genes (Wu and Hill 2009).

7.6.3 Requirement for TGF-β Signals in Germ Layer Formation

Loss of function studies indicated that signaling through the Activin/TGF-β pathway is required for germ layer formation. A truncated version of the Type II Activin Receptor, ActR2b/ACVR2b can bind Activin but this mutant receptor cannot activate the cognate Type I receptor and downstream Smad effector proteins (Hemmati-Brivanlou et al. 1992). Expression of this mutant receptor blocks the ability of exogenous molecules to induce mesoderm in *Xenopus* animal caps, and blocks the formation of all mesodermal derivatives in whole embryos (Hemmati-Brivanlou and Melton 1992; Schulte-Merker et al. 1994). Similar results were obtained by overexpression of a dominant negative version of the Type I Activin Receptor, ActR1b/ACVR1b (Chang et al. 1997). Expression of the dominant negative Activin-receptor inhibits endoderm formation in vegetal explants, indicating that this pathway is also required for endoderm formation in frogs (Cornell et al. 1995). Expression of a dominant negative variant Type II Activin receptor also blocks formation of mesoderm and endoderm in zebrafish (Rodaway et al. 1999). In addition, *actR1b/aCVR1b*$^{-/-}$ mutant cells are unable to contribute to mesoderm in chimeric mice, consistent with idea that TGF-β signaling is essential for germ layer formation (Gu et al. 1998). These studies show that activation of the Activin receptor complex is required for mesoderm and endoderm formation in vertebrates, but do not identify the essential ligand(s).

7.6.3.1 Nodal

Mutations in mouse and zebrafish demonstrate that *nodal* is required for mesoderm formation. The primitive streak and Node fail to form in mouse *nodal* mutants (Conlon et al. 1994). *nodal* mutants lack expressionof *goosecoid* (*gsc*) and *brachyury* (*T*) expression, indicating that the prechordal plate and notochord are absent. Mosaic analysis showed that *nodal* function is required in the visceral endoderm (VE) for formation of prechordal plate mesoderm (Varlet et al. 1997). Similarly in zebrafish, *ndr-1/sqt* and *ndr-2/cyc* single mutants display mild defects in the formation of axial mesoderm (Thisse et al. 1994; Heisenberg and Nusslein-Volhard 1997; Erter et al. 1998; Sampath et al. 1998). *ndr-1/sqt*; *ndr-2/cyc* double mutants lack all derivatives of the head and trunk mesoderm (Feldman et al. 1998). In the absence of *ndr-1/sqt* and *ndr-2/cyc*, marginal cells that would normally populate the mesoderm and endoderm of the head and trunk instead adopt ectodermal fates (Feldman et al. 2000). Nodal signaling is required in a dosage-dependent manner to pattern the animal–vegetal axis (Dougan et al. 2003). Cells in the dorsal margin are more sensitive to reductions in Nodal levels than cells in the ventrolateral margin (Dougan et al. 2003; Harvey and Smith 2009). Expression of Nodal antagonists inhibits formation of mesoderm and endoderm in a dosage dependent manner in both frogs and fish, and blocks formation of the primitive streak in chicken (Piccolo et al. 1999; Thisse and Thisse 1999; Agius et al. 2000; Bertocchini and Stern 2002). Mesoderm forms normally in frog *xnr1* morphants, suggesting that the *xnr* genes have redundant requirements during mesoderm formation (Toyoizumi et al. 2005). These experiments indicate that Nodal signals are the endogenous mesoderm and endoderm inducing signals in vertebrates, and that these signals act in a dosage dependent manner to pattern the mesoderm and endoderm.

7.6.3.2 Activin/Vg1

Formation of axial mesoderm in frogs is disrupted when Activin B function is reduced by injection of antisense morpholino oligonucleotides (Piepenburg et al. 2004). Similarly, expression of a dominant negative version of Activin in the Medaka fish showed that maternally expressed Activin is required in a dosage dependent manner for mesoderm formation (Wittbrodt and Rosa 1994). In zebrafish, Activin regulates oocyte maturation, but its function in mesoderm formation has not been directly tested (Tan et al. 2009a, b). This role of Activin in mesoderm formation may not be conserved, however, since mesoderm forms normally in *activin* null mutant mice, consistent with its late expression pattern (Matzuk et al. 1995). Depletion of Vg1/Gdf1 in *Xenopus* results in embryos with reduced head structures, and a lack of notochord (Birsoy et al. 2006). Thus, Vg1/Gdf-1 is required for dorso-anterior development in frogs. In zebrafish, depletion of maternal Dvr1 results in trunk defects of varying severity which have not been well characterized (Ye et al. 2010; Li et al. 2012). Posterior mesodermal fates do not form when Derriére/Gdf-3 activity was inhibited by overexpression of a dominant negative Derriére/Gdf-3 ligand (Sun et al. 1999a). Thus in frogs, ActivinB, Vg1/Gdf1 and

Derriére/Gdf-3 are all required for different aspects of mesoderm formation and patterning. In mice, the Vg1 orthologues *gdf1* and *gdf3* are required for mesoderm formation, suggesting that, unlike Activin, the role of Vg1 in mesoderm formation may be conserved in mammals (Chu and Shen 2010).

7.6.4 Fibroblast Growth Factor in Frogs and Fish

There are 23 members of the Fibroblast Growth Factor (Fgf) family, grouped into seven subfamilies based on their phylogeny: The Fgf1 Subfamily, Fgf4 Subfamily, Fgf7 Subfamily, Fgf8 Subfamily, Fgf9 Subfamily, Fgf11 Subfamily, and the Fgf19 Subfamily (Itoh and Ornitz 2004). Many of these ligands and their cognate Receptor Tyrosine Kinases are expressed during embryogenesis, which has presented a significant challenge for biologists trying to decipher their functions. The first hint that these proteins may have a role in mesoderm induction came when basic Fgf (bFgf/Fgf2), a member of the Fgf1 Subfamily, was identified in a screen for purified growth factors with the ability to induce mesoderm in *Xenopus* animal cap explants (Slack et al. 1987). Low doses of purified bFgf/Fgf2 induce ventral mesoderm in animal cap explants, while higher doses induce ventrolateral mesoderm (Slack et al. 1987; Kimelman and Maas 1992). bFgf/Fgf2 is not able to induce dorsal mesoderm. By contrast, other growth factors tested in this screen were unable to induce mesoderm even when used at very high, non-physiological concentrations. In frogs, bFgf/Fgf2 is expressed both maternally and zygotically through the end of gastrulation (Kimelman and Maas 1992). Fgf3, a member of the Fgf7 Subfamily, Fgf4 and Fgf8 are also expressed in *Xenopus* embryos during gastrulation and can induce mesoderm in animal cap explants (Isaacs et al. 1992; Tannahill et al. 1992; Christen and Slack 1997; Lombardo et al. 1998; Hardcastle et al. 2000; Fletcher et al. 2006).

In zebrafish, Fgf2 and Fgf4 are expressed maternally and zygotically during gastrulation, while Fgf3 is expressed zygotically in the presumptive mesendoderm (Phillips et al. 2001; Yamauchi et al. 2009; Lee et al. 2010). The zebrafish Fgf8 subfamily consists of six genes, *fgf8a*, *fgf8b* (formerly *fgf17a*), *fgf17* (formerly *fgf17b*), *fgf18a*, *fgf18b*, and *fgf24* (Itoh and Konishi 2007; Jovelin et al. 2007). Three of these genes, *fgf8a*, *fgf17*, and *fgf24* are co-expressed in the presumptive mesendoderm during gastrulation (Reifers et al. 1998; Fischer et al. 2003; Cao et al. 2004). bFgf/Fgf2, Fgf8a, or Fgf17 can all induce mesendoderm when overexpressed in embryos by mRNA injection (Griffin et al. 1995; Reifers et al. 1998; Cao et al. 2004).

Trunk and tail mesoderm does not form in frog or zebrafish embryos in which Fgf signaling is blocked by overexpression of a dominant negative mutant or by exposure to an Fgf antagonist (Amaya et al. 1991; Griffin et al. 1995; Fletcher et al. 2006). Interestingly, these embryos still form head mesoderm. Similar defects were reported in medaka *headfish* (*hdf*) mutants, which are mutant for Fgfr-1 (Yokoi et al. 2007). These results show that Fgf signaling is necessary and sufficient to specify certain types of mesoderm in frogs. They also implicate Fgf ligands of four different subfamilies as prime candidates for the ventral mesoderm-inducing signal predicted by the Three Signal Model (Fig. 7.6b, yellow arrows) (Dale and Slack 1987b; Slack et al. 1987).

A series of loss-of-function experiments identified Fgf4 and Fgf8 as endogenous mesoderm inducing signals in frogs and fish. Morpholino knockdown experiments in the frog showed that *fgf4* and *fgf8b*, an alternatively spiced isoform of Fgf8, are both required for to specify mesodermal cell fates (Fisher et al. 2002; Fletcher et al. 2006). At high doses, the *fgf8b* morpholino phenocopies embryos expressing the dominant negative Fgf receptor (Amaya et al. 1991). Interestingly, the Fgf8a isoform differs from Fgf8b by only 11 amino acids is necessary and sufficient to induce neural fates, but lacks the ability to induce mesoderm (Fletcher et al. 2006). In zebrafish, *fgf8* single mutants have defects in the somites associated with later roles in mesoderm differentiation (Reifers et al. 1998). When *fgf24* function is reduced in *fgf8* mutants, the embryos lack all posterior trunk mesoderm (Draper et al. 2003). This indicates that Fgf8 and Fgf24 function redundantly to specify posterior/ventral mesoderm in zebrafish.

Convergence-extension movements are blocked in frogs and fish when Fgf signaling is inhibited (Amaya et al. 1991; Griffin et al. 1995; Nutt et al. 2001). Expression of the Fgf antagonist, Sprouty2 inhibits the movements of gastrulation without affecting the specification of mesoderm and endoderm (Nutt et al. 2001). This suggests that the role of Fgf signaling in controlling cell movements is independent of its role inducing mesoderm.

7.6.5 Fibroblast Growth Factor in Amniotes

In chicken, *fgf2*, *-3*, *-4*, *-8*, *-12*, *-13*, and *-18* are all expressed in the primitive streak with slightly different spatial and temporal dynamics, while *fgf8* is initially expressed broadly in the primitive streak before being excluded from the node and anterior streak at later stages (Shamim and Mason 1999; Lawson et al. 2001). *fgf8* is also expressed in Koller's sickle before the primitive streak is apparent (Lawson et al. 2001). *fgf4* and *fgf8* label distinct subpopulations of cells within the streak (Karabagli et al. 2002). Cells close to the midline of the streak express *fgf4*, while cells express *fgf8* as they leave the streak and migrate laterally. Misexpression of Fgf8b or Fgf4 induces a secondary primitive streak that contains both mesoderm and a node (Bertocchini et al. 2004). Injection of Fgf4 into the subgerminal space induces all marginal cells to adopt mesodermal fates (Alev et al. 2013). In addition, mesoderm does not form when Fgf signaling is blocked, and Fgf antagonists prevent the ability of cVg1 to induce an ectopic body axis (Mitrani et al. 1990a; Bertocchini et al. 2004). This indicates that Fgf is necessary and sufficient to induce mesoderm in the chicken. Fgf4 can also induce mesoderm when overexpressed in turtle embryos, suggesting that this function is conserved in reptiles (Alev et al. 2013).

Fgf signaling also controls the polonaise movements during gastrulation by chemotaxis (Yang et al. 2002; Hardy et al. 2011). A fluorescent assay to detect cell migration revealed that cells within the epiblast were attracted towards a bead soaked with Fgf4 protein (Yang et al. 2002). Cells were not attracted to beads soaked with Fgf2 and were repelled by beads soaked with Fgf8. This suggests that Fgf4 acts as a chemoattractant drawing cells in the epiblast toward the primitive streak where

they ingress, while Fgf8 acts as a chemorepellant, driving cells away after they exit the streak. Cell movements are blocked when the Fgf pathway is pharmacologically inhibited (Hardy et al. 2011). Since Fgf4 can induce mesoderm even in the absence of polonaise movements, these results indicate that Fgf signals have two independent roles during gastrulation. They direct the movements of cells into and out of the streak and they specify cells to adopt mesodermal fates.

Similar results were obtained in the mouse. Fgf2 can induce cultured ectodermal cells to become mesoderm (Burdsal et al. 1998). Paraxial mesoderm fails to form in *fgfr-1*$^{-/-}$ mutants, and cells accumulate in the primitive streak due to defects in EMT, resulting in an expansion of axial mesoderm (Deng et al. 1994; Yamaguchi et al. 1994; Ciruna and Rossant 2001). The failure of EMT is caused by persistent E-cadherin expression in *fgfr-1*$^{-/-}$ mutants. In chimeras, *fgfr-1*$^{-/-}$ mutant cells display defective movements within a wild type primitive streak and an inability to delaminate from the epiblast (Ciruna et al. 1997). This indicates that Fgf signaling is required for the specification of mesodermal fates and for the migration of presumptive mesoderm and endodermal cells into the primitive streak in the mouse, as it is in the chicken. These roles are also conserved in teleosts, since medaka *hdf* mutants, which lack both the maternal and zygotic function of *fgfr1,* display defects in the migration of axial mesoderm during gastrulation in addition to the lack of trunk and tail mesoderm (Shimada et al. 2008).

Both *fgf4* and *fgf8* are expressed in the primitive streak (Niswander and Martin 1992; Crossley and Martin 1995). *fgf4* null mutant embryos had severe proliferation defects and failed to develop post-implantation, whereas *fgf8* mutants die after gastrulation, failing to form mesoderm (Feldman et al. 1995; Meyers et al. 1998). The early phase of *fgf4* expression is expressed normally in *fgf8*$^{-/-}$ mutants, but it is lost in the primitive streak during gastrulation (Sun et al. 1999b). In these embryos, cells move into the primitive streak and ingress, but fail to exit the streak or become mesoderm. This indicates that Fgf8 and/or Fgf4 are required both for mesoderm formation and to direct the cell movements of gastrulation into and out of the primitive streak. A role for Fgf4 in mesoderm formation and epiblast migration has not been firmly established in the mouse, however, because its early requirement in the blastocyst complicates analysis of its later functions.

7.6.6 Conclusion

These studies showed that the molecular mechanism of germ layer formation is highly conserved in vertebrates despite the dramatically differently morphologies of the blastula and gastrula stage embryos. Experiments in frogs, fish, chicken, and mice all converged to identify activation of the Activin/TGF-β and RTK pathways as important steps in vertebrate germ layer formation. In all vertebrates, members of the Nodal-related gene family are expressed in the presumptive mesoderm and endoderm. These factors can induce mesoderm and endoderm in a dosage dependent manner when overexpressed, and are required in all vertebrates for formation

of the germ layers. In addition, the orthologues of Activin-B and Vg1 are expressed maternally in both frogs and fish, and can induce mesoderm and endoderm when overexpressed. Maternal Activin is required for mesoderm formation in frogs and Medaka fish, and is involved in oocyte maturation in zebrafish. Vg1 is required for axis formation in both frogs and zebrafish, although the defects in zebrafish DVR1 morphants have not been well characterized.

Fgf signals have a dual role during mesoderm formation. Several members of the Fgf family can induce mesoderm when overexpressed in vertebrate embryos, and are expressed in the presumptive mesoderm. bFgf/Fgf2 is expressed maternally in frogs, and bFgf/Fgf2 and Fgf4 are expressed maternally in zebrafish. Fgf3, Fgf4, and Fgf8 are expressed zygotically in the blastula and gastrula stages in frogs and fish. These expression patterns suggest that Fgf ligands act redundantly in mesoderm formation. Experiments in frogs, fish, chicken, and mice indicate that both Fgf4 and Fgf8 are required to specify mesoderm. Fgf signals also act independently of its role inducing mesoderm to direct cell movements during gastrulation in frogs, fish, chicken, and mice. Overexpression of the Fgf antagonist Sprouty2 blocks convergence-extension movements in frogs without affecting mesoderm formation. In the chicken epiblast, Fgf4 acts as a chemoattractant, while Fgf8 act as a chemorepellant. Fgf4 may play a similar role attracting cells to the primitive streak in mice, but this has not been tested due to the early lethality of *fgf4*$^{-/-}$ mutants. *fgf8* and/or *fgf4* are required in the mouse for ingression at the primitive streak.

7.7 Hierarchy of Mesoderm Inducing Signals

7.7.1 TGF-β and Fgf

The identification of such a large number of secreted factors presented researchers with the challenge of understanding how these pathways interact with each other to induce and pattern the germ layers. A series of experiments in *Xenopus*, zebrafish, chicken, and mice indicated that Fgf signals acts in parallel to the TGF-β signals to induce mesoderm, and are interdependent on each other for mesoderm formation. In the chicken, Fgf signaling is required for formation of the endogenous streak, and for the induction of ectopic primitive streaks by misexpressed Vg1 or Nodal (Lawson et al. 2001; Skromne and Stern 2002; Bertocchini et al. 2004). In the normal embryo, *cNR-1* expression precedes that of Fgf8 in the primitive streak. Finally, Fgf8b and Nodal are more potent inducers of a secondary axis when expressed together than when either protein is expressed alone (Bertocchini et al. 2004).

The synergistic action of Fgf and TGF-β is conserved in *Xenopus*, since animal cap cells exposed to Fgf respond to lower doses of purified Activin or TGF-β than cells not exposed to Fgf, and adopt mesodermal fates at lower concentration thresholds (Kimelman and Kirschner 1987; Green et al. 1992). Animal cap cells expressing a dominant negative Fgf receptor are unable to respond to Activin and other mesoderm inducing signals (Cornell and Kimelman 1994; LaBonne and Whitman 1994;

Schulte-Merker et al. 1994). Conversely, endogenous Fgf signals are not able to induce mesoderm in embryos expressing a truncated Activin receptor (Hemmati-Brivanlou et al. 1992). Finally, vegetal explants adopt mesodermal fates in the presence of exogenously added bFgf/Fgf2 and endogenous Activin-like signals (Cornell et al. 1995). This indicates that in the frog, Fgf signals act as a competence factor that directs cells to adopt mesodermal fates instead of endoderm in response to TGF-β signals. The Fgf and Activin pathways act in parallel, at least initially, since Fgf ligands and Activin are both expressed maternally. Crosstalk between the two pathways may be mediated at the level of their transcriptional effectors. p53 is required for the expression of mesodermal markers in *Xenopus*, and induces mesoderm when overexpressed in animal caps (Cordenonsi et al. 2003, 2007). Activation of the RTK/Ras pathway results in an N-terminal phosphorylation of p53. This form of p53 binds to Smad2 and forms a complex that activates TGF-β responsive target genes in presumptive mesodermal cells. Expression of *fgf3* and *fgf8* depends upon the maternally provided transcription factor, VegT, and Fgf expression can be rescued in VegT depleted embryos by expression of *xnr2* (Fig. 7.9a) (Kofron et al. 1999).

In zebrafish, Fgf signaling is required for Nodal signals to induce mesoderm, but not to induce endoderm (Rodaway et al. 1999; Mathieu et al. 2004). Nodal signals induce expression of *fgf3* and *fgf8* in the presumptive mesendoderm (Mathieu et al. 2004). Activation of Fgf inhibits expression of the endodermal marker *casanova* (*cas*), whereas *cas* expression is expanded when Fgf signaling is blocked (Mizoguchi et al. 2006). Thus in zebrafish, Fgf signals act downstream of Nodal signals to control the balance between mesoderm and endoderm. In addition, Fgf8 or Fgf3 can induce expression of components of the Nodal signal transduction pathway to maintain mesodermal identity (Mathieu et al. 2004). This indicates that Fgf and Nodal signals participate in a positive feedback loop that amplifies expression of both mesoderm-inducing signals (Fig. 7.9b).

7.7.2 Control of Nodal-Related Gene Expression

7.7.2.1 Mouse

There is strong evidence that *nodal* gene expression is maintained by positive feedback loop in all vertebrates. In the mouse, expression of a *nodal*-reporter gene in the epiblast depends upon the function of a conserved Activin/Nodal response element in the first intron (Brennan et al. 2001; Norris et al. 2002). Nodal expression is induced in the proximal region of the epiblast by unknown signals emanating from the extraembryonic ectoderm (ExE). Nodal expression rapidly spreads throughout the entire epiblast and eventually in the VE, where it is required for primitive streak formation in the epiblast (Fig. 7.10a) (Varlet et al. 1997). Several experiments indicate that *nodal* expression in the epiblast and the VE depends on a positive feedback loop. First, *nodal* expression is expanded in embryos lacking the Nodal antagonist, Antivin/Lefty2, resulting in an expansion of mesoderm (Meno et al. 1999). Secondly,

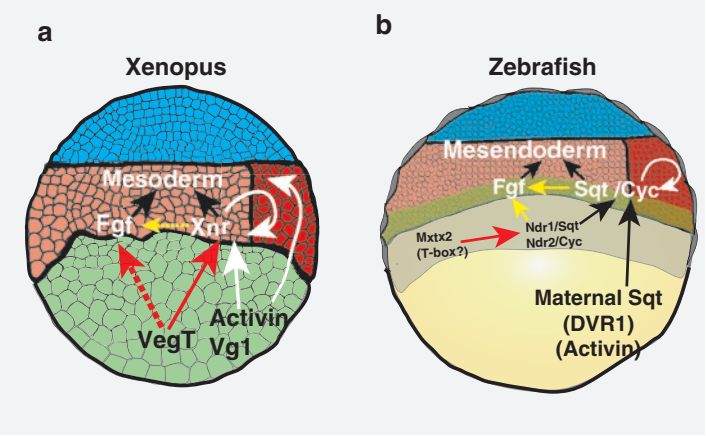


Fig. 7.9 Mesoderm and endoderm inducing pathway in frogs and fish. (**a**) In *Xenopus*, the maternally expressed T-box transcription factor, VegT induces expression of the *xnr* genes (*solid red arrow*), acting in parallel with maternally expressed Activin and Vg1 (*solid white arrow*). Xnr signals induce a positive feedback loop (*curved white arrow*), which acts via a conserved Activin/Nodal Response Element in the first intron of *xnr1*. Xnr signals act in parallel with Fgf signals to specify mesoderm (*black arrows*). Activin acts independently of the Xnr genes to induce mesodermal gene expression (*long white arrow*). Direct regulation of Fgf by VegT (*dashed red arrow*) and the Xnrs (*dashed yellow arrow*) is possible, but has not been established. (**b**) In zebrafish, Maternal Ndr1/Sqt, perhaps acting in parallel with maternally expressed Activin and DVR-1, induces zygotic expression of *ndr1/sqt* (*large black arrow*). The transcription factor Mxtx2 is also required to induce *ndr1/sqt* and *ndr2/cyc* expression in the YSL (*red arrow*). Ndr1/Sqt and Ndr2/Cyc in the YSL induces expression of *ndr1/sqt* and *ndr2/cyc* in the overlying marginal blastomeres (*short black arrow*), in a manner that requires a conserved Activin/Nodal Response Element in the first intron of both genes. Ndr1/Sqt and Ndr2/Cyc expression induces a positive feedback loop (*curved white arrow*), and expression of Fgf3 and Fgf8. Ndr1/Sqt, Ndr2/Cyc and Fgf signals act in parallel to specify mesendodermal fates (*short black arrows*). Fgf signals also induce expression of components of the Nodal signal transduction pathway, creating a self-reinforcing loop of expression between Nodal and Fgf signals. It is possible that Ndr1/Sqt and Ndr2/Cyc in the YSL also act directly to induce expression of Fgf3 and Fgf8 in the margin (*dashed yellow arrow*)

expression of a *nodal*-transgene in the distal epiblast depends on FoxH1, a transcriptional effector of Nodal signaling that binds to and activates an Activin/Nodal Response Element in the first intron (Saijoh et al. 2000; Yamamoto et al. 2001; Norris et al. 2002). Finally, *nodal* expression is restricted to the proximal epiblast in the absence of the essential Nodal co-receptor, Cripto (Liguori et al. 2008). The residual expression of *nodal* in *Cripto*$^{-/-}$ mutants depends upon a related co-receptor called Cryptic, since *Cripto*$^{-/-}$*;Cryptic*$^{-/-}$ double mutants are indistinguishable from *nodal* null mutants (Chu and Shen 2010). Nodal expression is restricted to the proximal epiblast of *gdf3*$^{-/-}$ embryos and *gdf1*$^{-/-}$*;gdf3*$^{-/-}$ double mutants, consistent with the idea that Vg1 orthologues act to induce *nodal* expression (Chen et al. 2006; Andersson et al. 2007). Another signal is likely acting in parallel to Gdf1 and Gdf3, since low levels of *nodal* are still expressed in *gdf1*$^{-/-}$*;gdf3*$^{-/-}$ double mutants, and neither gene is expressed in the ExE, the source of the initial *nodal*-inducing signals (Fig. 7.10a).

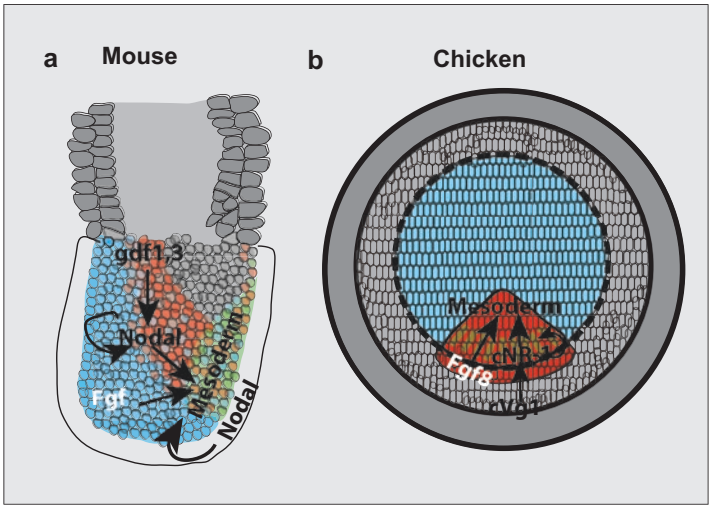


Fig. 7.10 Mesoderm and endoderm inducing pathway in amniotes. (**a**) In the mouse, a signal from the extraembryonic ectoderm induces Nodal expression in the epiblast. The Vg1 orthologues Gdf1 and Gdf3 act in parallel with this signal to induce Nodal expression (*black arrow*). Nodal expression in the epiblast establishes a self-reinforcing loop that expands Nodal expression throughout the epiblast and induces Nodal expression in the visceral endoderm. Nodal expression in the visceral endoderm is required to induce mesendodermal fates, acting in parallel with Fgf signals. (**b**) In the chick, Vg1 is expressed in a crescent in the PMZ and induces expression of cNR-1 in the epiblast. cNR-1 induces a positive feedback loop that expands the Nodal expression domain as the primitive streak extends. cNR-1 and Fgf8 act in parallel to induce mesodermal fates. In both the mouse and chick, Fgf signals act as a chemoattractant, drawing epiblast cells toward the primitive streak and repelling cells away from the primitive streak as they exit the streak

7.7.2.2 Chicken

In the chicken, *cNR-1* is expressed in the elongating primitive streak (Levin et al. 1995). COS cells expressing *cVg1* induce an ectopic primitive streak when implanted in the marginal zone (Seleiro et al. 1996; Shah et al. 1997). *cNR-1* expression is induced within 6 h after misexpressing cVg-1 (Skromne and Stern 2002). Formation of the ectopic primitive streak is blocked by expression of a Nodal antagonist in the area pellucida but not by a Nodal antagonist expressed in the marginal zone (Bertocchini and Stern 2002). This indicates that Nodal signaling is necessary for extension of the primitive streak, but is not required for inducing the primitive streak. The chick hypoblast secretes Cerberus, an endogenous Nodal antagonist (Piccolo et al. 1999; Foley et al. 2000). Surgical removal of the hypoblast results in the formation of supernumerary primitive streaks expressing *cNR-1* (Bertocchini and Stern 2002). This data is consistent with a model in which endogenous cVg-1 in the PMZ induces *cNR-1* expression in the posterior area pellucida and initiates formation of the primitive streak (Fig. 7.10b). *cNR-1* activity in the nascent streak is initially blocked by Cerberus expressed in the hypoblast. Elongation of the

primitive streak begins when the hypoblast is displaced by invasion of the definitive endodermal cells from the KS (Lawson and Schoenwolf 2003). These movements displace the Cerberus expressing cells in the hypoblast and relieves the inhibition of Nodal in the overlying epiblast, thereby activating the positive feedback loop of *cNR-1* expression.

7.7.2.3 *Xenopus*

The *xnr* genes are expressed in the margin, in the presumptive mesoderm (Jones et al. 1996; Joseph and Melton 1997; Takahashi et al. 2000). An Activin/Nodal response element from the first intron of *xnr1* is highly active in the marginal blastomeres in *Xenopus*, where *xnr1* is normally expressed, and can drive expression of a reporter gene in the mouse primitive streak (Osada et al. 2000). Expression of *xnr1*, *-2*, and *-4* is induced in response to Nodal signals, and lost when Nodal-signaling is inhibited by a Nodal antagonist (Agius et al. 2000; Cheng et al. 2000; Takahashi et al. 2000). *xnr1, -2, -4, -5, -6* expression is expanded in embryos lacking Antivin/Lefty, and reduced when Antivin/Lefty is overexpressed (Cha et al. 2006). Thus, *nodal-related* gene expression is controlled by an autoregulatory loop in frogs as it is in mammals and birds. Maternal TGF-β signals act to reinforce zygotic expression of the *xnr* genes. In overexpression assays, the *xnr* genes are upregulated in response to Activin, are required for Activin to induce mesoderm in animal caps and are reduced when Activin B is depleted (Osada and Wright 1999; Agius et al. 2000; Piepenburg et al. 2004). This provides strong evidence that Activin is required to induce the expression of the *xnr* genes (Fig. 7.9a). Although Activin B is required for *xnr* expression, some Activin target genes are induced independently of Nodal (Ramis et al. 2007). *xnr* gene expression is induced in response to Vg1, but their expression has not been fully characterized in embryos lacking Vg1 (Joseph and Melton 1998; Osada and Wright 1999; Agius et al. 2000; Birsoy et al. 2006). The phenotype of Vg1 depleted embryos is consistent with a reduction in Nodal signaling (Joseph and Melton 1998; Birsoy et al. 2006). Therefore, it is likely that Vg1 acts in parallel to Activin to induce *xnr* expression (Fig. 7.9a). The T-box transcription factor, VegT, is also required to induce expression of *xnr* genes (Clements et al. 1999; Kofron et al. 1999; Xanthos et al. 2001). Thus, in frogs, maternally provided TGF-β signals act in parallel with the VegT transcription factor to induce *xnr* gene expression, which is subsequently maintained by an autoregulatory loop mediated in part by an Activin/Nodal Response element in the first intron of *xnr1* (Fig. 7.9a).

7.7.2.4 Zebrafish

ndr1/sqt and *ndr2/cyc* are expressed in the marginal zone and in the YSL in the pregastrula stages (Erter et al. 1998; Feldman et al. 1998; Rebagliati et al. 1998a; Fan et al. 2007). *ndr2/cyc* is expressed a bit later than *ndr1/sqt,* and its expression largely depends on *ndr1/sqt* function (Dougan et al. 2003). The maintenance of *ndr1/sqt*

and *ndr2/cyc* in the margin depends upon the function of the EGF-CFC/Cripto/FRL co-receptor, One-Eyed Pinhead (Oep), and is expanded in embryos lacking the secreted Nodal antagonist, Antivin/Lefty (Gritsman et al. 1999; Meno et al. 1999; Chen and Schier 2002; Feldman et al. 2002). Overexpression of Antivin/Lefty inhibits expression of *ndr1/sqt* and *ndr2/cyc* (Thisse and Thisse 1999). Expression of a *ndr1/sqt* transgene depends on a conserved Activin/Nodal response element in the *ndr1/sqt* first intron (Fan et al. 2007). Thus, as in other vertebrates, expression of the zebrafish *nodal-related* genes depends upon an autoregulatory feedback loop (Fig. 7.9b). Nodal signals expressed in the extraembryonic YSL are required to induce expression of both *ndr1/sqt* and *ndr2/cyc* in the margin (Fan et al. 2007). Nodal expression in the YSL is independent of Nodal signals, however, since the YSL does not express the EGF-CFC co-receptor, Oep, or have activated Smad2 (Fan et al. 2007; Harvey and Smith 2009). Expression of *ndr1/sqt* and *ndr2/cyc* in the YSL is controlled in part by the Mix/Bix transcription factor Mxtx2, although evidence suggests that a T-box containing transcription factor may also be involved (Fig. 7.9b) (Hong et al. 2011; Xu et al. 2014a). *sqt/ndr1*, *zDVR-1* and *activin* are all expressed maternally in zebrafish, and drug inhibition studies indicate that embryos develop normally when these maternal signals are blocked before the mid-blastula transition (Hagos et al. 2007). Elimination of extraembryonic *ndr1/sqt* and *ndr2/cyc* in maternal *sqt/ndr1* mutants phenocopies *ndr1/cyc*$^{-/-}$; *ndr2/sqt*$^{-/-}$ double mutants (Hong et al. 2011). This indicates that maternal Activin-like signals and zygotic Nodal signals are required to boost expression of *nodal*-related genes in the embryo, acting in parallel to transcription factors in the YSL (Fig. 7.9b).

7.7.3 Conclusion

Taken together, these experiments show that the regulatory logic of *nodal-related* gene expression is remarkably consistent. In all vertebrates, expression of Nodal signals is maintained by a positive feedback loop, and their expression is induced by other Activin-like signals acting in parallel to transcription factors. This feedback loop acts through an Activin/Nodal Response Elements in the first introns of *nodal* genes in mice, frogs, and fish. In both *Xenopus* and zebrafish, the Activin-like signals are provided maternally. In frogs, the maternally provided Activin and Vg1 act in parallel to the T-box transcription factor, VegT transcription factor to induce *xnr* expression (Fig. 7.9a). In zebrafish, maternal Ndr1/Sqt acts in parallel with the zygotically expressed Mix/Bix transcription factor, Mxtx2, which is controlled by the maternal T-box gene, Eomesa (Fig. 7.9b) (Bruce et al. 2005). The transcription factors that control Nodal expression in mice and chicken have not been identified.

Another common feature of Nodal regulation is that signals from extraembryonic tissues induce Nodal expression in mice, chicken, and fish. In the mouse, *nodal* expression is induced by signals from the extraembryonic ectoderm (Exe) (Brennan et al. 2001). Nodal expression is partially dependent on the Vg1 orthologues, *gdf1 and gdf3*, but neither of these genes can be detected in the ExE at this stage

(Fig. 7.10a). Furthermore, *nodal* is expressed, albeit at low levels, in the proximal epiblast of *cripto*$^{-/-}$*;cryptic*$^{-/-}$ double mutants. This indicates that the signal that induces nodal expression acts independently of the Nodal co-receptor. This would appear to rule out Vg1 and Nodal family members as the inducing signal. In the chicken, cVg1 in the PMZ induces cNR-1 in the epiblast (Fig. 7.10b). In zebrafish, Nodal signals in the extraembryonic YSL are required to induce *ndr1/sqt* and *ndr2/cyc* in the overlying blastomeres (Fig. 7.9b).

7.8 Interpreting Activin/TGF-β Signals

In 1952, the famous mathematician and founder of computer science, Alan Turing (1912–1954), built a model to explain how a reproducible pattern could emerge from an initially homogenous population of cells (Turing 1952). His Reaction-Diffusion model proposed that two diffusible factors, morphogens, could generate reproducible patterns in embryos. The features of this model are that one of the factors, called the Inducer, can induce its own expression as well as that of the second factor, called the Repressor. The Repressor diffuses faster than the Inducer, and inhibits production of the Inducer. Turing showed that over time, these two factors form graded distributions that resolve into a stable pattern of peaks and troughs. The pattern generated by this model can be changed dramatically simply by altering a few parameters, such as the rates of production, degradation and diffusion of the signals (Kondo and Miura 2010).

7.8.1 The Nodal Morphogen Gradient

Several lines of evidence in frogs, fish, and mice indicate that Nodal signals behave as morphogens to pattern the germ layers. In all species examined, *nodal* genes are expressed at higher levels in dorsal cells than in ventral cells (Zhou et al. 1993; Jones et al. 1995; Joseph and Melton 1997; Rebagliati et al. 1998a; Takahashi et al. 2000; Skromne and Stern 2002). Dorsal cells have higher levels of activated Smad2 than ventral cells, indicating that they experience an elevated level of Nodal signaling (Harvey and Smith 2009). Nodal proteins are diffusible and act directly on cells at a distance (Chen and Schier 2001). Of the three *nodal-related* genes in zebrafish, only Ndr1/Sqt acts over long distances and is considered a candidate morphogen. In overexpression assays, Nodal signaling acts in a concentration dependent manner, with high doses specifying endodermal fates and lower doses specifying ventral and lateral mesoderm (Smith et al. 1988; Green and Smith 1990; Jones et al. 1995; Agius et al. 2000). Conversely, inhibition of Nodal by overexpressing Antivin/Lefty suggests that Nodal signaling is required in a dosage dependent manner to pattern the dorsoventral axis (Agius et al. 2000; Thisse et al. 2000). Genetic analysis in the mouse supports the idea that Nodal signals are also required in a dosage dependent to pattern the epiblast (Robertson 2014).

Other studies indicate that Nodal signaling is required to pattern the animal–vegetal (anteroposterior axis) instead of the dorsoventral axis. Fate mapping and gene expression analysis in *ndr1/sqt;ndr2/cyc* double mutants revealed that marginal cells adopt neural fates in the complete absence of Nodal signaling (Feldman et al. 2000). Similar results were observed in mutants in which the levels of the Nodal co-receptor Oep are reduced or completely eliminated (Gritsman et al. 1999, 2000). Finally, fate mapping of $sqt^{-/-};cyc^{+/+}$ and $sqt^{-/-};cyc^{+/-}$ embryos, in which Nodal signaling is reduced to different extents, showed that marginal cells adopt more animal (anterior) fates when Nodal levels are abnormally low (Dougan et al. 2003). This analysis also showed that dorsal cells require higher levels of Nodal signaling to adopt marginal fates than do cells in the ventrolateral margin.

7.8.2 The Nodal Reaction-Diffusion Mechanism

Nodal signaling fits all the criteria laid out by Turing's Reaction-Diffusion mechanism (Solnica-Krezel 2003). As predicted by Turing, Nodal proteins induce their own expression as well as that of a Nodal antagonist, Antivin/Lefty (Fig. 7.11a) (Meno et al. 1999; Thisse and Thisse 1999; Cheng et al. 2000). Consistent with the Reaction-Diffusion Model, Nodal-related proteins diffuse farther than normal in the absence of Antivin/Lefty, and the mesoderm is expanded (Meno et al. 2001; Branford and Yost 2002; Chen and Schier 2002; Feldman et al. 2002; Sakuma et al. 2002). The prediction of the Reaction-Diffusion model that the antagonist should diffuse faster than the inducer has been more difficult to test. Fluorescently tagged versions of Nodal and Lefty2 showed that Lefty had a longer range than Nodal when overexpressed in *Xenopus* animal caps (Sakuma et al. 2002). The different ranges of the two proteins could be explained by differential degradation or by differential rates of diffusion. To distinguish between these possibilities, Ndr1/Sqt, Ndr2/Cyc, and Lefty were tagged with GFP or a photoconvertable fluorescent protein, Dendra2.5 (Muller et al. 2012). Cells overexpressing the tagged proteins were transplanted into a wild type host embryo, resulting in a localized source of the signaling molecule in the animal pole. The kinetics of diffusion and degradation were determined by measuring the rates of recovery after photobleaching or photoconversion. These studies ruled out the idea that Nodal and Lefty proteins were differentially degraded, and demonstrated that Antivin/Lefty proteins diffuse at a faster rate than either tagged Ndr1/Sqt or Ndr2/Cyc in the zebrafish blastoderm. Differential regulation of translation by microRNA-430, which delays translation of Antivin/Lefty, contributes to the different ranges of Nodal and Antivin/Lefty in the zebrafish (van Boxtel et al. 2015). This data supports a model in which diffusion of a Nodal protein establishes a spatial gradient within the responding tissue (Fig. 7.11b). At short distances from the source of Nodal signals, Nodal activity predominates and Nodal signaling induces its own expression, the expression of Antivin/Lefty and mesodermal and endodermal target genes. At long distances from the source of Nodal signals, the concentration of Antivin/Lefty increases above the threshold required to inhibit Nodal autoregulation and mesoderm induction. Cell

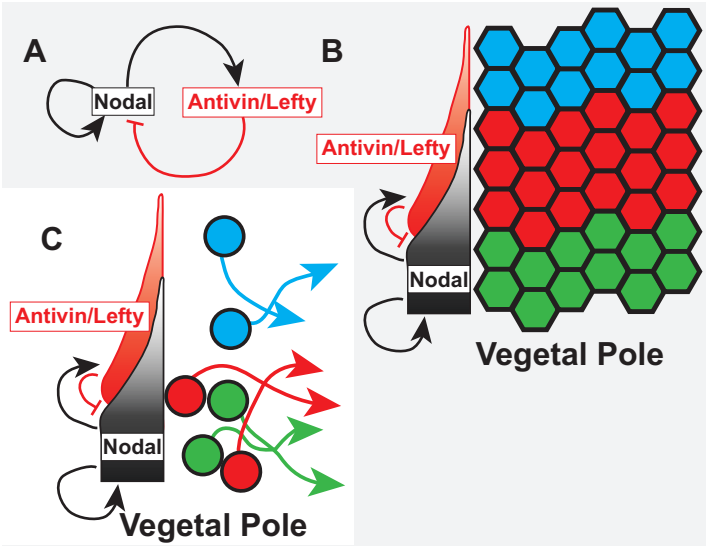


Fig. 7.11 Nodal reaction-diffusion mechanism. (**a**) Nodal signals induce their own expression as well as that of their antagonist, Antivin/Lefty. (**b**) In the classic Morphogen Model, Antivin/Lefty diffuses at a faster rate than Nodal, creating a gradient of Nodal signaling activity. Cells interpret this gradient according to their position within the gradient, with high doses specifying endoderm and lower levels specifying mesoderm. Cells adopt ectodermal fates in the complete absence of Nodal signals. (**c**) The Temporal Gradient Model takes the cell movements of the blastoderm and gastrula into account. The Reaction-Diffusion mechanism establishes a zone of Nodal signaling, in which cells are exposed to different Nodal doses. Cells are exposed to Nodal signals for different lengths of time depending on their movement path with respect to the Nodal gradient during the blastoderm and gastrula stages. Cells adopt different fates according to the total cumulative dose of Nodal signals to which they are exposed, as a function of the duration of their exposure and their position within the Nodal gradient

fates are determined by the absolute dosage of Nodal to which a cell is exposed as a function of their distance form the source of Nodal (Dyson and Gurdon 1998).

The classic morphogen gradient model could explain how the germ layers are induced and patterned in amphibians, but is difficult to reconcile with certain features of amniote and teleost embryos. The fate maps of fish, chicken, and mice show that mesoderm and endodermal precursors are intermingled before gastrulation (Fig. 7.5b-d) (Lawson et al. 1991; Warga and Nusslein-Volhard 1999; Lawson and Schoenwolf 2003; Hagos and Dougan 2007; Hagos et al. 2007). In zebrafish at this stage, cells move in random directions as far as 70 μm (Bensch et al. 2013). Cells are first exposed to Nodal signals at the margin during epiboly and conditional inactivation experiments showed that Nodal signals are required during this window to specify cell fates (Rebagliati et al. 1998a; Hagos and Dougan 2007; Hagos et al. 2007). In the mouse and chicken, cells are first exposed to Nodal signals when they enter the primitive streak and they are continually exposed to these signals as long as they remain in the streak (Joubin and Stern 1999; Kinder et al. 2001). In both fish and chicken, germ layer cell fates only become irreversibly determined during internalization, as shown by transplant experiments (Ho and Kimmel 1993; Kimura et al. 2006).

7.8.3 The Temporal Gradient Model

Some recent experiments indicate that cells interpret the length of time they are exposed to Nodal signals (Gritsman et al. 2000; Hagos and Dougan 2007; Hagos et al. 2007). Late expression of the Oep/Cripto co-receptor can rescue notochord formation in null *oep* mutants, but does not rescue formation of the prechordal plate (Gritsman et al. 2000). This suggests that transient Nodal signaling is sufficient to specify notochord, but continuous Nodal signaling is necessary to induce the prechordal plate. Experiments using the drug SB431542 to conditionally inactivate or activate the Type I Nodal receptor, ActRIb/ACVR1b at different times showed that transient Nodal signaling can induce notochord at any point in the blastoderm stage, and that specification of the prechordal plate requires prolonged exposure to Nodal signals (Hagos and Dougan 2007; Hagos et al. 2007). Similar results were obtained with other mesodermal cells types, indicating that this phenomenon is not restricted to the shield. In a separate set of experiments, the embryo was exposed to uniformly high levels of Nodal signals and the Nodal receptor was inactivated at different times. When the receptor was blocked at early stages, all cells adopted a low threshold response and expressed the notochord marker, *no-tail* (*ntl*). When the Nodal receptor was blocked at later stages, cells adopted a high threshold response and expressed the prechordal plate marker, *goosecoid* (*gsc*) (Hagos and Dougan 2007). Experiments using microfluidic chambers to culture embryonic stem cells revealed that mouse cells measure the rate of increase in Nodal signals as well as measuring the absolute dose (Sorre et al. 2014).

These results suggest a Temporal Gradient Model of Nodal signaling (Fig. 7.11c). According to this model, the Reaction-Diffusion interaction between Nodal and Antivin/Lefty may act to establish a domain in which cells are exposed to Nodal signals. Nodal signals from extraembryonic tissues, such as the zebrafish YSL or the mouse VE may act to maintain a stable domain of Nodal expression despite the cell movements in the blastula and gastrula stages. The Temporal Gradient Model could explain how Nodal signals pattern dynamically moving and proliferative tissues, such as the zebrafish margin or the amniote primitive streak. Cells that are next to each other at any given point in time may have different histories of exposure to Nodal signals based on their movement paths in the blastoderm and adopt different fates. This could explain the juxtaposition of mesoderm and endoderm precursors in many blastoderm fate maps, which can be viewed as a snapshot of the distribution of precursors at a single moment of time. If the cell movements in the blastoderm are random, then the Temporal Gradient Model predicts that mesodermal and endodermal cell identities are specified stochastically. Precedent for stochastic specification of cell fates comes from the invertebrate retina, in which cone cell identities are chosen at random (Wernet et al. 2006; Perry et al. 2016). The Temporal Gradient Model also predicts that long-term exposure to a low dose of Nodal signals will have the same effect on cells as a short-term exposure to a high dose, which has been verified experimentally (Hagos and Dougan 2007). More experiments are necessary to determine if cell movements can actually influence cell fates, and a refined Reaction-Diffusion Model needs to be developed to take into account these movements.

7.8.4 Conclusion

In some ways, the Temporal Gradient Model resembles John Saunders' (1919–2011) Progress Zone Model for limb development (Saunders 1948). Saunders proposed that limb bud mesenchymal cells in close proximity to an ectodermal structure called the Apical Ectodermal Ridge (AER) are maintained in a proliferative state and that cells adopt more distal fates the longer they remain under the influence under the AER. The AER acts as a stable source of signals adjacent to a highly dynamic cell population, analogous to the role proposed for the extraembryonic tissues of teleosts and mammals in the Temporal Gradient Model of mesoderm induction. The main difference between the two models is that in the Progress Zone Model, cell divisions push older cells away from the influence of the AER, whereas in the Temporal Gradient Model, active or passive cell movements bring cells into and/or out of the influence of Nodal signals from the extraembryonic tissues, though cell division could also influence a cell's position in the Nodal gradient. The Progress Zone Model has fallen out of favor recently, and other models may better explain proximo-distal axis formation in the limb (for review, see Mariani 2010). Nonetheless, the model has provided a firm intellectual framework to understand limb patterning. The Temporal gradient model provides a similar intellectual framework to understand how a reproducible pattern is imposed on dynamic tissues, such as the teleost margin or amniote primitive streak. The model may also apply to how other signaling molecules, like Sonic Hedgehog or BMP, act to pattern other dynamic tissues such as the anteroposterior axis in the limb or the dorsoventral axis of the embryo (Harfe et al. 2004; Tucker et al. 2008).

The classic Reaction-Diffusion model may better explain germ layer induction and patterning in species like the amphibians. In these embryos, the presumptive mesoderm and endoderm are spatially segregated at the blastoderm stage, and cell movements are limited before gastrulation. The spatial gradient of Nodal-related proteins could then impose a pattern on a static field of responding cells. Cells close to the source of Nodal expression are exposed to a high Nodal-to-Lefty/Antivin ratio and adopt endodermal fates while cells further from the source are exposed to low Nodal-to-Lefty/Antivin ratio and adopt mesodermal fates. This suggests that the Nodal signal transduction pathway can be adapted to pattern both static fields of cells as well as dynamic tissues. How this occurs is not clear, but in theory the same mechanism that interprets the absolute dosage of Nodal can also be used to interpret the total cumulative dose over time. It has already been well established that cells constantly assess their exposure to a gradient of Activin-like signals using a "ratchet-like" mechanism, in which cells can modulate to a higher threshold response, but not to a lower threshold response (Gurdon et al. 1995).

7.9 Systems Biology of Germ Layer Differentiation

The differentiation of the germ layers is one of the earliest morphogenetic changes that can be investigated quantitatively. There is a fundamental difference between the initial process of germ layer cell differentiation, in which molecular processes are dominant via the action of gene regulatory networks (GRNs), and the events after gastrulation, in which physics also plays a major role.

Genes are expressed and regulated in a structured cascade of events, thus forming a network with well-defined switching patterns of activation and inhibition. The complexity and differences observed during development are a function of the total number of gene patterns (rather than the total number of genes), and the interactions between those genes. As the number of gene patterns increase, the likelihood of interaction between protein products increase as well, therefore increasing the complexity of the network of gene interactions.

The first step in understanding a GRN is to characterize the regulation of gene patterns. The transcription of genes involved in morphogenesis is regulated by noncoding DNA regions known as cis-regulatory elements (CREs). CREs perform their function by acting as binding sites for transcription factors. A major development function of CREs is to respond to spatially diverse driver inputs so that the output pattern differs from the individual inputs, and thus CREs allow different genes to respond in multiple ways to similar regulatory states (Davidson 2010). A dramatic example of this is the comparison of *Drosophila melanogaster* with its approximately 14,000 genes, versus the significantly morphologically less complex *Caenorhabditis elegans*, which nevertheless has a greater number of genes, approximately 20,000. The difference is that *D. melanogaster* has between two and three CREs per gene, producing about 50,000 gene expression patterns, while *C. elegans* has between one and two CREs per gene, producing about 40,000 gene expression patterns (Markstein and Levine 2002).

Experimental validation of CREs is laborious process; the alternative is to use computational methods. An approach to determine CREs computationally is through the identification of clusters of high-affinity motifs within a given window of a determined gene; for example, in the case of the *zen* gene, the high-affinity consensus sequences representing 106 binding sites GGGWWWWCCM and GGGWDWWWCCM (W = A or T, M = C or A, D = A or T or G) were found within a window of 400–660 bp of *zen*. A survey of the *Drosophila* genome informed 15 novel clusters in addition to the *zen* cluster. This approach has been successfully used to study regulation of other genes (Markstein and Levine 2002; Markstein et al. 2002).

Given a set of gene patterns, as informed by CREs regulation, the value of understanding a GRN is to reveal the sequence of events that originate complexity. This knowledge can be used to formulate hypothesis about the outcomes of perturbations to the gene network, and even to control development to reach a desired outcome (or avoid an undesirable one). The study of GRNs during development is confounded by the large number of changes that occur simultaneously. Traditional network reconstruction approaches can only elucidate structure in the presence of a single or a few simultaneous changes (Laubenbacher and Stigler 2004; Kholodenko et al. 2005); when successful, these methods can represent a GRN as a systems of ordinary differential equations (ODEs). However, the use of ODEs is often thwarted by lack of data to determine kinetic parameters, and absence of mechanistic details; an alternative is to use Boolean networks, i.e., a set of nodes with binary states determined by other nodes in the network (Wang et al. 2012).

In the case of vertebrate embryos, the GRN for the establishment of the germ layers is complex. Morphogens are extracellular signal ligands propagated via diffusion, cells are development units, and the activation of regulatory responses is a function of the intensity of signaling thus activating different genes in different parts of the embryo.

The effect of diffusion on multispecific morphogen gradients might result in the formation of spatial patterns. At a fundamental mathematical level, this occurs when locally asymptotically stable equilibria become unstable with the addition of diffusivity. This is contrary to intuition in the sense that diffusion usually results in smoothing of concentrations, so on a first approach it seems unlikely that adding diffusion to equilibria can cause instability. But it does, and Alan Turing was the first to notice in 1952 (Turing 1952). This phenomenon has since been known as Turing pattern formation. The most spectacular Turing patterns are observed in nonlinear systems, e.g., the reaction-diffusion system of FitzHugh-Nagumo type. However, at a very fundamental level, Turing patterns are simply a property of linear systems (Malchow et al. 2008).

Consider the following systems of diffusion-reaction equations:

$$\frac{\partial U}{\partial t} = D_1 \nabla^2 U + a_{11} U + a_{12} V, \tag{7.1}$$

$$\frac{\partial V}{\partial t} = D_2 \nabla^2 V + a_{21} U + a_{22} V, \tag{7.2}$$

where U and V are morphogens, $\frac{\partial U}{\partial t}$ and $\frac{\partial V}{\partial t}$ represent the rate of change of the morphogens with respect to time, ∇^2 represents the Laplacian operator of second derivatives with respect to space (i.e., acceleration of change of morphogen in every direction), D_i represents diffusion coefficients, and a_{ij} are constant coefficients. When diffusion is negligible, then (0, 0) is a steady state, and the Jacobian of the system is

$$J = \begin{pmatrix} a_{11} & a_{12} \\ a_{21} & a_{22} \end{pmatrix}.$$

The conditions of stability are simply that both eigenvalues of the Jacobian have negative real parts, which are equivalent to

$$a_{11} + a_{22} < 0,$$

$$a_{11} a_{22} - a_{12} a_{21} > 0.$$

Now, we consider what happens when we introduce diffusivity. Since we are concerned only with a finite domain of size $0 < x < L_x$, and $0 < y < L_y$, resulting in wavenumbers $k_n = \pi n / L_X$ and $k_m = \pi m / L_y$, then Eqs. (7.1) and (7.2) can be solved explicitly using Fourier series so that

$$U = \sum_{n.m=0}^{\infty} \alpha_{nm} \sin(\mathbf{kX}), \tag{7.3}$$

$$V = \sum_{n.m=0}^{\alpha} \beta_{nm} \sin(\mathbf{kX}), \tag{7.4}$$

where $\mathbf{k} = (\boldsymbol{k}_n, \boldsymbol{k}_m)$, and $\mathbf{X} = (x, y)$. Substituting Eqs. (7.3) and (7.4) into (7.1) and (7.2), we obtain

$$\frac{d\alpha_{nm}}{dt} = (a_{11} - D_1 k^2)\alpha_{nm} + a_{12}\beta_{nm},$$

$$\frac{d\beta_{nm}}{dt} = \alpha_{21}\alpha_{nm} + (a_{22} - D_2 k^2)\beta_{nm}.$$

where $\mathbf{K}^2 = \boldsymbol{k}_n^2 + \boldsymbol{k}_m^2$.

The Jacobian of the system is

$$A = \begin{pmatrix} a_{11} - D_1 \boldsymbol{k}^2 & a_{12} \\ a_{21} & a_{22} - D_2 \boldsymbol{k}^2 \end{pmatrix}.$$

The conditions for stability are analogous to the previous case:

$$\hat{a}_{11} + \hat{a}_{22} < 0,$$

$$\hat{a}_{11}\hat{a}_{22} - a_{12}a_{21} > 0.$$

where $\hat{a}_{11} = a_{11} - D_1 \boldsymbol{k}^2$, and $\hat{a}_{22} = a_{22} - D_2 \boldsymbol{k}^2$.

Some general properties can be inferred immediately from this solution: (1) a change in stability only occurs if $a_{12}a_{21} < 0$, i.e., if they have opposite signs. (2) Destabilization of the asymptotically locally stable steady state is possible only if the diffusivity coefficients D_1 and D_2 are different.

When we extend this simple 2-morphogen example to multiple morphogens, the conditions for absolute stability are given by the Routh–Hurwitz stability criterion. If any of these conditions are violated, then instability occurs, and a Turing pattern is formed (Qian and Murray 2001).

A simple computational exercise illustrates well the differences between a stable and an unstable system. Figure 7.12a shows an arbitrary initial condition with compact support, i.e., a value greater than zero in an isolated region, and zero everywhere else. When the conditions for stability are met, the system evolves with smooth gradients (Fig. 7.12b). However, when the conditions for instability are violated, Turing patterns appear in ways that resemble some morphological processes (Fig. 7.12c).

Turing pattern formation is a vehicle with which to study the activation of regulatory responses influenced by spatial diffusion of morphogens. More complex nonlinear functions can be linearized about a point of interest so that the previous analysis can be applied. Given the simplicity in the structure of a multi-morphogen system, Turing pattern formation can be studied both analytically and with numerical simulations. Analysis informs fundamental patterns, and simulations are useful to

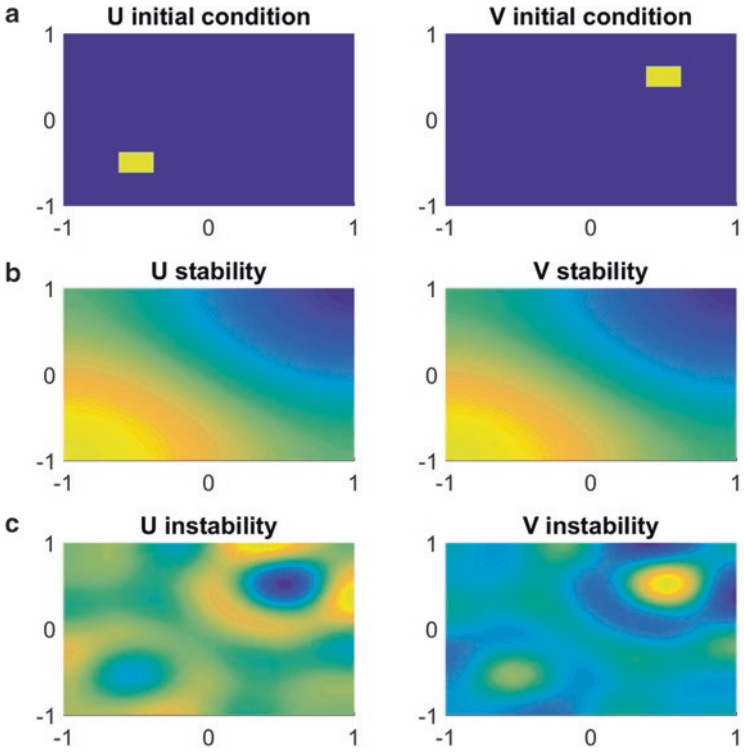


Fig. 7.12 (**a**) Initial conditions for a numerical implementation of Eqs. (7.1) and (7.2) in a square domain. The initial condition was set at 0.5 in square sub-domains at different locations for the two variables, and zero everywhere else. (**b**) When the conditions for stability are met, the numerical solution to the PDE problem in Eqs. (7.1) and (7.2), with Neumann boundary conditions, after 10,000 iterations with time step size of 0.08, shows a simple gradient with $D_1 = 0.003$, $D_2 = 0.001$, $a_{11} = 0.03$, $a_{12} = -0.05$, $a_{21} = 0.03$, $a_{22} = -0.04$. In this case stability conditions are met with $a_{11} + a_{22} < 0$, and $a_{11}a_{22} - a_{12}a_{21} > 0$. (**c**) A Turing instability appears when the conditions for stability are violated. The figure shows pattern formation with $D_1 = 0.003$, $D_2 = 0.001$, $a_{11} = -0.03$, $a_{12} = -0.05$, $a_{21} = 0.03$, $a_{22} = 0.04$. In this case stability conditions are violated with $a_{11} + a_{22} > 0$, and $a_{11}a_{22} - a_{12}a_{21} < 0$

represent a given instance, thus bridging molecular and cellular scales in the study of germ layer differentiation.

7.10 Future Prospects

The Hourglass Model is instructive because it provides a broad context in which to understand the similarities and differences of early vertebrate development. According to the Hourglass Model, embryos within a phylum strongly resemble

each other at the phylotypic stage, but there is considerable morphological diversity at earlier and later stages. Vertebrate embryos fall into one of two broad categories at the blastoderm stage, depending largely upon the amount of yolk contained in the eggs. Embryos with internal yolk platelets, like amphibians, or a relatively small-sized extraembryonic yolk cell, like the teleosts, have a spherical blastoderm. Embryos with a large extraembryonic yolk cell, like birds, reptiles, and monotremes, have a disc shaped blastoderm. In most eutherian mammals, the epiblast is disc-shaped despite the loss of yolk, suggesting that other morphological constraints act in the uterus to maintain this morphology. The exception to this is the rodent family, which has an inverted cup shaped epiblast.

Vertebrate embryos employ a variety of different mechanisms to internalize the presumptive mesoderm and endoderm. For example, the mesoderm and endoderm are internalized primarily by involution in basal vertebrates, sturgeon and amphibian embryos, and by ingression in birds and mammals. In reptiles, mesoderm and endoderm are internalized by involution through a blastopore, despite their disc-shape. Ingression occurs in a subset of mesodermal populations in some amphibians and reptiles. Thus, reptilian embryos seem to represent an intermediate state between the amphibian and avian forms of gastrulation. In teleosts, mesoderm and endoderm internalize by synchronous ingression, which differs from involution because the cells in a teleost blastoderm are not tightly connected to each other, as they are in an amphibian embryo. These different mechanisms may reflect the amount of cell movement in the pre-gastrula stages. Amphibians have relatively little cell mixing before gastrulation, whereas teleosts, birds, and mammals have a high degree of cell mixing. It is not clear how much cell mixing there is in the reptile blastodisc, or in the blastula of basal vertebrates, because no high-resolution fate maps have been produced for these early stages.

Despite these different mechanisms of internalization, the molecular mechanisms underlying germ layer induction are quite similar across the vertebrate phylum. Signals of the Nodal subclass of the TGF-β superfamily are required to induce and pattern the mesoderm and endoderm. These signals act in parallel with signals of the Fgf family, typically Fgf4 and Fgf8 to induce mesoderm. Despite some progress in *Xenopus*, we know little about how cells integrate their response to these two signaling pathways, or about how Fgf signals can act both to instruct cell fate and to direct cell movements. The allocation of cells to the three germ layers is only the beginning of the process of germ layer formation. We have not discussed the events downstream of Nodal and/or Fgf signaling that result in the expression of mesoderm or endodermal fate in vertebrates. There has been significant progress in elucidating the events immediately downstream of Nodal signals in frogs and fish, but further work is necessary to fully understand how mesoderm is distinguished from endoderm (Kimelman 2006; Kiecker et al. 2016). The events upstream of Nodal signaling are also an area of future investigation. In frogs, expression of the *xnr* genes is induced by the T-box transcription factor, VegT and maternally provided Activin, but the upstream factors in other vertebrates are not known. It is likely a T-box protein is required in zebrafish, acting in parallel to maternally provided Ndr1/Sqt signals to induce zygotic expression of *ndr1/sqt* and *ndr2/cyc*, but this factor has not

been identified. Even less is known about how Nodal signals are controlled in chicken and mammals.

Significant questions remain about how a morphogen gradient of Nodal signals could specify cell fates when the positions of the responding cells are not fixed. The primitive streak in birds and mammals, and the embryonic margin of teleosts are characterized by dramatic cell movements at the time they respond to Nodal signals. In these cases, germ layer identities may be determined by the total cumulative dose of Nodal signals to which cells are exposed, as a function of absolute dosage and the length of time to which the cells are exposed to Nodal. Cells in a moving population could be exposed to Nodal signals for different lengths of time by virtue of their different movement paths. Those cells that remain near a Nodal source for longer times would be exposed to a higher Nodal dosage than cells which move away—or are pushed away—more quickly.

The molecular mechanism by which cells could interpret such a temporal gradient is not understood. Elucidating this mechanism will certainly involve the development of more sophisticated mathematical models, variations on Turing's Reaction-Diffusion Model that take into account subtle changes in Nodal concentration over time and the movements of cells in the blastoderm stage. Our success depends upon the development of new techniques and strategies to image the response to Nodal signaling in vivo and in vitro. One area of progress has been the invention of techniques to culture single embryonic stem cells in microfluidic chambers (Warmflash et al. 2014). In these chambers, the Nodal dosage can be controlled over time with great precision, and the response to Nodal can be assessed at single cell resolution in real time. These studies can be used to generate models for the time-dependent response to Nodal signaling, which can be tested in whole wild type embryos as well as in various mutant backgrounds. In combination with more established embryological techniques, these new computational and imaging tools will provide an increasingly granular view of the molecular and cellular basis of germ layer formation in vertebrates.

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Chapter 8
Mechanisms of Vertebrate Germ Cell Determination

Tristan Aguero, Susannah Kassmer, Ramiro Alberio, Andrew Johnson, and Mary Lou King

Abstract Two unique characteristics of the germ line are the ability to persist from generation to generation and to retain full developmental potential while differentiating into gametes. How the germ line is specified that allows it to retain these characteristics within the context of a developing embryo remains unknown and is one focus of current research. Germ cell specification proceeds through one of two basic mechanisms: cell autonomous or inductive. Here, we discuss how germ plasm driven germ cell specification (cell autonomous) occurs in both zebrafish and the frog *Xenopus*. We describe the segregation of germ cells during embryonic development of solitary and colonial ascidians to provide an evolutionary context to both mechanisms. We conclude with a discussion of the inductive mechanism as exemplified by both the mouse and axolotl model systems. Regardless of mechanism, several general themes can be recognized including the essential role of repression and posttranscriptional regulation of gene expression.

Keywords Germ line primordial germ cells • Zebrafish • Xenopus • Ascidians • Mouse • Axolotl • Cell-autonomous • Inductive • Gene network or genetics

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8.1 Introduction

Successful formation of the germ line in each individual is required for genetic information to proceed into future generations. During development, the precursors to the gametes, the primordial germ cells (PGCs) segregate from somatic cell lineages. At the proper moment, PGCs migrate a significant distance to enter the developing gonads. Here they divide, acquire their sexual identity, enter into meiosis, and fully differentiate into haploid eggs or sperm. At fertilization, the genetic information from each gamete recombines and a genetically unique individual is formed. Continuity of the germ line from generation to generation is thus established. It follows that two hallmarks of the germ line is its ability to avoid death, the inevitable fate of somatic cells, and to retain full developmental potential. A major goal of research on the germ line is understanding how this unique lineage comes to be specified within the context of a developing embryo that allows it to both differentiate yet retain full potential.

Germ cell specification proceeds through basically two different mechanisms that have been broadly characterized as inherited or inductive. In this chapter, we discuss how germ plasm driven germ cell specification (inherited) occurs in both zebrafish and the frog Xenopus. To provide an evolutionary context, we discuss both the segregation of germ cells during embryonic development of solitary and colonial ascidians, as well as the regeneration of germ cells in colonial species, since these organisms provide salient examples of both inherited and inductive specification patterns. Ascidians are marine chordates closely related to vertebrates that also contain germ plasm. Finally, we examine the mechanism of germ cell specification via induction, as exemplified by mouse and axolotl. Although these model systems are highlighted because more is known about them, it is important to point out that there are important differences between mouse and human germ cell specification.

Inherited mechanisms operate by assembling, during oogenesis, a molecular "tool kit" of proteins and RNAs into a specialized subcellular domain called germ plasm. Cells that inherit germ plasm will enter the germ cell lineage and if they survive, will give rise to the gametes. No other lineage can do this in vertebrates. Germ plasm, although not membrane bound, retains a subset of proteins and RNAs that are both required and sufficient to confer germ line identity. The known components of germ plasm have been conserved across a wide variety of species and include endoplasmic reticulum, germinal granules, mitochondria, and lipid droplets. Such similarity is especially interesting when one considers that germ plasm has been reinvented repeatedly during evolution. The inheritance mechanism of germ cell specification apparently derived from the basal inductive mechanism (Johnson et al. 2001, 2003a; Extavour and Akam 2003). In both solitary and colonial species of ascidians, maternally specified cells containing germ plasm segregate early in development and, in colonials, give rise to gonads in asexually developing bodies. In some colonial species, however, segregation of germ line and soma is not absolute, as germ cells can be generated de novo at postembryonic stages. The inductive mechanism for germ cell formation in both mammals and axolotls involves the selection from a population of pluripotent cells formed at the time of gastrulation. Not all of these pluripotent cells will give rise to PGCs, but have the potential for

other cell lineages. Those cells that receive signals from the Bone Morphogenic Protein (BMP) family of growth factors originating from a proximal germ layer ultimately specify which cells will enter the definitive germ line.

Regardless of whether germ cells are specified by the inherited or inductive mechanism, several general themes in vertebrate germ cell specification have emerged. (1) Germ cell lineages are, in part, determined through a process of repressing programs of somatic differentiation. A fast block to somatic gene programs occurs by globally repressing mRNA transcription and thus preventing a response to any somatic differentiation signals in the embryo. A slow, long-term repression occurs at the level of chromatin modifications. (2) A reliance on posttranscriptional regulation of gene expression, both repressing somatic determinants and promoting gene programs required for germ cell characteristics such as migration, immortality and meiosis. Many of the genes just downstream of pluripotency factors such as Oct3/4 are translational repressors like Nanos/Pumilio or activators like Dazl. (3) Germ line stem cells are protected from expressing their potential for somatic differentiation within the context of niches. Although it is possible to describe linear pathways that lead to specific somatic fates such as pancreas or muscle, the germ cell lineage has eluded such a characterization; there is no master switch. It appears that there is no one pathway but several combinatorial pathways that may function independently, but all are required. The molecular and genetic underpinnings of these general themes are understood to varying degrees. Clearly, much remains to be learned.

8.2 Zebrafish

8.2.1 Background

Only relatively recently, in the 1980s, did the zebrafish (*Danio rerio*) emerge as a genetic model organism for studies in vertebrate developmental biology. It offers many advantages including transparent embryos, external development, and rapid life cycle facilitating direct visualization of cellular processes and genetic approaches. In the 1990s, thousands of mutant strains were obtained due to high throughput mutagenesis, becoming one of the leading models to perform genetic manipulations (Mullins et al. 1994; Kimmel 1989; Ingham 1997). At that time, genetic approaches to study germ cells were based mainly in *Drosophila melanogaster* (Williamson and Lehmann 1996) and *C. elegans* (Kimble and White 1981; Sulston et al. 1983; Ellis and Kimble 1995) putting zebrafish in the limelight as an inexpensive vertebrate genetic model to study germ cell development (Haffter et al. 1996; Westerfield 2000). Originally, information regarding primordial germ cells (PGCs) specification relied almost entirely on morphological identification of PGCs. In 1997, Yoon and coworkers identified, for the first time, a specific germ cell lineage marker in zebrafish (Yoon et al. 1997). They cloned the zebrafish homologue of the *Drosophila* vasa gene. This discovery allowed the design of new techniques for genetic manipulation and analysis in zebrafish germ cells, providing opportunities for researchers to obtain and analyze embryonic development without

surgical procedures. In addition to vasa, other conserved germ line genes between invertebrates and *Danio* were subsequently identified including nanos1 and dazl (boule in *Drosophila*) (Koprunner et al. 2001; Hashimoto et al. 2004).

The most significant biological processes that distinguish germ cell development are early segregation of the germ cells precursors (pPGCs) from somatic cell lineages at extragonadal locations, migration of PGCs to the developing gonadal primordium and finally, differentiation into germ cells influenced by the somatic gonadal environment (Braat et al. 1999c). Across different species, PGCs are morphologically very similar. Ultrastructural analysis shows the presence of RNAs and protein aggregates that appear as discrete, electron dense cytoplasmic inclusions, in close association with mitochondria, endoplasmic reticulum, and Golgi (Eddy 1975). In *Danio rerio*, germ plasm assembly starts before the end of oogenesis (Olsen et al. 1997). Many of the components that constitute the germ plasm and specify germ cell identity are conserved evolutionarily (Table 8.1). PGCs lineage separates from somatic cell lineages early in development and display a unique regulation over their basic cell functions. These include a selective responsiveness to cell signaling, RNA stabilization, and posttranscriptional regulation. In this way, PGCs avoid being differentiated into somatic cells and thus preserve the capacity to give rise to the next generation. It follows that the major goals of research into the germ line are to understand the mechanisms that allow PGCs formation, directional migration, and differentiation into mature gametes within the gonads.

8.2.2 Germ Cell Development in Zebrafish

Danio rerio belongs to the class Pisces and displays partial or meroblastic cleavage. The blastodisc undergoes division as it forms on top of a large yolky mass which does not divide (Gilbert 2006). The precursor of primordial germ cells (pPGC) refers to blastomeres containing germ plasm that, during early cleavages, divide unequally as stem cells. One daughter cell will receive germ plasm and maintain the germ stem cell character while the other blastomere will enter the somatic lineages. PGCs correspond to the founders of the germ line that will give rise to two daughter cells, both of them contributing to the germ cell lineage. The term germ cell (GC) is reserved for the stem cells of the germ line that reside in the gonad producing cells that will eventually give rise to gametes (Braat et al. 1999a).

Oogenesis: The process of oogenesis has been divided into four different stages as described by Selman et al. (1993). The earliest stage Ia oocyte appears as a transparent ball of cytoplasm surrounding a central germinal vesicle (GV) with no yolk and forming nests of interconnected oocytes. At Stage Ib the germ plasm aggregates to form the Balbiani body. In situ hybridization analysis shows that the germ plasm mRNAs vasa, nanos1, and dazl are localized to the Bb (Braat et al. 1999c; Kosaka et al. 2007). One important feature at this stage is that the axis marked by the Bb and nucleus (germinal vesicle) is the first indicator of the animal–vegetal polarity that will determine the anterior–posterior axis later on during

Table 8.1 Genes related to germ cell development in zebrafish

Germ name	Protein	Expression/Phenotype	Function	Reference
Nanos1	RNA binding zinc-finger proteins	Nanos-1 mRNA is expressed in the germ plasm and in the germ cells until 5 dpf. PGCs with lower levels of Nanos-1 have migration defects and eventually die	Acts as a translational repressor that regulates translation of specific mRNAs by forming a complex with Pumilio that associates with the 3′-UTR of mRNA targets	Koprunner et al. (2001)
Dazl	RNA binding proteins	Depletion of maternal Dazl mRNA in early embryos results in significant depletion of PGCs in Zf larva. DAZL overexpression strongly induces somatic protein expression	DAZL activates the translation efficiency of the target mRNAs through direct binding to cis-elements in their 3′ UTRs. Interacts with poly(A)-binding proteins, which are critical for the initiation of translation. Protect mRNAs against miRNA repression in germ cells	Takeda et al. (2009) and Hashimoto et al. (2004)
Dead end 1 (dnd1)	RNA binding proteins	Knockdown of dnd1 blocks confinement of PGCs to the deep blastoderm and results in failure of PGCs motile behavior and to actively migrate thereafter. PGCs subsequently die	Protect mRNAs against miRNA repression in germ cells	Weidinger et al. (2003)
Vasa	A DEAD-box RNA helicase	Vasa RNA expressed in germ plasm, PGCs and germ cells during gametogenesis. Protein is initially uniformly distributed but becomes restricted to the PGCs during gastrulation. Mutations disrupting vasa cause sexually dimorphic infertility, with impaired development of the ovary testis	Crucial role during germ-line specification, migration, survival, and maintenance. Involved in female meiosis, differentiation, and maintenance of germ-line stem cells	Hartung et al. (2014)
Cxcr4b	Seven transmembrane G-protein coupled receptor	cxcr4b RNA is maternally provided and during gastrulation becomes expressed in specific tissues including the PGCs, which migrate towards domains of sdf-1a RNA expression	Cxcr4b receptor for the chemokine Sdf1a. Critical role in PGC migration, required specifically for their chemotaxis	Knaut et al. (2003)
Sdf-1a (stromal cell-derived factor-1)	Chemokine	Mutant are able to activate the migratory program, but fail to undergo directed migration towards their target tissue, resulting in randomly dispersed germ cells	Sdf1a guides migrating PGCs towards their intermediate and final targets	Knaut et al. (2003) and Mizoguchi et al. (2008)
Staufen1/2	RNA binding proteins	Knock down embryos affect expression of the PGC marker *vasa*. Zf Stau proteins have evolutionarily conserved functions in germ cells and in neurons	Stau1 and Stau2 proteins are involved in survival and maintenance of germ cells. PGC migration is aberrant, and the mis-migrating PGCs do not survive in Stau-compromised embryos	Ramasamy et al. (2006)

(continued)

Table 8.1 (continued)

Germ name	Protein	Expression/Phenotype	Function	Reference
Ziwi/Zili	RNA binding proteins	Proteins specifically expressed in germ cells. Loss of Ziwi function results in a progressive loss of germ cells due to apoptosis during larval development	Ziwi proteins associate with a class of germ-cell-specific small RNA molecules named Piwi-Interacting RNAs (piRNAs). Have a role in germ cell maintenance and transposon silencing	Houwing et al. (2007)
Buc (Buckyball)	Novel protein (unrelated to any known family protein)	Buc protein localizes within the Balbiani body. Mutants present defects in germ plasm localization and polarity. Overexpression generates ectopic germ cells in the zebrafish embryo	Germ plasm defect in bucky ball mutants precedes the loss of polarity, indicating that Buc primarily controls Balbiani body formation	Bontems et al. (2009) Heim et al. (2014)
Tdrd7 (Tudor domain containing protein-7)	RNA binding proteins	Tdrd7 transcripts are localized in germ plasm and are expressed in PGCs for at least the first days of development. Despite normal PGC specification and migration observed in *Tdrd7* morphants, germ cell granule structure integrity is abnormal	Tdrd7 has a critical role for structural integrity of granules in PGCs	Strasser et al. (2008)
Mgn (Magellan microtubule actin crosslinking factor 1 [macf1] gene	Spectraplakin family of cytoskeletal linker proteins	Zebrafish magellan mutant exhibits a novel enlarged Balbiani body phenotype and a disruption of oocyte polarity	In mgn mutants, the oocyte nucleus is mislocalized; and the Balbiani body, localized mRNAs, and organelles are absent from the periphery of the oocyte, consistent with a function for macf1 in nuclear anchoring and cortical localization	Gupta et al. (2010)
Pum 1/2 (Pumilio2)	Puf family of RNA-binding proteins	*pumilio-2* are distributed in all blastomeres from the 1-cell stage to Gastrulation. Then accumulate in head and tail during early segmentation. Pumilio-2 protein is strongly expressed in the ovary, testis and brain	Translational repression of target mRNA. Pumilio proteins regulate the translation of specific proteins required for germ cell development and morphogenesis	Wang et al. (2009) and Kotani et al. (2013)
Puf-A	Puf family of RNA-binding proteins	Knockdown of *puf-A* resulted in microphthalmia, a small head, and abnormal PGC migration	*puf-A* plays an important role not only in eye development, but also in PGC migration and the specification of germ cell lineage	Kuo et al. (2009)
Brul (Bruno-like)	Elav-type RNA-binding protein that belongs to the Bruno-like family	mRNA protein of brul are localized to the germ plasm at the ends of the cleavage furrows	Brul genes may have a conserved function in axis specification (Dm analogy) and germ cells maintenance	Hashimoto et al. (2006)

embryogenesis. In stage II, germ plasm bearing specific mRNAs is moving towards the vegetal pole. Some RNAs like cyclinB1, pou-2, and notch1a are localized to the animal pole cortex until embryogenesis. They represent the earliest molecular markers for the animal pole (Howley and Ho 2000). At stage III, the oocytes increase in size due to vitellogenesis (yolk uptake), while the GV remains in the center of the oocyte. The animal–vegetal asymmetries become more visible with the formation of the micropylar cell within the follicular layer at the animal pole. The mRNAs localized at the animal and vegetal pole are distributed underneath the oocyte plasma membrane and will stay at the cortex until embryogenesis. At stage IV, oocytes undergo maturation, including GV migration to the future animal pole, subsequent germinal vesicle breakdown (GVBD) and the completion of first meiosis. After maturation, mature ovulated oocytes in second meiotic metaphase, are transported through the oviduct, and are now capable of being fertilized (the mature oocyte is now called egg). The micropylar forms the micropyle, an actin canal, permitting sperm passage through the chorion (Howley and Ho 2000).

Cytoplasm rearrangements: After fertilization, cytoplasm segregates from the yolk and starts to migrate towards the animal pole to form the blastodisc. Profound reorganization occurs during this time. Germ plasm mRNAs are redistributed and positioned at the animal pole, driven by the rearrangement of the cytoskeleton, particularly through the action of the microtubules and microfilaments (f-actin function) (Fig. 8.1). Some RNAs, like vasa, dead-end1, and nanos1, become enriched at the base of the blastodisc. Other components of the germ plasm, including dazl and bruno-like mRNAs, are delayed at the vegetal cortex, and are not observed at the blastodisc until almost one hour after fertilization, concomitantly when the first cleavage furrow starts. These observations suggest an alternative route or mechanism for RNA transport (Hashimoto et al. 2004, 2006). Differential regulation of dazl and bruno-like mRNAs is likely through binding elements present in their 3′ untranslated regions (3′ UTR). Binding elements present in the 3′ UTR of germ plasm RNAs play a key role in localization and posttranscriptional regulation during oogenesis and embryogenesis (Kosaka et al. 2007). Aggregation within the animal region and translocation from the vegetal cortex lead to the recruitment of RNAs at the furrow during germ plasm aggregation (Theusch et al. 2006). Notably, germ plasm co-segregation in the furrow presents a nonoverlapping pattern. The selective localization defines a compartmentalization within the germ plasm, with dissimilar RNA composition at the furrow. Where a medial domain is enriched in nos1, vasa, and dead-end (dnd), an intermediate domain that contains both, early mRNA and dazl, and a distal domain enriched in dazl RNA. As development proceeds, germ plasm mRNAs are segregated asymmetrically during cell divisions until the activation of the zygotic genome at gastrula stage (Figs. 8.1 and 8.2).

Germ plasm formation: The Balbiani body (Bb) is a transient and complex assemblage of mitochondria, endoplasmic reticulum, Golgi, proteins, and RNA that forms next to the nucleus. The initial components that will form the Bb originate from the nucleus and can be detected ultrastructurally as a complex assortment of mRNA and proteins called nuage (Figs. 8.1 and 8.5a, b). In zebrafish and frogs, the Bb acts as a vehicle to transport the germ plasm components to the vegetal pole of the oocyte.

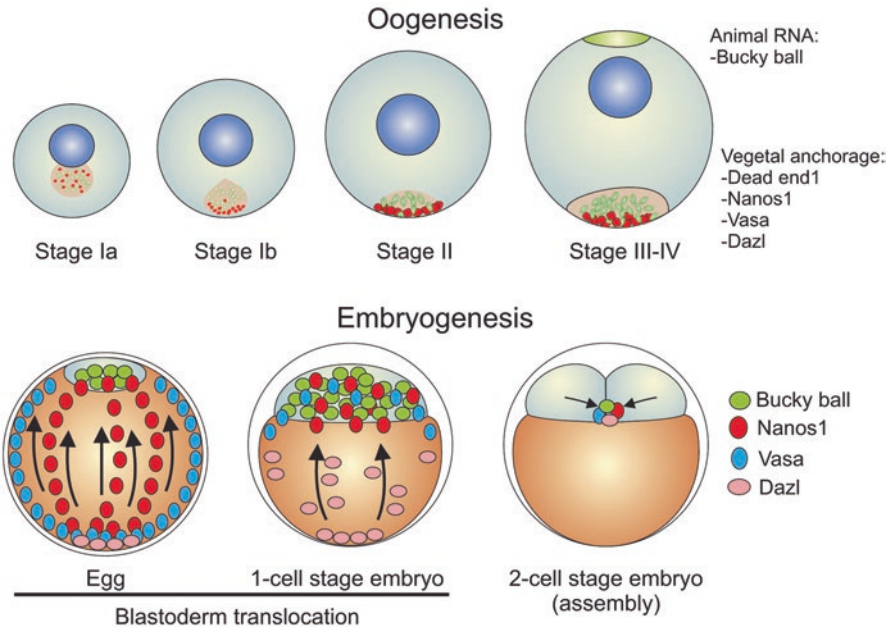

Fig. 8.1 Germ plasm localization during Zebrafish oogenesis and early embryogenesis: During oogenesis (*top row*), the Balbiani body (Bb) or mitochondrial cloud, shown in *light brown*, embedded with mitochondria, shown in *green*, marks the vegetal pole of the oocyte (stage I) and indicates the future vegetal pole of the egg and embryo (zygote). In stage I oocytes, newly synthesized RNAs enter the Bb as either a component of nuage or later by a diffusion/entrapment mechanism. In stage Ib and II oocytes, the Bb moves to the vegetal cortex and begins to disperse. Bb continues to disperse during the next two stages. Germinal granules (*red balls*) and germ plasm RNAs form a disc at the vegetal cortex. Other RNAs are localized at the animal pole (*light green disc*). Bucky ball originally localizes to the vegetal pole, but then translocates to the animal pole during St. III. After egg activation (*bottom row*), germ plasm mRNAs undergo a redistribution. Cytoplasm segregates from the yolk and starts to migrate towards the animal pole to form the blastoderm. At that time, vasa, dnd, and nos1 mRNAs translocate towards the animal pole (*blue* and *red circles*). *Arrows* indicate direction of germ plasm movement. After reaching the blastoderm, the rearrangement of the cytoskeleton (F-actin and microtubules) recruit germ plasm RNAs to two domains of the first cleavage furrow. In this way, the germ plasm becomes concentrated in a cytokinetic ring. dazl mRNA is recruited slightly later to the germ plasm suggesting a separate route of RNA transport (*pink circles*)

This structure properly positions germ plasm within the protective environment of the future endoderm. Endoderm also provides the proper location from which PGCs can correctly migrate and reach the presumptive gonads (Gupta et al. 2010). The majority of the mRNA present in the Bb corresponds to germ plasm components, nevertheless some mRNA involved in zygotic patterning including wnt11 and syntabulin (Makita et al. 1998; Nojima et al. 2010), also utilize the early Bb-mediated vegetal pathway. It has been proposed that the Bb has a basal role protecting RNAs from degradation as well as preventing any premature translation. The fact that maternal mRNA and translational regulators, required for germ line specification, are components of the Bb supports this idea (Collier et al. 2005; Marlow 2010).

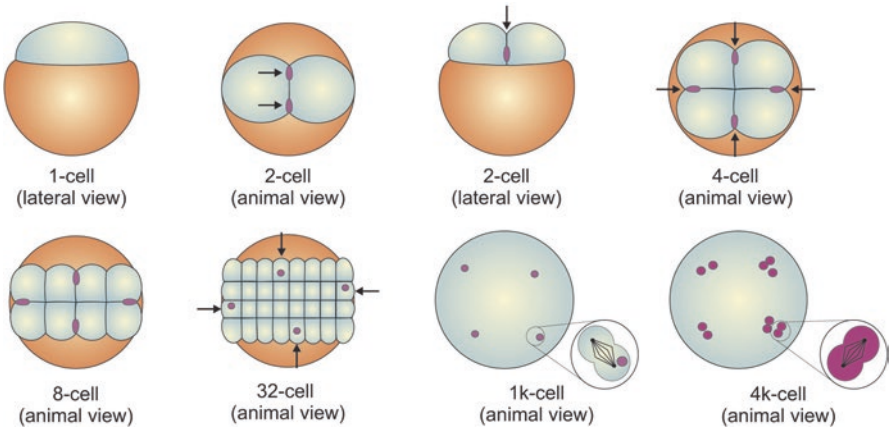

Fig. 8.2 Specification of zebrafish primordial germ cells: Schematic summary of zebrafish germ line development and segregation during early embryogenesis based on the distribution of vasa and nanos1 RNA. Germ plasm mRNAs are recruited to the forming furrows of the first and second cleavage planes (*arrows*). Upon furrow maturation, the recruited mRNAs form a tight aggregate at the distal end of the furrow, a microtubule dependent process. The resulting four subcellular aggregates ingress into four cells at the 32-cell stage and segregate asymmetrically during subsequent cell divisions (insert at 1K-cell stage). Until this point, these cells are called precursors of PGCs. At early gastrula stage (~4k-cells), germ plasm mRNAs become evenly distributed within cells and segregate symmetrically during cell divisions (insert). At this point cells are called PGCs

Recently, new information has become available on the regulation of Bb formation, its function, and disassembly (Heim et al. 2014). Large-scale mutagenesis screens in zebrafish (Wienholds et al. 2003; Dosch et al. 2004), uncovered a mutant line showing defects in oocyte polarity (Marlow and Mullins 2008). Further experiments demonstrated that a nonsense mutation in the *buckyball* (buc) gene disrupts maintenance of oocyte polarity and affects germ plasm assembly (Bontems et al. 2009). *Buckyball* is the only gene known to be required for Bb formation in vertebrates. During zebrafish early oogenesis *buc* mRNA and protein are vegetally localized in close association with the germ plasm. By mid to late oogenesis, buc protein is repositioned in the animal pole cortex where it persists until embryogenesis. During development, buc transcripts are lost but its protein remains localized. Remarkably, misexpression of BUC during embryogenesis induces germ plasm and PGC formation (Bontems et al. 2009). In *buckyball* mutants, the Bb fails to form and vegetal RNAs like vasa, nanos1 and dazl are not localized, reflecting a defect in the animal–vegetal polarity of the oocyte (Bontems et al. 2009). Although Buc is an evolutionary conserved protein it is unrelated to any known family protein, with no conserved protein motifs that would provide awareness into its biological function.

Large-scale mutagenesis screens discovered another gene, Magellan, required for animal–vegetal polarity of the oocyte and egg (Dosch et al. 2004). Magellan (mgn) mutants exhibit an asymmetric localization of the oocyte nucleus, a novel enlarged Bb phenotype and an absence of vegetally localized RNAs at the oocyte cortex (Gupta et al. 2010). The mutation was identified as a deletion in the coding

sequence of the zebrafish microtubule actin crosslinking factor 1 (macf1). macf1 is a member of the highly conserved spectraplakin family of cytoskeletal linker proteins that play diverse roles in polarized cells. Distinct from buc mutants, primary oocytes of mgn mutants are capable of recruiting germ plasm RNA and mitochondria to form a bona fide Bb (Gupta et al. 2010). However, eventually the Bb grows abnormally large and persists into later-stage oocytes with the particularity that the Bb fails to transit to the oocyte cortex. Accordingly, germ plasm mRNAs are not transferred properly to the vegetal cortex, being trapped in the persistent and enlarged Bb. In mgn mutants, the oocyte nucleus is mislocalized. Bb-localized mRNAs and organelles are absent from the oocyte periphery, consistent with a role of macf1 in nuclear anchoring and cortical localization. Both buc and mgn regulate animal–vegetal polarity and are critical for delivery of mRNAs to the vegetal cortex. buc promotes Bb assembly, whereas mgn is required for Bb translocation and dispersal via regulation of the microtubule cytoskeleton (Gupta et al. 2010).

8.2.3 Posttranscriptional Regulation in PGCs

Posttranscriptional regulation plays a critical role during germ cell development. Several maternal mRNAs, such as vasa (Knaut et al. 2002), nanos1 (Koprunner et al. 2001), dnd (Weidinger et al. 2003), and Tudor domain-containing protein 7 (tdrd7) (Mishima et al. 2006), are known to be localized to the germ plasm. However, during early cleavage stages, these RNAs are detected outside the germ plasm as well and are subsequently incorporated into somatic cells in addition to PGCs. vasa, nanos1, and tdrd7 mRNAs are rapidly degraded in somatic cells but are stabilized in PGCs in a process mediated by cis-acting elements in their 3′ untranslated region (UTR) (Wolke et al. 2002). Somatic cell must eliminate germ plasm mRNAs in order to preserve their somatic cell fates. Small noncoding RNAs (snRNA) have emerged as important players in early zebrafish development. One kind of snRNAs is micro RNA (miR) which functions to negatively regulate target genes by binding to their 3′UTR, promoting deadenylation, translational repression, and/or ultimately degradation of the transcript (Bushati and Cohen 2007). In zebrafish, miRNA-430 is the principally expressed miRNA during early embryogenesis. It is worth mentioning that currently there are no maternal miRNAs reported in the zebrafish embryo. In fact, miR-430 is first expressed at the mid-blastula transition (Schier and Giraldez 2006). Because of miRNAs are also present in PGCs, inhibition of miRNA function plays a vital role in germ cell development. For example, nanos1 is resistant to miRNA repression in the germ line, but not in somatic cells. This resistance is granted by the maternally expressed binding protein Dnd, which interacts with the nanos1 3′ UTR, blocking the binding of miR-430 (Kedde et al. 2007). This protection from miRNA repression is also found in other germ plasm mRNAs, Tdrd7 and dazl, suggesting a general protecting mechanism in germ lineage (Kedde et al. 2007; Takeda et al. 2009). Intriguingly, the fact that another germ plasm RNA such as vasa is eliminated from the somatic cells in a miR-430 independent mechanism, suggests the existence of additional modes of modulating

maternal transcripts (Mishima et al. 2006). Conversely, germ cells are protected from entering somatic fates by blocking somatic mRNA expression through promoting their deadenylation and subsequent degradation. RNA binding proteins Nanos and Pumilio play a key role in this process (Vasudevan et al. 2006; Collier et al. 2005).

Discovering the regulatory networks within germ plasm that are responsible for specifying the germ cell lineage is a major goal of current research. Germ plasm mRNAs like vasa, dnd and nanos1 exhibit an intricate and precise expression pattern during embryonic development. They are present in small cortical aggregates that become enriched in the cytokinetic ring (Braat et al. 1999b). Consequently, cytoskeleton components (microtubules and microfilaments) and germ plasm RNAs become recruited to the forming cleavage furrow (Yoon et al. 1997; Pelegri et al. 1999). Notably, zebrafish nebel mutant embryos, which have defects in furrow microtubule array formation, exhibit defects in the peripheral aggregation of germ plasm mRNA. At the 32-cell stage, four germ plasm aggregates ingress into four cells, where they remain subcellularly localized (Yoon et al. 1997). Between 32-cell and 8k-cell stage (cell cycle 13), these aggregates segregate asymmetrically during cell division, so that only four descendant cells inherit the germ plasm (Braat et al. 1999c). In each of these cells, germ plasm forms a cup-shaped structure that is often in apparent association with one of the spindle poles (Knaut et al. 2000) (Fig. 8.2, inset A). This program of asymmetric segregation changes at late blastula stage (8k-cell stage), when germ plasm is distributed equally to both daughter cells, now called PGCs (Knaut et al. 2000). At this time, the germ plasm appears to disintegrate and spread throughout the cytoplasm. Briefly before gastrulation (30 % epiboly), the germ plasm is entirely dispersed and fills the cytoplasm evenly in little patches (Knaut et al. 2000).

8.2.4 PGC Migration

In zebrafish, as in other vertebrates, PGC are specified before gastrulation in a different position from where the gonads develop. PGCs have to acquire directional motility and migrate towards the site where PGCs associate with somatic gonadal cells to subsequently differentiate into gametes (Raz and Reichman-Fried 2006). Zebrafish, PGCs are specified before gastrulation in four different locations within four different somatic environments. Therefore they have to traverse a complicated trail to reach the developing gonads (Fig. 8.3). As in amphibians and mammals, somatic tissues provide directional cues that help PGCs to reach their final destination.

The principal signaling system driving zebrafish PGC migration is Stromal-derived factor 1 (SDF1A) and its receptor, the GPCr chemokine (CXC motif) receptor 4b (CXCr4B), which is expressed in PGCs (Knaut et al. 2003). The PGCs migratory pathway is closely associated with the somatic expression of SDF1A that indicates the intermediate and final targets of migration (Reichman-Fried et al. 2004). Ectopic expression of SDF1A is sufficient to cause PGCs to mis-migrate to those positions in the embryo. In loss-of-function mutants of either SDF1A or CXCr4B, PGCs randomly migrate through the embryo (Richardson and Lehmann 2010).

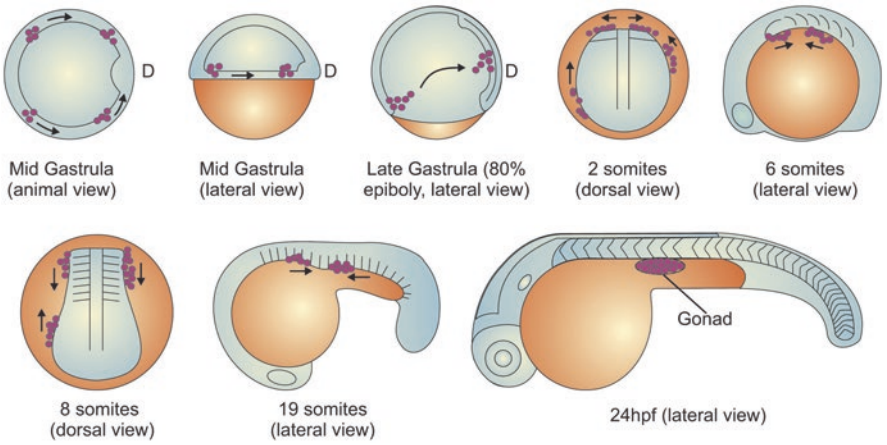

Fig. 8.3 Migration of zebrafish primordial germ cells: Schematic representation of embryos from early gastrula stage (shield) to 24 hpf showing the positions and movements of the PGC clusters. During gastrulation, four clusters of PGCs are found close to the blastoderm margin. (1) Concomitantly with gastrulation, lateral and ventral clusters move towards the dorsal region, ventral clusters migrate more slowly. (2) Clusters located very close to the dorsal side migrate away from the midline. (3) Ventrally located clusters align at the lateral border of the mesoderm. (4) In the early somite stages, most PGCs have arrived in two lines at the level of the first somite. These anterior located PGCs migrate towards the lateral region, forming two lateral PGC clusters. Cells that were initially located ventrally migrate towards the anterior along the anlage of the pronephros. (5) At the 8-somite stage, all anterior PGCs are found lateral to the paraxial mesoderm in a cluster extending from first to third somites. These clusters start to move towards the posterior area, while the ventral cells tightly align on the lateral border of the pronephros and continue to migrate anteriorly. (6) At the 19-somite stage, the main clusters have shifted to more posterior positions. (7) At 24 hpf, the PGC clusters are located at the genital ridges in the anterior end of the yolk extension, which corresponds to the eighth to tenth somite level

During the first 24 h of development, the PGCs follow a route that can be divided into six migration steps where they seem to have a certain association with specific embryonic structures. In the end, two cell clusters form in the region where the future gonad will be (Fig. 8.3). PGCs appear to leave certain regions and migrate in the direction of several intermediate target sites before reaching their final destination (Raz 2003). The use of tissue-specific mutants facilitated understanding the nature of the signals that guide the migrating cells. For example, the absence of some mesodermal tissues does not interfere with the ability of PGCs to leave the medial positions and reach their intermediate target. But a change in the position of their target leads to alterations in PGC migration, strongly suggesting that PGCs actively migrate towards their targets (Weidinger et al. 1999, 2002).

Interestingly, isolated PGCs undergo apoptosis after prolonged incubation periods suggesting that the extracellular matrix and cell surface signals may play important roles in PGC development (Di Carlo and De Felici 2000). In addition, Dnd activity is required for PGCs to begin their migration (Raz and Reichman-Fried 2006). Specific knockdown of dnd blocks the polarization and migration of PGCs.

This effect seems to be mediated through E-cadherin. E-cadherin is normally downregulated as PGCs begin to polarize and disperse, but this does not occur in PGCs depleted of Dnd. PGCs remain clustered in groups and maintain close cell–cell contacts (Richardson and Lehmann 2010). During migration to the gonadal ridges, PGCs remain in close contact with neighboring somatic cells. Such cell-cell interaction is governed by extracellular matrix components. Particularly important are the heparin sulfate glycosaminoglycans (HS-GAGs), which are present in the surrounding area of migrating PGCs. They play a critical role in guidance, survival, and localization of the migrating PGCs (Wei and Liu 2014). During germ cell specification, zebrafish PGCs have a smooth, round morphology and lack migratory activity (1k-cell stage or 3 hpf). PGCs begin to extend small cellular projections in multiple directions at 3.5 hpf. These projections disappear during mitosis. At the end of blastula (4.5 hpf), PGCs become polarized and extend broader projections at the guiding edge. This step is dependent on transcription and the Dead-end protein, and is necessary for the cells to respond to SDF1A (Richardson and Lehmann 2010).

8.2.5 Genetic Approaches

Perhaps the main advantage of using zebrafish to study germ cell specification is the availability of transgenic and mutant lines that allow functional approaches at any time point along the zebrafish life-cycle. In order to make any genetically manipulated organism, changes must be made in germ cells. Therefore it is of critical importance to establish an efficient technology for high specific PGC-targeted gene manipulation in vertebrates. New, sophisticated and affordable genetic approaches have recently emerged, allowing the manipulation of virtually any gene in a diverse range of organisms, including zebrafish (Auer and Del Bene 2014; Gaj et al. 2013; Dong et al. 2014). This core technology exploits different approaches, permitting the generation of transgenic and mutant lines. One well known strategy to obtain transgenic zebrafish is using the Cre/loxP recombinase and the Gal4/UAS transcription system for induction and regulation of genes of interest. PGCs are ideal target cells for genetics since they can be specifically labeled and manipulated during early development (Ciruna et al. 2002; Weidinger et al. 2003; Hsiao and Tsai 2003). Most importantly, they will generate adult gametes that provide genetic materials necessary to form a whole organism. The use of the Gal4/UAS system was optimized in zebrafish, allowing a tissue-specific expression of an improved Gal4 transcriptional activator (KalTA4), thus promoting gene expression driven by upstream activation sequence (UAS) (Distel et al. 2009).

The use of a specific germ cell promoter, allowing the localized expression of Gal4 in the germ cell lineage, or the fusion of the gene of interest to the 3′ UTR of the germ cell marker nanos, promoting the targeting of the newly transcribed mRNA mainly in germ cells, are new approaches used to improve transgenesis efficiency (Blaser et al. 2005; Xiong et al. 2013). Novel technologies will allow investigators not only to generate transgenic line with labeled PGCs, but will also have the

potential to introduce genes of interest as well as mutated versions in order to do functional studies during PGC development. TALEN (Transcription Activator-Like Effector Genome Editing) and CRISPR/Cas9 have been shown to be successful in generating loss-of-function alleles in zebrafish (Auer and Del Bene 2014; Irion et al. 2014). Both, by disruption of coding sequence by indel mutations or by deletion of whole loci from the genome, these approaches represent powerful new tools for future generations of targeted mutagenesis and DNA integration strategies (Hwang et al. 2013; Hruscha and Schmid 2015).

Future directions in genome-edited zebrafish protocols should be to improve germ line transmission of induced mutations and allelic insertions, high efficient screening of knock-out and knock-in progeny and the conditional regulation of manipulated genes. The use of the 3′ UTR region of some germ line genes allows the specific protection of mRNAs from degradation in germ cells while promoting their degradation in the soma. This approach could be extremely useful in making a recombinant Cas9 mRNA that would be expressed exclusively by germ cell precursors in order to improve the rate of germ line transmission. Genetically modified zebrafish lines will provide powerful tools to conduct conditional gene activation and inactivation approaches for studies of gene function in PGCs.

8.3 Xenopus

Similar to zebrafish, the germ cell lineage in the frog *Xenopus laevis* is specified by the inheritance of maternally formed germ plasm. Ultrastructural characterization of germ plasm in Xenopus has underscored how similar this aggregate of organelles is across both vertebrate and invertebrate species. Germ plasm contains dense aggregation of some 500,000 maternal mitochondria, endoplasmic reticulum, lipid droplets, and several hundred electron dense "germinal" granules, a hallmark of germ plasm (Marinos and Billett 1981; Kloc et al. 2001) (Figs. 8.4 and 8.5). Such similarity is especially interesting when one considers that germ plasm has been reinvented repeatedly during evolution as the inheritance mechanism of germ cell specification apparently derived from the basal inductive mechanism (Extavour and Akam 2003).

8.3.1 The Germ Line Life Cycle: Continuity of the Germ Plasm

In Xenopus, as in zebrafish, germ plasm assembles during the earliest stages of oogenesis within a structure called the Balbiani body (Bb). Kloc et al. (2004b) have shown that the developing Bb contains a centriole that likely facilitates microtubule-based transport of germ plasm components, at least in part, driving the formation of this body. The formation of the Bb and germ plasm is a lengthy and complex process (reviewed in King 2014). Studies at the ultrastructural level indicate the first

visible signs of nuage, widely believed to be the precursors to the germinal granules, appear in the nuclei of PGCs within the indifferent gonad (al-Mukhtar and Web 1971). Nuage can clearly be seen passing through the nuclear pores and into the cytoplasm where it becomes intimately associated with mitochondria (Fig. 8.4b). Aggregates of mitochondria become embedded within nuage material now called mitochondrial cement at oogonia stages (Fig. 8.5a–d). What initiates this interaction is unknown. Interestingly, mitochondria in germ plasm have a lower respiratory activity than other mitochondria (Kogo et al. 2011). There are precedents to suggest that the physiological state of mitochondria can determine position within the cell (Cox and Spradling 2009). It is possible that nuage contains proteins similar to Clueless in *Drosophila* that help recruit mitochondria based on their respiratory activity. Mitochondria cement is thought to be the direct precursor of granulofibrillar material (GFM) that forms from it but within the Bb. The GFM eventually matures into germinal granules enriched in maternal RNAs and proteins required for germ cell specification (Fig. 8.4b). However, in Xenopus the molecular identity of germinal granules is only partially known, but includes nanos, deadsouth, xdazl, large and small subunits of mitochondrial rRNA and xpat RNAs distributed either in or on the surface of these granules (Kloc et al. 2002). The RNA binding protein Hermes is the only known protein within the granules, but it is also found in nuclei, germ plasm, and ooplasm (Chang et al. 2004). Hermes likely has several different functions largely unknown, but recent work identifying Hermes interacting partner, Xvelo, offers a clue. Xvelo is the homologue of zebrafish Buckyball, the gene required for Bb formation (Marlow and Mullins 2008; Nijjar and Woodland 2013b). Two splice variants of Xvelo1 were found and both RNAs produce a protein product expressed in previtellogenic oocytes. However, only the larger isoform is found in the Bb whereas the smaller protein variant has a cortical localization. The larger variant Xvelo protein is too stable to deplete by degrading its RNA by antisense DNA oligonucleotides. However, successful depletion of the shorter splice variant from fully grown oocytes resulted in a decrease in germ plasm aggregates and a loosening of associated mitochondria (Nijjar and Woodland 2013b). These results suggest a role for Xvelo as a key scaffolding protein that interacts with RNA-binding proteins. Interestingly, both Xvelo RNA variants are present in previtellogenic oocytes, but are not localized within the Bb. Both RNAs localize later to the vegetal cortex using the Late pathway. Because so few studies have been done characterizing the proteins in germ plasm, this pattern of protein distribution being different from its corresponding RNA may be much more common than previously appreciated. Making assumptions about protein expression based on RNA patterns can be misleading. Vasa and Xpat proteins may offer another example (Machado et al. 2005; Lasko 2013). Two uncharacterized RNA binding proteins were also identified as interacting with Hermes. Whether these Hermes interacting proteins are in germinal granules remains to be determined. In addition to germinal granules, germ plasm accumulates a distinct set of RNAs including Xdazl, Xlsirts, Xwnt11, Xpat, many of which encode RNA binding proteins (see Table 8.2 for a complete list).

One intriguing and unanswered question is what keeps the Bb as a separate membraneless structure. It contains a very dense accumulation of endoplasmic reticulum;

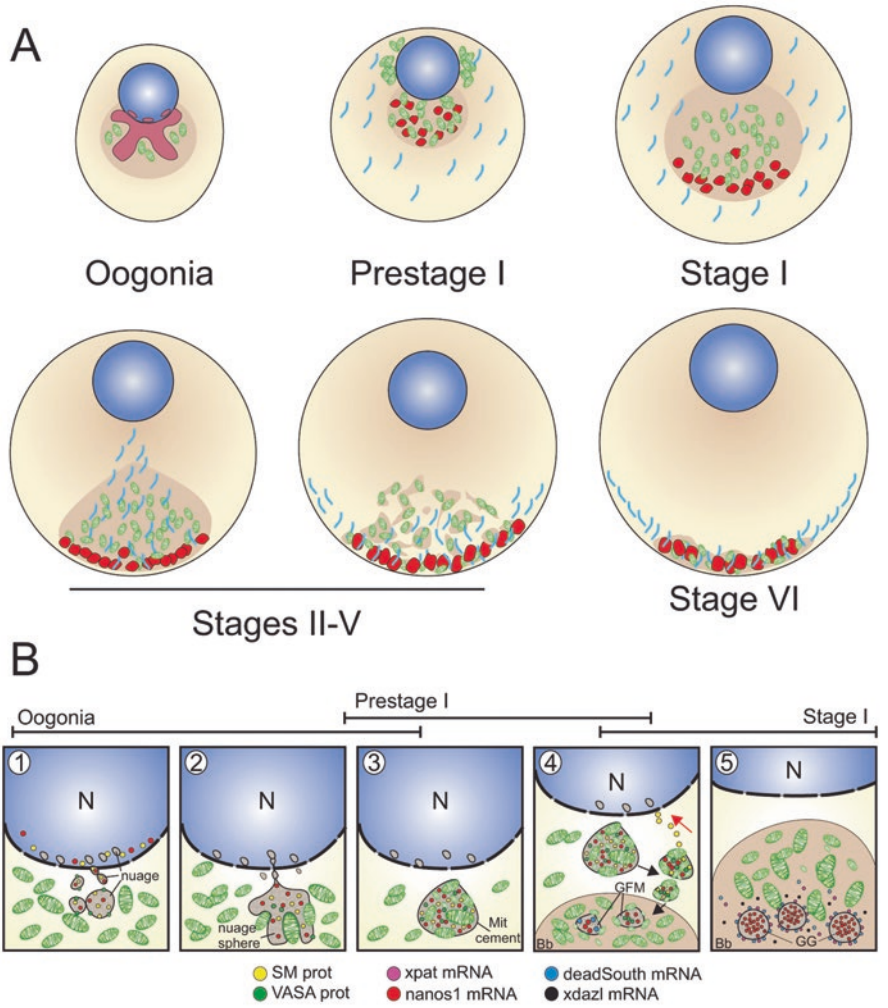

Fig. 8.4 Germ plasm formation and RNA localization during Xenopus oogenesis. (**a**) Schematic showing different stages in oogenesis in relation to the Balbiani body (Bb, tan). Oogonia show beginning of mitochondria (*green*) clustering in relation to nuage (*red*) in close nuclear apposition. Prestage I shows further development of germ plasm as germ line RNAs accumulate within Bb and germinal granules (*red circles*), late stage RNAs are uniformly distributed (*blue lines*). Stage I oocytes with germ plasm (*red circles*) at tip of mature Bb. Stages II begins expansion of Bb towards the vegetal pole with germ plasm reaching cortical region followed by fragmented Bb containing late pathway RNAs. Stage VI oocyte germ plasm RNAs in a small cortical region while late pathway RNAs overlap with germ plasm but extend to a larger cortical domain. (**b**) Scheme of germ plasm formation. In pre-stage I and early stage I oocytes an electron dense material called nuage (40 nm), leaves the nucleus via nuclear pores (*1*). In the cytoplasm, nuage associates with specific germ plasm components including nanos1 mRNA (*red dots*), Vasa protein (*green dots*), and the spliceosomal protein Sm (*yellow dots*). Nuage aggregates coalesce into larger spherical accumulations (200–600 nm), or nuage spheres (*2*) become surrounded by mitochondria, forming a large nuage/mitochondria complex, called mitochondria cement (*3*). As this complex translocates toward the Balbiani

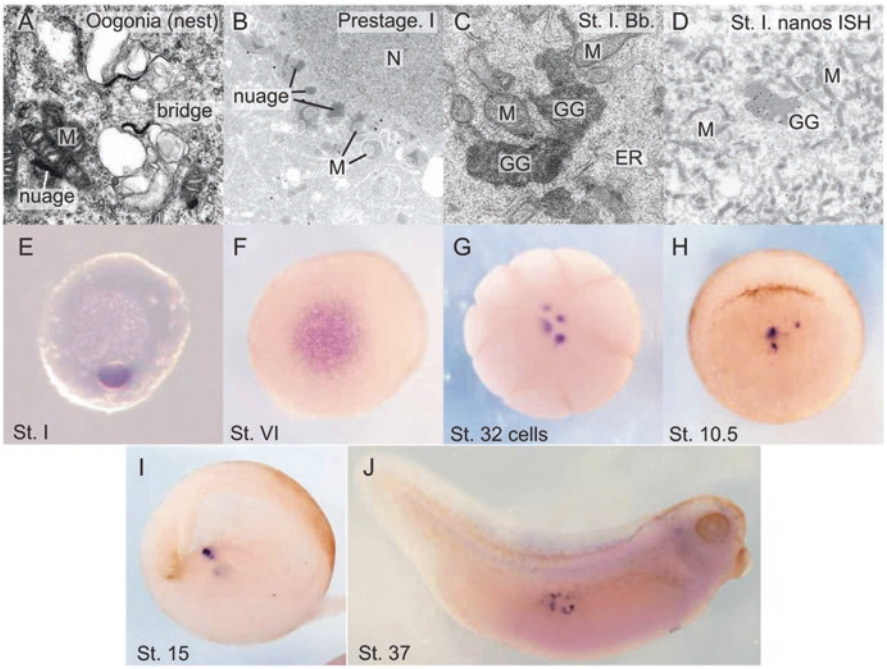

Fig. 8.5 Germ plasm formation and distribution in Xenopus embryo. (**a**–**d**) Ultrastructural analysis of germ plasm formation. (**a**) Interconnected oogonia at nest stage showing nuage closely apposed to mitochondria. (**b**) Inter-mitochondrial cement or nuage, closely apposed to nucleus, pre-stage 1 oocyte; (**c**) Germ plasm within Bb in Stage I oocyte; note the electron dense germinal granules, mitochondria, smooth endoplasmic reticulum, ribosomes; (**d**) EM in situ hybridization showing labeled nanos RNA within germinal granule, stage 1 oocyte. (**e**–**j**) Whole mount in situ hybridizations showing Xpat expression in germ plasm. (**e**) Stage I oocytes showing clear nucleus and closely apposed Bb; (**f**) Vegetal pole of stage VI fully grown oocyte; (**g**) Xpat staining within germ plasm at tips of vegetal pole blastomeres of 32-cell embryo; (**h**) Gastrulation showing PGCs within vegetal mass, closely apposed PGCs are likely mitotic sisters; (**i**) Sagittal cut of early neurula stage showing PGCs close to the posterior floor of the archenteron; (**j**) Tailbud with PGCs clustering at the dorsal aspect of endoderm. *M* mitochondria, *N* nucleus, *GG* germinal granules, *ER* endoplasmic reticulum. (Malgorzata Kloc and Kenneth Dunner, unpublished micrographic data)

Fig. 8.4 (continued) body (Bb), it fragments into small clusters composed of several mitochondria surrounding the mitochondrial cement (4). At this point, Sm proteins leave the mitochondrial cement and are recycled back into the nucleus (red arrow). At stage I, the mitochondrial cement, releases the mitochondria and transforms into granulo-fibrillar material (GFM, 4), the precursor of the germinal granules (GG). At this stage, the germ plasm is located at the vegetal tip of the Bb. Germ plasm RNAs present in the GFM includes nanos1 (red dots), xpat (purple dots), xdazl (black dots), and deadsouth (blue dots). Germinal granules (in gray) are mainly concentrated at the vegetal tip of the Bb (5)

Table 8.2 Germ line mRNAs in Xenopus

mRNA	Protein classification	Phenotype	Function	Reference
Dead-end (dndl)	RNA-binding (RRM)	Knockdown of dnd1 in oocyte interfered with formation of vegetal cortical microtubule arrays. Mis-migration of PGCs in tailbud stages	Blocks miRNA repression in germ cells, PGC survival. Dnd1 anchors trim36 to vegetal cortex	Horvay et al. (2006) and Mei et al. (2013)
Xdazl (dazl)	RNA-binding (RRM)	Knockdown of Xdazl inhibited migration from the ventral to the dorsal endoderm	Positive regulator of translation; promotes polyadenylation	Houston et al. (1998) and Houston and King (2000a, b)
XGrip (grip2)	Glutamate Receptor-Interacting Protein	Knockdown and overexpression reduced average PGC number; PGCs mis-localized into anterior endoderm at tailbud stages. PGC migration to germinal ridges impaired	PGC development and migration	Tarbashevich et al. (2007), Claussen et al. (2011), and Kirilenko et al. (2008)
Sybu	Adapter protein binds KIF5b, syntaxin	Knockdown mRNA sybu caused loss of dorso-anterior axis formation in embryos	May play a role in protein that bridges microtubule motors and membrane vesicles, during dorsoventral axis formation in the vertebrates	Colozza and Robertis (2014)
Xpat (PGAT)	Unknown	Major constituent of germ plasm throughout oogenesis and early development. Also present in oocyte and embryo (i.e., nuclei)	May play a role in germ plasm formation, position and maintenance	Hudson and Woodland (1998) and Machado et al. (2005)
DeadSouth (ddx25)	DEAD box RNA helicase	Knockdown of DEADsouth inhibited migration of germ plasm from the cortex to perinuclear region and inhibited PGC division at stage 12. PGC number reduced after stage 20 when ddx25 overexpressed	ATP dependent RNA helicase	MacArthur et al. (2000) and Yamaguchi et al. (2013)
Germes	Unknown; associates with dynein light chains	Overexpression of germes resulted in number of primordial germ cells and abnormalities in germ plasm distribution	Translocation of germ plasm from cortex to perinuclear region during gastrulation; germ plasm organization	Berekelya et al. (2003, 2007) and Yamaguchi et al. (2013)
Trim36	E3 Ubiquitin-Protein Ligase	Trim36 mRNA found in mitochondrial cloud of stage I oocytes. Vegetal microtubule polymerization and cortical rotation were disrupted in trim36-depleted embryos	Regulates microtubule array formation during cortical rotation involved in dorsal axis formation	Cuykendall and Houston (2009), Yoshigai et al. (2009), and Mei et al. (2013)

Kif13B	Kinesin/ATPase	Mis-migration occurred with modulations of KIF13B. Knockdown inhibited blebbing and accumulated PIP3	Germ cell migration	Tarbashevich et al. (2011)
Xlsirts	Noncoding and coding RNAs containing tandemly repeated elements	Destruction of the localized Xlsirts results in the release of Vg1 vegetal cortex. Xlsirt needed for proper localization of Vg1. Removal of Xlsirts disrupts cytokeratin cytoskeleton	Localize RNA and anchors mRNA at the vegetal cortex	Kloc et al. (1993), Kloc and Etkin (1994), and Kloc et al. (2007)
Xdsg	Myosin ATPase	Xdsg faintly detected in germ plasm at cleavage stages. Found in cytoplasm of germ line cells at stage 12–40 and in PGCs at stage 46 PGCs at stage 46	Formation of PGCs by regulating the production of XVLG1 (Vasa) protein	Yamakita et al. (2004) and Ikenishi et al. (2006)
Poc1b /pix1 And poc1 a/pix2	W40 repeat containing protein/ esterase	Colocalizes with Xpat in the mitochondrial cloud in stage 1 oocytes and in cortical germ plasm islands in stage VI oocytes. Pix proteins also localize to centrosomes, mitochondria and microtubules. Interference by antibody microinjection leads to a cell division failure	Possible microtubule dependent germ plasm organizer	Hames et al. (2008)
Wnt 11	Receptor	Wnt11 partially rescues UV ventralized embryos by inducing dorsal tissue formation allowing formation of neural tube and somatic muscle (lacks notochord and anterior head structures)	Dorsal ventral axis specification through Wnt signaling; function in germ line is unknown	Ku and Melton (1993)
Nanos1 (Xcat2)	RNA binding, zinc finger	Morpholino knockdown of maternal Nanos1 decreased PGCs and a loss of germ cells from the gonads; PGCS fail to migrate out of endoderm; somatic genes mis-expressed in PGCs	Translational repressor; functions with Pumilio to repress RNAs; Represses VegT endoderm determinant	Mosquera et al. (1993), Zhou and King (1996), Lai et al. (2011), and Lai et al. (2012)

(continued)

Table 8.2 (continued)

mRNA	Protein classification	Phenotype	Function	Reference
Xvelo1	Unknown	Velo1 found in cytoplasm in stage l oocyte. Localization of velo1 to vegetal cortex starts during late stage II oocytes. Depletion of xvelo results in decrease of germ plasm islands and loosing of associated mitochondria	Homologue of Bucky ball; Germ plasm formation and maintenance	Claussen and Pieler (2004) and Nijjar and Woodland (2013a)
Pcsk6/XPACE4	Subtilisin-Like Protease/proprotein convertase	Depletion XPACE4 results in delayed formation of blastopore and disrupted gastrulation. XPACE4 depleted embryos resulted in ventralization and small heads	Regulates production of TGF-beta proteins. Required for the endogenous mesoderm inducing activity of vegetal cells before gastrulation	Birsoy et al. (2005)
Plin2/fatvg	Perilipin	Depletion of the maternal fatvg mRNA results venetralized embryos that lack primordial germ cells. Inhibits germ cell determinants to move to their proper locations. Localizes via METRO and late pathway (intermediate pathway)		Chan et al. (1999; 2007)
Gptl1	Noncoding	Unknown	In germ plasm until stage 30; found in dorsal tissues. Unknown function	Cuykendall and Houston (2010)
Exd2	3′–5′ Exonuclease	Exd2 found in the germ plasm until gastrulation. Exd2 is not expressed outside the early germ line and not detectable in later stage embryos. Exd2 expression robust in spermatogenic cells of the adult testis	Unknown	Cuykendall and Houston (2010)
Xnoa 36/znf330	Zinc finger protein	Xnoa36 localizes to the nucleoli and the mitochondrial cloud	mRNA anchoring and microtubule organization	Vaccaro et al. (2010, 2012)
Hermes	RNA binding, RRM	Depletion of hermes accelerates maturation resulting in cleavage defects in vegetal blastomeres of embryo	Germ cell development, maturation and cleavage events; role in translation(?)	Song et al. (2007)
Centroid/ddx59	ATP dependent helicase	Localized via intermediate pathway	Regulation of germ plasm stored messenger ribonucleoprotein and/or germ plasm function	Kloc and Chan (2007)

how does the Bb maintain this concentration? The Bb includes all three members of the major cytoskeletal protein families: actin, microtubules and intermediate filaments (Gard et al. 1997). The F-actin crosslinking protein spectrin is a germ plasm component contributing to its integrity. As a scaffolding protein, spectrin also likely facilitates the concentration of specific components within the germ plasm matrix. Recently it has been proposed that RNA-binding proteins with low complexity sequences can form GFM-like hydrogels at high concentrations (Kato et al. 2012). A reasonable hypothesis is that germ plasm is such a hydrogel formed by the specific accumulation and high density of these germ line RNA-binding proteins.

The Bb lies in close apposition to the nucleus and faces the future vegetal pole where endoderm will form (Fig. 8.4a and b). Germ plasm formation occurs almost entirely while oocytes are in first meiotic prophase when maximum transcription is occurring. In stage I oocytes, the germ plasm segregates to the vegetal tip of the Bb by an unknown process. Morphologically, germ plasm assembly appears to be complete at this stage, although additional molecular components are likely added throughout oogenesis (al-Mukhtar and Web 1971; Heasman et al. 1984). In stage II oocytes, the Bb expands to the vegetal cortex region perhaps driven by the intense endocytosis (Wilk et al. 2004). The expansion pushes the germ plasm into the vegetal subcortical region where it becomes anchored by a poorly understood process that involves both the actin and intermediate filament systems (Kloc et al. 2007) . In stage II of oogenesis the Bb becomes irregularly shaped and eventually fragments in stage III/VI oocytes but these fragments do not contain germ plasm. In fact, these fragments likely contain RNAs involved in somatic patterning such as Vg1 and VegT (Kloc and Etkin 1998; Wilk et al. 2004).

The localization of germ plasm to the vegetal cortex has been referred to as the Early or METRO RNA localization pathway while the Late pathway predominantly translocates somatic determinants via microtubules. There are exceptions. Fatvg protein functions in both somatic and germ cell lineages. Consistent with this duality, Fatvg RNA uses both the early and late pathway (Chan et al. 1999, 2001, 2007). In contrast, Dead-end RNA localizes only through the late pathway but its RNA persists only in the germ plasm during embryogenesis. All known RNAs localized as part of the Bb have been found later within germ plasm containing blastomeres of early staged embryos. At the end of oogenesis (VI oocytes), germ plasm is observed as hundreds of small yolk-free islands in a disc-shaped domain within the vegetal subcortex while somatic determinants are distributed over a larger but overlapping cortical area (Figs. 8.4 and 8.5). The question remains, when is germ plasm assembly complete? Injected nanos RNA remains capable of entering the germ plasm even in stage VI oocytes, indicating that it is possible for additional components to become incorporated even at the end of oogenesis (Nijjar and Woodland 2013a).

Germinal granules are believed to serve as long-term storage for RNAs that will not be translated until after fertilization in the embryo. *Nanos* is a good example. Since oogenesis can last 6 or more months in Xenopus, it is critical that such protection be failsafe. RNAs that are not sequestered within granules must also be protected from degradation. Key questions then are how are these RNAs protected? When are germ line RNAs translated and are they translated at different times? Xdazl, Dead-end, and DeadSouth proteins have only been detected in ooplasm, not

germ plasm per se (Padmanabhan and Richter 2006; Mei et al. 2013; Mora and King unpublished). As discussed below, these proteins appear to have a function in oogenesis separate from their role during embryogenesis in PGCs. Thus, their RNAs may be stored within germ plasm for later use, although also translated during oogenesis. Clues as to when germinal granule components become active may be found in the changing appearance of the granules going from round and compact in early oogenesis to irregular and less electron dense late in oogenesis. After fertilization, germinal granules form irregularly shaped branching structures and we know that *Dead-end* is translated then and soon after, *nanos*. Between late neurula (st 18) and early tailbud (st 25) they change into irregular string-like bodies and finally into round granular bodies again at the feeding tadpole (st 46) (reviewed in Kloc et al. 2001).

After fertilization events, the islands of germ plasm begin a continual process of aggregation driven by actin contractions during divisions and later, ingression along cleavage planes via microtubules. The end result is that by the 32-cell stage, germ plasm is concentrated into ~32 large pools within the vegetal most blastomeres (Savage and Danilchik 1993). During cleavage, germ plasm is found close to the plasma membrane and segregates asymmetrically into one of the two daughter blastomeres, thus preserving the amount of germ plasm inherited by the one, presumptive primordial germ cell (pPGC). The other daughter cell enters the somatic endoderm lineage. This changes during gastrulation when the germ plasm moves in a microtubule dependent manner, to a perinuclear position. Subsequent cellular divisions then generate cells both of which contain germ plasm and hence, if they survive, are fated to give rise to gametes. These cells are now called primordial germ cells (PGCs). Gastrulation, then, marks an important time period when the germ line segregates from the future somatic lineages. PGCs will divide approximately three times, once each at gastrulation, tail bud, and late tailbud stages to yield between 20 and 50 PGCs (Dziadek and Dixon 1977; Kamimura et al. 1980). The wide range in numbers of PGCs is likely the result of the random distribution of germ plasm into early vegetal blastomeres and the chance that some cells will not receive enough germ plasm to preserve germ line fate (Fig. 8.5).

PGCs remain in the endoderm from their inception until late tailbud stages (40–41), 3 days post-fertilization. During this time, PGCs have a characteristic migration pattern, discussed later, that brings PGCs to the dorsal most midline of the endoderm, and subsequently, along both sides of the dorsal mesentery. A few days later, PGCs arrive at the genital ridge or presumptive gonads, squeeze in between the gonadal epithelial cells and there begin to divide mitotically. What regulates the number of divisions these cells undergo before they enter meiosis, is not known (al-Mukhtar and Web 1971; Kloc et al. 2004a, b). PGCs are sexually indifferent and will develop into either female or male gametes depending on the levels of estradiol present in the somatic gonad (Villalpando and Merchant-Larios 1990). Frogs reach sexual maturity some 6 months later. With the fusion of the gametes at fertilization and subsequent embryogenesis, the process of segregating the germ line begins again. The “life-cycle” of the germ line from gametes to PGCs and back again highlights the continuity of the germ cell lineage through generations while somatic cell lineages are terminated with each individual’s death (Fig. 8.6). The functional significance of these morphological changes must await a better understanding of translational control in the germ line.

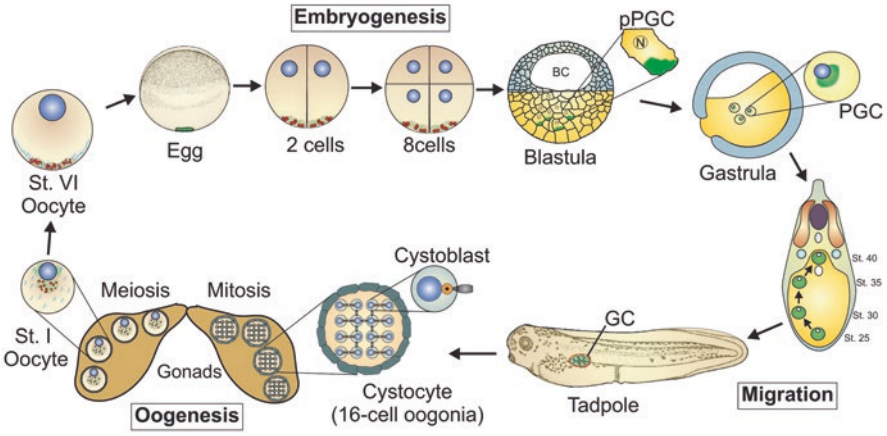

Fig. 8.6 Continuity of germ plasm in Xenopus (female cycle is depicted). Schematic showing germ plasm (*red circles*) with mitochondria (*green*) within the Bb. The Bb moves to vegetal pole later in oogenesis. During early cleavage stages, germ plasm is asymmetrically inherited by a few vegetal pole blastomeres and remains peripheral (at this point called pPGC). At gastrula, germ plasm becomes perinuclear (PGCs). During tailbud stages, PGCs begin to migrate laterally and dorsally and exit the endoderm at tadpole stage 40, migrating along the dorsal mesentery to the presumptive gonads. PGCs enter developing gonads at tadpole stages; here PGCs divide mitotically to form 16-cell interconnected nests of oogonia. These cells enter meiosis becoming oocytes. At all stages either nuage or germ plasm is present

8.3.2 Cell Autonomous Specification of Germ Line

Although germ plasm was shown to be both required and sufficient for determination of the *Drosophila* germ line, the question remained whether this was also true in vertebrate systems. Early on, Smith (1965) had found that nuclei from PGCs, but not other cells, remain totipotent upon nuclear transplantation into enucleated eggs of the frog *Rana pipiens* (Smith 1965). His results suggested that germ plasm components conferred a totipotent "status" to the genetic material within the nucleus. But whether germ plasm contained all the determinants to convert a naïve cell into the germ cell lineage remained untested. Tada and coworkers took advantage of the unique abundance of mitochondria in germ plasm to generate a transgenic frog in which EGFP was fused to a mitochondrial transport signal to selectively label germ plasm (Tada et al. 2012; Taguchi et al. 2012). At the eight-cell stage, the EGFP labeled germ plasm was removed by micropipette and injected into an ectopic location, an animal pole cell. Interestingly, the injected germ plasm behaved, as it would have in vegetal pole cells, by becoming localized to the peripheral cytoplasm early on and migrating to a perinuclear location by gastrulation (Fig. 8.6). These ectopic EGFP-germ plasm cells, referred to as T-GP, did not migrate properly into the genital ridges, but continued to express germ plasm markers such as nanos, xdazl, and xpat at least until tailbud stage 28. For proper migration into the genital ridges, perhaps T-GP cells had to begin within the endoderm. To test that idea, at

gastrulation and tailbud stages, T-GP cells were selected and transplanted back into the endoderm. While most of them failed to migrate, 46% did reach the genital ridges. These results were very encouraging and led to the next key experiment that asked if functional gametes could be produced from animal pole cells receiving EGFP labeled germ plasm. To distinguish these cells, EGFP-labeled germ plasm was injected into recipient transgenic frogs in which every cell expressed DsRed2. Embryos with DsRed2 labeled animal pole cells containing EGFP-labeled germ plasm were allowed to reach gastrula stage. These labeled cells were then transplanted into the blastocoel of a wild-type host frog where they could enter the endoderm and eventually migrate into the gonads. Both genetically marked DsRed2 sperm and oocytes were produced in gonads of wild-type hosts. These wild-type host animals were then mated to generate embryos. A very small number of resulting embryos carried the DsRed2 genetic marker. Despite these small numbers, the results do demonstrate that the presence of germ plasm alone was sufficient to redirect cells fated to become somatic ectoderm or neural ectoderm, into the germ cell lineage. The change in fate was accomplished despite what other cytoplasmic or nuclear cues were present and despite cell signaling from other somatic cells. These results also imply that germ plasm contains dominant repressive mechanisms to shield pPGCs from somatic fates. Thus germ plasm has evolved in vertebrates as well as in invertebrates and functions as autonomous determinants of the germ line.

Earlier approaches to settle the issue of germ plasm sufficiency had ectopically placed either whole vegetal pole blastomeres, presumably containing germ plasm, or migrating rhodamine (TRITC)-labeled PGCs within the blastocoel of unlabeled host embryos. Using these methods, Wylie et al. (1985) found that TRITC labeled cells were not detected within the host's gonadal tissue, but that a very small number were detected histologically in somatic tissues (Wylie et al. 1985). Furthermore, the TRITC labeled cells appeared to have differentiated into somatic cells representative of all three primary germ layers. There are several reasons why Wylie et al. (1985) may not have detected germ line TRITC labeled cells. These include the limited persistence of TRITC, migration failure, loss of cell due to apoptosis, or insufficient germ plasm. However, Tada and coworkers did not document the fate of the T-GP cells that failed to enter the germ line, making it difficult to compare the results of the two studies. Did any of the T-GP cells differentiate into somatic cells and survive? These are important questions as they concern the ability of germ plasm to preserve full potential within ectopic environments and outside any germ line niche.

It has been known for a long time that in order for *Xenopus* PGCs to migrate correctly into the genital ridges they must be correctly situated in posterior endoderm (Fig. 8.7). If labeled pPGCs are implanted into anterior endoderm, the number of PGCs reaching the genital ridge declines severely (Ikenishi and Tsuzaki 1988). The Tada et al. (2012) study goes further and confirms that the endodermal environment is only required for correct migration and not for survival or germ line maintenance. In fact, if PGCs are isolated and cultured in a simple buffer on fibronectin, they autonomously undergo the correct cellular movements required for directional migration and at the normal developmental times (Morichika et al. 2010). What initiates such a program and what types of cues are provided by the endoderm in

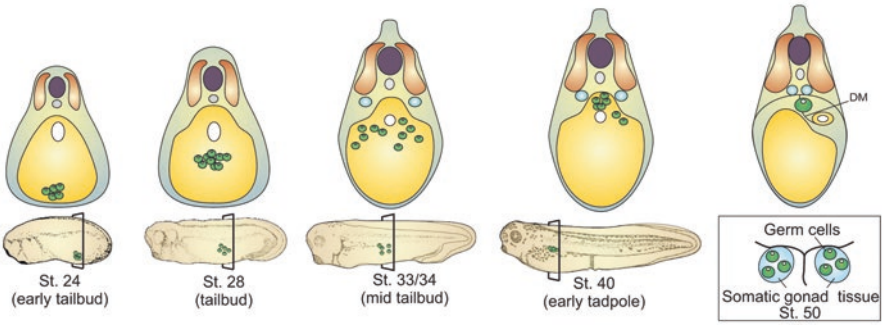

Fig. 8.7 Migration of Xenopus Primordial Germ Cells within the endoderm. Migration within the endoderm has been divided into four steps: clustering at stage 24, dispersing laterally at stage 28, directionally migrating dorsally at stages 33/34 and aggregating at the dorsal-most aspect of the endoderm at stage 40. Migration stages according to Terayama et al. (2013). *Inset* shows schematic of PGCs within somatic gonadal tissue

terms of direction remains unclear. There is evidence for three different cell signaling systems operating to guide PGCs to their targeted location: CXCR4/SDF1 (Takeuchi et al. 2010), Notch/Delta2 (Morichika et al. 2010), and KIF13B-PIP3-GRIP2.1 (Tarbashevich et al. 2007). Notch 1 and Delta-1 are expressed in the endoderm and PGCs, but only delta-2 and Serrate-1 are expressed in PGCs at tailbud stages. CXCR4 is expressed in PGCs and its ligand, SDF1 is expressed in the dorsal mesentery, implicating this signaling system in providing the directional cues for correct migration. Whether the endoderm secretes any signal that repulses migrating PGCs from the endoderm is unknown. PGC proteins Dead-End and Xdazl have been implicated in normal PGC migration (Horvay et al. 2006; Houston and King 2000a). These RNA-binding proteins are involved in promoting mRNA translation, suggesting that level of control is important.

8.3.3 Mechanisms Preserving Full Potential Through Protection

As previously described, during oogenesis both germ cell and somatic cell determinants become localized at the vegetal cortex. After fertilization, cellular divisions partition somatic determinants equally to the vegetal blastomeres, but germ plasm is asymmetrically inherited by only one of the two dividing daughter cells. To complicate matters, some of these localized RNAs, such as Fatvg, have functions in both the endoderm and PGCs (Chan et al. 2001, 2007; Table 8.2). Given that the genetic programs for endoderm are not activated in PGCs, despite the presence of the endoderm determinant VegT, repressive mechanisms must be operating. In Xenopus, as well as in other organisms, both translational repression and the transient suppression of transcription are critical to preventing expression of somatic fate in pPGCs (Lai et al.

2012; reviewed in Lai and King 2013). In fact, the onset of transcription in PGCs occurs some 10 or more hours after it is initiated in somatic cells at the mid-blastula transition (MBT). The delay allows PGCs to avoid inappropriately responding to signals patterning the early embryo and initiated by the growth factor Vg1 and the transcription factor VegT (reviewed in Heasman 2006). At the same time, PGCs have their own unique gene program that is initiated during mid-gastrulation and early neurula stages (Venkatarama et al. 2010). Genes transcribed at this time likely support a migration program and preserve the potential for meiosis and totipotency. Recent evidence suggests that Nanos and Pumilio may be the lead players in translational repression while Dead-end and Xdazl in translational activation in the Xenopus germ line.

Virtually all characterized mRNAs within the germ plasm are sequestered in a translationally repressed state throughout oogenesis (Houston and King 2000b; Kloc et al. 2001; 2002; Voronina et al. 2011; Schisa 2012) (Table 8.2). Nanos RNA is repressed not only by being sequestered in germinal granules, but by steric hindrance via secondary structure just 4 nt downstream of the AUG site (Luo et al. 2011). What activates the translation of these mRNAs is important to understand. Several candidate RNA-binding proteins with ATPase activity have been implicated including Vasa, DeadSouth, and Dnd (Liu and Collodi 2010; Lasko 2013). Among the first RNAs to be translated in pPGCs is nanos where Dnd appears to play a critical role relieving repression at the pre-initiation step (Luo 2011). Nanos is a broadly conserved protein found in species as divergent as sponges and humans (Lai et al. 2011). The only known function for Nanos is as a translational repressor in partnership with the RNA-binding protein Pumilio (Lai et al. 2011, 2012). Pumilio recognizes very specific nucleotide binding sites (PBE: UGUA(N)AUA). Pumilio provides the selectivity for repression by recruiting Nanos to only those RNAs. VegT and other vegetally localized RNAs contain PBEs in their 3′ UTR required for translational repression (Lai et al. 2012; Lai and King 2013). In nanos mutants, the endoderm specific transcription factors and downstream targets of VegT, Xsox17, and Bix4, are now expressed in PGCs. Furthermore, the delay in transcription is lost and PGCs initiate transcription at the same time as somatic cells (Lai et al. 2012). PGCs lacking Nanos subsequently undergo apoptosis. Interestingly, nanos mutants in *Drosophila* and *C. elegans* also display premature transcription and loss of the germ line (Schaner et al. 2003; Deshpande et al. 2005). How can translational repression by Nanos/Pumilio in PGCs be related to the transient genome-wide suppression of mRNA transcription in these cells at the MBT?

Recently, a link between the translation and accumulation of maternal transcription factors, including VegT, has been found to be critical to the normal onset of the MBT (Skirkanich et al. 2011). In zebrafish, pluripotency factors Oct91 and Sox were also found to be important in this regard (Harvey et al. 2013; Lee et al. 2013). The working model that has emerged from these studies suggests that first genomic DNA must be released from a repressed state. Such an event is correlated with the nuclear to cytoplasmic ratio and depletion of a repressor (reviewed in Langley et al. 2014). Cell cycle regulation therefore, is one component, driving changes in the N/C ratio. One target of nanos/Pumilio repression, apparently conserved across species, is cyclin B1. Cyclin B1, in a concentration dependent fashion, regulates the

cell cycle (Mendez and Richter 2001; Kotani et al. 2013). By repressing the cyclin B1 mRNA, the levels of Cyclin B1 protein would be expected to decline in PGCs. Consistent with this observation, whereas endodermal cells can divide every 40–75 min, PGCs undergo cell divisions less frequently, dividing during three discrete time periods. A second requirement for correct initiation depends on de novo translation and accumulation of key maternal transcription factors, factors that are in limited supply. In Xenopus, as mentioned above, VegT is one of these. Translational repression by Nanos/Pumilio of VegT, cyclinB1 and other maternal factors in PGCs may contribute to the observed delay in transcription because sufficient levels of core factors have not been reached. In PGCs, VegT is degraded by gastrulation (Lai et al. 2012).

Xenopus Oct60 and Oct25 are maternally expressed and play key roles in maintaining pluripotency in the early cleaving embryo by blocking signaling pathways that promote differentiation including Activin/Nodal, BMP, and WNT (Whitfield et al. 1993; Cao et al. 2004, 2006, 2008). While Oct25 is not detected in PGCs, Oct60 is and expression persists until gastrulation. Oct60 expression and function have primarily been investigated in somatic cells and its role in PGCs remains unknown. The maternally expressed transcription factor, Sox7, localizes to the vegetal cortex as well as the germ plasm (Zhang and Klymkowsky 2007; Owens and King, unpublished observations). Sox7 may also have a role in PGCs in addition to its known role in somatic cells (Zhang et al. 2005). Interestingly, maternal expression of Oct60 depends on an Octamer-Sox binding motif in its promoter region that Oct60 has been shown to bind (Morichika et al. 2014). Thus Oct60 and Sox7 may form a positive feedback loop that promotes pluripotency in the germ line. PGCs are transcriptionally active at gastrulation/early neurula. An early transcript is Oct91 (Venkatarama et al. 2010). A TRANSFAC promoter analysis identifies putative oct and sox binding motifs within the Oct91 promoter region. Although the maternal factors that activate the zygotic program in PGCs are unknown, it seems reasonable to suggest that Oct60, with Sox7, are those factors, and that together they activate Oct91. In this way, totipotency in the germ line could be preserved (Hinkley et al. 1992; Venkatarama et al. 2010; Owens and King, unpublished observations). Further research is required to determine exactly what maternal factors are required to initiate the zygotic program in PGCs and what the downstream targets are of Oct91.

While nanos and Pumilio repress translation in the germ line, the RNA-binding protein Xdazl promotes translation (Collier et al. 2005; reviewed in Brook et al. 2009). Almost certainly, Xdazl does this by interacting with PABP (Poly A Binding Protein), a translation initiation factor that binds poly A in the 3′ end, thereby facilitating efficient translation. Xdazl may recruit PABP to specific germ line mRNAs, but the identity of those RNAs in PGCs remains unknown. Whatever mRNAs Xdazl regulates, its function is essential in PGCs. Depletion of Xdazl from PGCs results in their failure to migrate properly within the endoderm and their eventual loss (Houston et al. 1998; Houston and King 2000a). In oocytes, Dazl has a critical function in progression of meiosis and gamete development perhaps providing hints as to what RNAs it regulates in PGCs (Padmanabhan and Richter 2006). In oocytes, Xdazl and Pumilio both bind cyclin B1 mRNA, suggesting the interesting paradigm

of having both negative and positive regulators on the same mRNA. After Pumilio is phosphorylated, cyclin B1 is translated, perhaps through Xdazl promoting its polyadenylation (Ota et al. 2011). Xdazl protein is detected in PGCs continuously from blastula stages until germ cells are within the gonads at stage 52 (Houston and King 2000a; Kataoka et al. 2006). The persistent expression of Xdazl protein in the germ line suggests that it may regulate the translation of RNAs responsible for many PGC functions including migration, meiosis, and proliferation. Interestingly, mouse PGCs lacking dazl fail to transcriptionally activate the Oct4 pluripotency program, a hallmark of the germ line (Haston et al. 2009). Thus, there may be a role for Xdazl in preserving the potential for totipotency in Xenopus PGCs.

Another level of translational control in the early embryo is mediated by microRNAs. Whereas germ line RNAs must be cleared from the soma to prevent abnormal development, they must also be protected in the germ plasm. Overexpression of nanos RNA in early staged embryos results in incomplete neural tube closure, most likely because it represses required VegT activity (Luo et al. 2011). Evidence supports a role for RNA binding proteins Dnd and Xdazl in protecting other germ line RNAs including their own RNAs, from degradation. xdazl, dnd, xpat, nanos and deadsouth, appear to be protected from miR-18 degradation by a combination of Dnd and ElrB1 binding the target region in the 3′ UTR (Koebernick et al. 2010). These studies need to be extended beyond using reporter assays to specific loss-of-function analyses of miR-18. Xdazl protein may also protect from miR-427 mediated degradation in the germ line if its role has been conserved with its predicted zebrafish ortholog miR-430. Dazl relieves miR-430-translation repression of tdrd7 mRNA by inducing its polyadenylation in zebrafish (Takeda et al. 2009). Caution is advised however in drawing too many parallels between Xenopus and zebrafish miR-functions (Koebernick et al. 2010). Exactly when miR-18 is transcribed in development is not clear, but in the case of miR-427 and other miRNAs in general, expression is dynamic during development, reaching significant levels only at the MBT and predominantly in the animal hemisphere (Lund et al. 2009; Harding et al. 2014). Thus a level of protection from miR-mediated degradation in the germ line is accomplished simply by keeping miR transcripts very low within the vegetal hemisphere before the MBT.

Another important component of germ plasm that plays a role in protection is a member of a subclass of Argonaute proteins called Piwi proteins. Piwi proteins interact with piRNAs, and both are very abundant as well as essential for germ cell development and function in mouse and zebrafish (Ketting 2011; Houwing et al. 2008). piRNAs are enriched in transposon-derived sequences and appear to silence transposons in the germ line by either cleaving transcripts or facilitating chromatin methylation of transposon sequences (Armisen et al. 2009; reviewed in Siomi et al. 2011). Xiwi, one of two Piwi proteins found in Xenopus oocytes, coimmunoprecipitated with nanos RNA in large RNP particles from egg extracts (Lau et al. 2009; Minshall et al. 2007). Because Xiwi1 was found to have putative nuclear localization and export signals, it has been proposed that it accompanies nanos RNA, along with Sm proteins, during nuclear transport of nuage into the ooplasm as germ plasm is formed (Bilinski et al. 2004; Lau et al. 2009). Clearly, much remains to be done before a mechanistic picture is completed of how germ plasm components specify and protect the germ line.

8.3.4 Summary

In summary, three critical activities appear to be required in PGCs to protect them from somatic differentiation: (1) translation of sequestered maternal germ line mRNAs, (2) translational repression of the endoderm determinant VegT, and perhaps other RNAs, and (3) transient genome-wide suppression of mRNA transcription at and before the MBT to ensure that somatic differentiation programs remain inactive when zygotic transcription is initiated in the rest of the embryo. Major outstanding questions remain: How are germ plasm maternal RNAs translationally activated and in what temporal order? What maternal transcription factors initiate the germ line gene program? What initiates different programs of migration in PGCs? What is the relationship between PGCs and adult ovarian germ stem cells? In this new age of deep-sequencing and proteomics, many RNAs and proteins will be discovered as germ plasm components. Putting these components into a viable gene network essential to specify the germ line remains the challenge. The hope is that understanding the mechanisms that suppress and reactivate the totipotent potential of germ cells will allow us to reprogram and program other stem cells for cell-based therapies.

8.4 Germ Cell Specification in Ascidians

Ascidians are marine invertebrate organisms that belong to phylum Chordata together with cephalochordates and vertebrates. They share many features of the ancestral Chordata, such as a notochord, dorsal hollow nerve tube and a post-anal tail. After fertilization, ascidian embryos undergo a typical form of mosaic development. Cell fate determination is regulated by maternal RNAs and proteins, which are deposited into specific regions of eggs and early embryos. Embryonic development gives rise to a tadpole larva with simple tissue organization. After settlement, the larva undergoes metamorphosis, the tail is absorbed, and after several days, the juvenile starts feeding (Fig. 8.8).

In both solitary and colonial ascidians, the germ line is segregated early in development, and the specification of primordial germ cells is dependent on maternally localized determinants. This process has been studied in greater detail in solitary ascidians, such as *Ciona intestinalis*, as their embryonic development is more suitable to observation and experimentation, but data from colonial species suggest that the mechanisms of germ cell specification are highly similar between both groups. However, in some ascidian species, the segregation of germ line and soma may not be absolute, as some observations suggest that germ cells can be regenerated later in development and even in adult animals.

In contrast to solitary ascidians, sexual reproduction and asexual reproduction occur simultaneously in colonial ascidians, a situation that requires regeneration of gonads in each asexual generation. To accomplish this, colonial species retain long-lived germ line progenitors that are specified during embryogenesis and persist throughout the life of the animal.

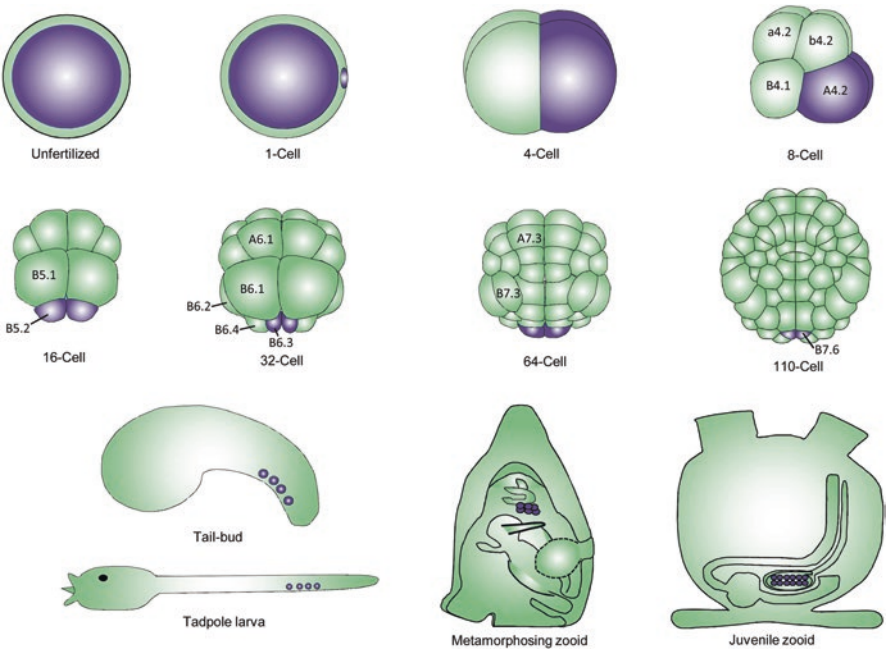

Fig. 8.8 Germ cell specification in Ascidians: distribution of *vasa*-expression during embryogenesis, metamorphosis and larval stages to juveniles. *Vasa*-mRNA is broadly distributed in unfertilized eggs, and accumulates at the posterior cortex of the fertilized one-cell stage embryo. During the 4 to 32-cell stages, it is incorporated into a pair of blastomeres located at the posterior pole. In 64- and 110-cell stage embryos, Vasa-protein is concentrated in the posterior-most cells. After gastrulation, four Vasa-protein-positive cells are formed by the late tailbud stage. In the tadpole larva, Vasa-positive cells in the endodermal strand are located in the posterior half of the tail. During metamorphosis, eight Vasa-positive cells align in the space between the intestine and stomach. In juvenile zooids, Vasa-positive cells increase in number and form a cluster next to the pyloric gland, and later give rise to the gonad rudiment

Here, we will outline mechanisms of germ cell formation in solitary and colonial ascidians, as well as the regeneration of germ cells in colonial species. We will also briefly discuss observations about de novo formation of germ cells during postembryonic development.

8.4.1 Embryonic Germ Line Specification in Solitary and Colonial Ascidians

In ascidians, germ cells originate from the part of the embryo that contains a specific cytoplasm, called the postplasm. The postplasm accumulates many maternal RNAs and proteins (Sardet et al. 2005), and contains a structure termed the centrosome attracting body (CAB), which shows morphological similarity to germinal granules. These granules are a hallmark of germ cells in a wide range of animal species. In the solitary ascidian *Ciona intestinalis*, RNA and protein of the *Ciona vasa homolog*

(*Civh*) are highly enriched in the postplasm (Fujimura and Takamura 2000; Takamura et al. 2002b). *Vasa* encodes an ATP-dependent RNA-helicase, and it is expressed by germ cells and primordial germ cells in most phyla studied to date (Mochizuki et al. 2001; Extavour and Akam 2003). *Vasa* is therefore a reliable marker for primordial germ cells in all animals.

Vasa-mRNA is distributed broadly in unfertilized eggs (Fig. 8.8), but starts to accumulate at the posterior cortex of the one-cell stage embryo (Fig. 8.8). It is then gradually segregated into the posterior-most blastomeres at the 4, 8, 16, 32, and 64 cell stages (Shirae-Kurabayashi et al. 2006). The postplasm-containing blastomeres at the 64 cell stage, termed the "B7.6 cells," undergo an asymmetric cell division during gastrulation, giving rise to two distinct daughter cells: B8.11 and B8.12. Most of the postplasmic components are distributed into the "B8.11" cells, which are never incorporated into the gonad. The "B8.12" cells accumulate CiVH-protein, which formes perinuclear granules (Fig. 8.8). This CiVH protein is produced by translation of inherited maternal *civh* RNA, which is released from the CAB prior to asymmetric division of the B7.6 cells. Although the postplasm in Ascidians is thought to act as the germ plasm, it is also highly enriched in several factors essential for somatic cell development. Most of the postplasmic RNA's and the CAB are inherited by the B8.11 cells, which become somatic cells. An ascidian-specific gene, pem-1 (posterior end-mark-1), was originally identified in *Ciona savignyi* as a postplasmic RNA (Satou 1999). In *Ciona intestinalis*, knockdown of Ci-pem-1 derepressed the transcription of zygotic genes in the germ line blastomeres, showing that in ascidians, Ci-Pem-1 functions to prevent somatic gene expression in the germ line. Ci-Pem-1 localizes to the nuclei of germ line blastomeres and forms a complex with two *Ciona intestinalis* homologs of the transcriptional co-repressor Groucho (Shirae-Kurabayashi et al. 2011).

The B8.12 cells then separate from the B.11 cells and divide to produce four CiVH-positive cells at the late tailbud stage (Shirae-Kurabayashi et al. 2006). A study investigating the location CiVH-positive cells from tailbud stage to juveniles in *Ciona* showed that the cells expressing Vasa-protein in middle tailbud embryos are located in the endodermal strand, where PGC will originate (Takamura et al. 2002b). At the larva stage, these Vasa-positive cells are located in the posterior half of the tail. During metamorphosis, these cells move into the trunk region and localize to the debris of the larval tail (Fig. 8.8). Eight CiVH-positive cells then align between the intestine and stomach (Fig. 8.8), and subsequently increase in number and form a mass next to the pyloric gland. These cells later get incorporated into the gonad rudiment, which contains numerous Vasa-positive cells (Fig. 8.8). The testis rudiment later separates from the gonad rudiment, the remainder of which differentiates into the ovary. In an interesting experiment, the authors observed that when the larval tail containing the CiVH-positive cells was surgically removed, the resulting juveniles did not contain any cells expressing Vasa-protein after metamorphosis, indicating that germ cells normally originate from the CiVH-expressing cells in the larval endodermal strand. However, even in such juveniles, normal germ cells did eventually appear at a later stage. Since a weak signal for *civh*-mRNA was detected in the anterior and middle parts of the endodermal strand at the tailbud stage in cells that do not express CiVH-protein, and the anterior part of the tail was not removed in the experiment, it is possible that cells in the endodermal strand have the potential to become germ cells.

Vasa localization during embryonic development is similar across solitary ascidians. *Vasa* mRNA is expressed in the posterior lineage and late the germ cells of the gonad in another solitary species, *Boltenia villosa* (Brown and Swalla 2007), similar to *Ciona intestinalis. Vasa* localized in two posterior cells at the eight-cell stage and later cleavage stages. A strong signal was detected in posterior cells of the cleaving embryo, which gave rise to B7.6 cells. Expression of *vasa* later became localized to two cells posterior to the endodermal strand of the tailbud embryo. These putative germ cells are most likely derived from the B8.12 cells, as reported for *Ciona* (Fig. 8.8).

Embryos in colonial species are brooded and lineage-tracing studies lag behind those in the more accessible free-spawning solitary species. Nevertheless, determinative development is a conserved characteristic in ascidians. As observed in solitary species, vasa-mRNA concentrates in granular structures of the fertilized egg cortex in the colonial ascidian *Botryllus schlosseri* (Brown et al. 2009). During the first cleavage, vasa-mRNA localized to the vegetal pole of the embryo. *Vasa* is then segregated in the cleavage furrow of the posterior-most B4.1 pair of cells of the eight-cell embryo and in the descendant B5.1 pair of cells of the 16-cell embryo. During gastrulation, vasa-mRNA was expressed in a pair of cells found posterior to the blastopore that are likely to correspond to the precursor germ line B7.6 pair, reminiscent of results in *Ciona* (Shirae-Kurabayashi et al. 2006). So far, only one study investigated the developmental stages of colonial ascidians later than gastrula. In *Botryllus primigenus*, both *vasa*-mRNA and protein were detected in embryos until the tailbud stage, but no signals were detected in the tail nor the trunk of tadpole larva (Kawamura et al. 2010). More studies are needed to investigate the fate of germ cells in tailbud stage embryos, tadpole larvae and during metamorphosis of colonial ascidians.

Taken together, all the available data strongly suggest that in both solitary and colonial ascidians the process of germ cell segregation during embryonic development is very similar, and that the number of germ line blastomeres and their descendants is constant during embryogenesis to the tailbud stage.

8.4.2 Gonad Regeneration During Asexual Reproduction in Colonial Ascidians

In colonial ascidians such as *Botryllus* and *Botrylloides*, asexual growth by budding is a continuously occurring process. Each new generation is interconnected to each other and the parent by a common vasculature, and germ cells are passed on between generations through this vascular network (Fig. 8.9a and b).

Vasa is expressed in small cells within the blood vessels, as well as in small, developing oocytes, maturing oocytes and testes both in *Botryllus schlosseri* and in another, related species, *Botrylloides violaceus* (Brown and Swalla 2007; Brown et al. 2009). Vasa-positive germ cell precursors are mobile, and can migrate to a niche in the developing new bodies (buds), then expand and differentiate into gametes (Sunanaga et al. 2006; Brown and Swalla 2007; Brown

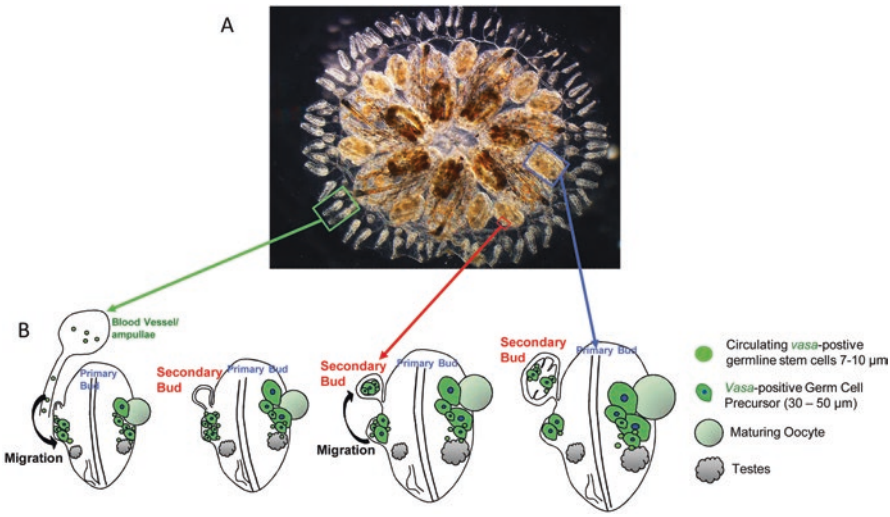

Fig. 8.9 Gonad regeneration during asexual reproduction in *Botryllus schlosseri*. (**a**) Ventral view of a colony of *Botryllus schlosseri* showing individual adult animals, each of which is connected to asexual propagating primary and secondary buds. Individual animals and buds are connected by a common extracorporeal vasculature, which ends in terminal projections known as ampullae. (**b**) During the asexual budding process, new secondary buds begin as a thickening of the peribranchial epithelium, which forms a pocket and eventually closes to form a double vesicle. Small, round germ line stem cells (GSC, 7–10 µm in diameter, *green*) expressing *Vasa* are present in the circulation. These Vasa-positive GSCs are also present in primary buds near developing gonads and larger Vasa-positive germ cell precursors (30–50 µm, *green*). When a secondary bud develops and closes to form a double vesicle, *Vasa*-positive germ cells (*green*) migrate into the secondary bud. As secondary bud development proceeds, more and larger germ cells are visible, indicating proliferation and differentiation. At later stages, *Vasa*-positive germ cells differentiate into testes and oocytes, as primary buds develop into the adult form

et al. 2009; Kawamura and Sunanaga 2011). Circulatory vasa-positive cells have been observed in all botryllid species examined to date (Sunanaga et al. 2006; Brown and Swalla 2007; Kawamura and Sunanaga 2010, 2011; Rosner et al. 2009). These cells are small, round, with large nuclei, and are present in the vasculature immediately following metamorphosis (Brown et al. 2009). Germ line precursors from juveniles contribute to gamete formation immediately upon transplantation into adults, demonstrating that these cells are germ line stem cells that were specified during embryonic development. Other evidence for the presence of mobile, long-lived germ line stem cells comes from naturally occurring vascular fusion between genetically distinct individuals. After fusion, germ line chimerism persists even after surgical separation of the fusion partners (Laird et al. 2005), indicating that long-lived, self-renewing germ line stem cells are actively transferred between the two colonies. These cells can be prospectively isolated based on expression of Aldehyde Dehydrogenase, express high levels of *vasa*, and can reconstitute the germ line upon transplantation (Laird et al. 2005).

8.4.3 De Novo Formation of Germ Cells

A handful of studies suggest that in some ascidians, germ cells can be regenerated at postembryonic stages of development. One example is *Ciona intestinalis*, where all cells expressing Vasa-protein were removed during embryogenesis. These cells are then formed de novo in juveniles post-metamorphosis (Takamura et al. 2002a). However, vasa-mRNA was detected in cells within a more anterior region of the tail that was not removed in the experiment, and it is possible that those cells might be the source of the germ cells at later stages.

In the colonial ascidian *Polyandrocarpa*, buds only develop organs after being separated from the parent. No *vasa*-mRNA was detected in buds that have separated from the parent, and *vasa*-positive cells appear only later in developing animals. These data suggest that in this particular species, germ cells might be formed de novo in each asexual cycle of reproduction (Sunanaga et al. 2007). More detailed studies are needed to test whether small, *vasa*-positive circulatory germ line stem cells are entering the bud before separation, or whether germ cells are indeed formed by epigenesis, and if so, by what mechanism.

When adult bodies and buds are removed from colonies of *Botryllus primigenus*, *Botrylloides violaceus* or *B. schlosseri*, new bodies can regenerate from the remaining vasculature in a process termed "vascular budding." One study in *Botryllus primigenus* suggested that vasa-positive cells are generated de novo during vascular budding, as *vasa*-mRNA expression was undetectable immediately after ablation of bodies and buds, but became detectable in 30 day specimens (Sunanaga et al. 2006). However, a later study in the same species showed that circulating *vasa*-positive cells are in fact entering the developing vascular buds (Kawamura and Sunanaga 2011). As described above, circulating vasa-positive cells are present in all botryllid species, and therefore, these cells are likely the source of the germ line in vascular buds.

Taken together, these studies suggest that de novo formation of germ cells may occur in some but not all ascidian species, and more detailed studies are needed to investigate this phenomenon.

8.5 The Enigma of PGC Specification in Urodeles

8.5.1 The Origin of PGCs in Urodele Amphibians

The origin of the germ line in urodele amphibians (salamanders) was a source of considerable controversy during the early twentieth century. By that time the endodermal origins of anuran (frog) primordial germ cells (PGCs) had been well established; however, conflicting reports placed the origins of urodele PGCs in either endoderm or mesoderm. To address this, Humphrey (1925) analyzed early development in a number of urodele species, and he identified PGCs in the dorsal-most aspect of the posterior lateral mesoderm adjacent to the mesonephric ducts (Humphrey 1925). He later showed that the PGCs and mesonephric ducts were eliminated following unilateral

extirpation of a strip of intermediate mesoderm from early tailbud stage embryos (Humphrey 1927, 1929). The absence of PGCs on the operated side of the embryo demonstrated that PGCs do not arise from a medial endodermal origin, as suggested by others (Bounoure 1925), and was thus consistent with their origination from within the mesodermal germ layer itself. Furthermore, these results provided the first conclusive evidence arguing against the possibility that PGCs arise de novo from differentiated somatic cells, which was also a prominent theory at the time. Nevertheless, because Humphrey's dissections were executed with embryos of a fairly advanced stage, it remained a possibility that PGCs migrate to the mesoderm from an earlier alternative location, such as the endoderm. This hypothesis was favored by workers who presumed that PGCs must arise from a common origin in urodeles and in anurans.

Nieuwkoop (1947) later extended investigation into the origins of urodelean PGCs using embryos from gastrula stages, at which time it is feasible to separate mesoderm precursors from endoderm experimentally (Nieuwkoop 1947). Focusing primarily on embryos from axolotls, he combined fate mapping with transplant and deletion studies to precisely map germ cell precursors within the ventral marginal zone of early gastrulae, where they reside adjacent to the presumptive blood cells. Specified progenitors of the germ line later pass over the lateral lips of the blastopore prior to taking residence in the dorsal lateral plate. Interestingly, presumptive germ cells are the last cells to internalize during gastrulation. They pass over the blastopore during mid to late gastrula stage (at about stage 11), which is after the somatic cell lineages are established. Moreover, removal of the germ cell domain during gastrula stages resulted in complete sterility in the majority of operated embryos. This finding ruled out the possibility that PGCs might originate within the endoderm and later migrate into the mesodermal layer, unequivocally establishing a mesodermal origin for the PGCs. Furthermore, these results provided confirmation that PGCs could not arise secondarily from differentiated somatic cells. However, in addition to establishing these fundamental principles of germ cell biology, Nieuwkoop also found that deletion of the caudal endoderm completely eliminated the germ line from developing embryos. He thus postulated, for the first time, that PGCs were formed in response to inducing signals. Yet, validation of this hypothesis would only come decades later (Fig. 8.10 for summary on PGC formation).

8.5.2 Induction of PGCs from "Totipotent" Cells

Weissman (1898) proposed the Germ Plasm Hypothesis in which the germ line is maintained across generations by maternally inherited germ cell determinants, and the subsequent discovery of material fitting this description in the eggs of species as diverse as frogs and fruit flies fostered the perception that germ plasm was a universal component of development in the animal kingdom (Blackler 1970; Mahowald and Hennen 1971). Germ plasm segregates PGCs from somatic cells in the earliest stages of development, thus it was conceivable that germ plasm, or an equivalent substance, segregates with a subpopulation of presumptive mesodermal cells in urodele embryos

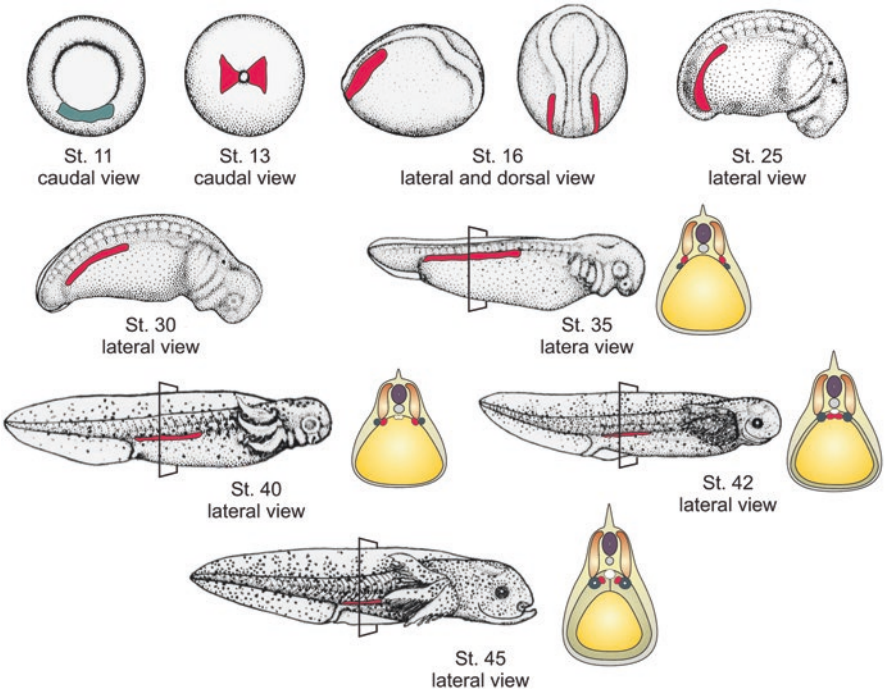

Fig. 8.10 Development of the germ line in axolotl embryos. The precursors of axolotl primordial germ cells develop from cells in the ventral lip of the blastopore (*blue*). They pass over the lateral lips of the blastopore during the final stages of gastrulation and reside in the presumptive intermediate mesoderm (*red*). Commitment to the germ line occurs at about stage 25. PGCs are then displaced medially by the expansion of the lateral plate. They develop in close association with the mesonephric ducts (*dark gray*), which come to occupy a more lateral position. By stage 45, the PGCs have reached the genital ridge, and they are positioned ventro-lateral to the dorsal aorta (*white*), and medial to the mesonephric ducts in the aorta–gonad–mesonephros (AGM) region of the embryo

to predetermine their fate as PGCs. Indeed, material with the properties of germinal granules, a major component of germ plasm, was found in the equatorial region of axolotl oocytes, suggesting such a possibility (Williams and Smith 1971).

However, an alternative hypothesis was that PGCs arise from common totipotent cells with the capacity to form germ cells or somatic cells. This was first suggested by transplantation experiments in the newt *Triturus* where the presumptive lateral mesoderm of gastrulae was replaced by primitive ectoderm of donors, without a noticeable reduction in the number of PGCs (Kotani 1957). Later, Kocher-Becker and Tiedemann (1971) demonstrated the induction of PGCs, along with mesodermal and endodermal structures, when explanted primitive ectoderm (animal cap) of embryos from *Triturus* was exposed to a "vegetalizing factor" isolated from extracts of chicken embryos. While the nature of this PGC inducing factor has never been determined, these studies laid the groundwork for subsequent studies to determine the mechanisms that underlie PGC induction.

Building on his seminal discovery of mesoderm induction (Nieuwkoop 1969), Nieuwkoop and colleagues began a series of studies that defined the basic parameters underlying the induction of urodele PGCs. First, they cultured the primitive ectoderm of early axolotl gastrulae with vegetal blastomeres from either the ventral or dorsal side of the embryo (Boterenbrood and Nieuwkoop 1973). When associated with ventral cells, the primitive ectoderm elicited a robust induction of PGCs, along with somatic ventral mesodermal derivatives, such as blood and mesonephric tissue. Dorsal vegetal blastomeres induced a very different response, resulting in the production of dorsal axial structures, notably including notochord. These studies represent the first demonstration that patterning of the mesodermal mantle in amphibians occurs in response to signals from the presumptive endoderm. Equally provocative, they prompted the hypothesis that PGCs are typical mesoderm derivatives, formed similar to somatic cells, blurring the fundamental distinction between germ cells and somatic cells that had been a fixture in the germ cell literature until that time. Nonetheless, these experiments could not exclude the possibility that PGCs are derived exclusively from cells in the animal cap that harbor germinal granules which might predispose them towards germ line development. Sutasurja and Nieuwkoop (1974) addressed this possibility. They dissected animal caps into discreet regions, and provided these with equivalent inducing stimuli. Their data showed that all regions within the animal moiety were capable of producing PGCs. Interestingly, from the data in this study the authors concluded that PGCs and somatic cells are derived from the same population of precursors, which in modern parlance is the functional definition for cells in the pluripotent ground state (Nichols and Smith 2009).

The hypothesis that PGCs might be derived from typical cells in the primitive ectoderm was difficult to reconcile with work from experimental models that contain germ plasm. However, Ikenishi and Nieuwkoop (1978) were unable to detect germ plasm in the PGCs of tailbud stage (stage 23) embryos. Germ–cell specific material, called nuage, which has some features of germinal granules, was first observed in PGCs in larval stage embryos (stage 40), as PGCs begin to approach the presumptive gonad. While Ikenishi and Nieuwkoop proposed that that their findings negate the likelihood of predetermined germ cells in urodeles, other workers considered the evidence insufficient to exclude a role for early acting germ cell determinants (Michael 1984; Smith et al. 1983). Therefore, the issue of whether or not urodele embryos contain germ plasm, or related material, was considered unresolved by the time the twentieth century came to an end. However, a related larger issue, which influenced contemporary interpretations of data up to that period, also remained unresolved: Could closely related species evolve divergent mechanisms to regulate cell fate specification in early development?

8.5.3 PGC Induction Is a Conserved Basal Trait of Vertebrates

The issue of whether urodeles and anurans employ shared, or divergent, modes of PGC specification led Johnson et al. (2001) to revisit germ line development in axolotls. They asked if axolotl oocytes contain germ plasm, and demonstrated that

RNA encoding the germ cell-specific protein dazl, which is a component of Xenopus germ plasm (Houston et al. 1998), is not similarly localized in axolotl oocytes. On this basis they concluded that urodele oocytes do not contain germ plasm, implying a fundamental difference in the development of anuran and urodele embryos. However, in contrast to previous work, these authors considered the disparate mechanisms for PGC specification within a modern phylogenetic context in which extant amphibians are known to have evolved from a common tetrapod ancestor. Because urodeles retain basal amphibian traits, relative to anurans, they proposed that PGC specification by induction, also known as epigenesis, is conserved in vertebrates. On the other hand, they postulated that germ plasm was an evolutionary innovation of frogs, and other organisms (Johnson et al. 2001). This hypothesis conflicted directly with the prevailing assumption that germ plasm, in some form or other, is conserved across the animal kingdom. In later work, Johnson and colleagues postulated that germ plasm has evolved by convergence throughout the animal kingdom in response to selective pressures (Johnson et al. 2003a, b, 2011). This controversial hypothesis enhances the concept that germ cells are not fundamentally different than somatic cells. Furthermore, it suggests a heretofore unappreciated ability of organisms to evolve novel mechanisms for the control of early development.

Using axolotl embryos as a model, Chatfield et al. (2014) defined the basal mechanism for PGC specification in vertebrates. By modulating gene expression in vivo, or in vitro (using the animal cap system), they showed that PGCs are induced by a combination of fibroblast growth factor (FGF) and bone morphogenetic protein (BMP) signaling. Moreover, it was possible to expand or eliminate the PGC pool by modulating FGF signaling levels, suggesting that presumptive somatic cells could be recruited to germ line development, or vice versa, in response to an appropriate signaling regime. This supports the hypothesis that PGCs are derived from pluripotent precursors. Accordingly, the cells that make up axolotl animal caps express conserved orthologs of the pluripotency genes oct4 and nanog (Bachvarova et al. 2004; Dixon et al. 2010), which are not found in Xenopus (Frankenberg and Renfree 2013; Hellsten et al. 2010). These transcription factors are required to establish the ground state of pluripotency in mammals, suggesting that the mechanisms which govern early vertebrate development are conserved between urodeles and mammals.

Chatfield et al. (2014) also showed that downstream of FGF, PGCs could be induced, along with related somatic cell types, by the mesodermal determinant Brachyury. In addition, they showed that pluripotent cells in the animal cap were directed to either a germ line or somatic fate by the competing effects of extracellular signaling. These data suggest that PGC specification in axolotls is a stochastic event, which does not involve the early acting germ line determinants found in other animal models. Further, these authors used a variety of methods to determine when cells undergo restriction to the germ lineage in axolotls. They showed that irreversible germ line commitment occurs during tailbud stages, which is days after the completion of gastrulation. Prior to this, PGCs can readily be converted to somatic cells. Up until lineage restriction, germ cell potential is maintained within a multipotent mesodermal domain by MAP kinase signaling. The mechanisms acting downstream of MAPK signaling are presently unknown; however, their abrogation eliminates the PGCs

without radically affecting the development of somatic cells. The paradigm established with the axolotl system is defined by the Last Cell Standing Hypothesis, which proposes that PGCs are derived from the last cells from the animal cap to engage in lineage commitment. This model for germ line development contrasts with that of experimental systems that contain germ plasm: In these organisms PGCs are the first cell lineage established, not the last. However, the Last Cell Standing Hypothesis is in accord with the late passage of PGCs over the blastopore, demonstrated independently by both Nieuwkoop (1947) and Smith (1964). Moreover, axolotl PGCs develop within a signaling niche that is conserved in large, non-rodent mammals, suggesting that the Last Cell Standing Model describes a conserved paradigm for vertebrate germ cell development (Johnson and Alberio 2015).

8.6 Germ Cell Development in Mammals

The first detailed accounts of PGC development in mammals were based on histological studies of early human and domestic animal embryos (Fuss 1913; Allen 1904). Although these reports showed that germ cells migrated through the gut mesentery before entering the primitive gonad, they provided details of where the cells originated (Fig. 8.11a). A few decades after these initial studies were published, Witschi described the presence of PGCs in 3.5-week-old human specimens in the posterior end of the embryo close to the base of the allantois (Witschi 1948). These histological observations were further validated when alkaline phosphatase activity was identified as a reliable method for identifying PGCs, and enabled researchers to track their origin back to the posterior primitive streak of E7 embryos (Chiquoine 1954; Ginsburg et al. 1990). Despite this progress the question of the mechanisms by which germ cell are specified in the mammalian embryo remained unexplained (Eddy et al. 1981). The intricate process of germ cell specification and epigenetic reprogramming of the mammalian germ line have been thoroughly studied in mice, and some progress has been made in other mammalian species. Here we present a summary of the current understanding of these processes and highlight some of the critical differences between species.

8.6.1 Germ Cell Specification Genes

Early lineage tracing experiments suggested that PGC did not originate from epiblast cells in E6.5 mouse embryos (Lawson and Hage 1994). Transplantation experiments also showed that when cells from the distal epiblast were introduced into the posterior proximal epiblast in E6.5 embryos they could contribute to the germ line, indicating that cells can be recruited to this lineage after the onset of gastrulation (Tam and Zhou 1996). However, in 2005, Ohinata et al. (2005) showed using a genetic labeling technique, that approximately six cells in E6.25 proximal epiblast

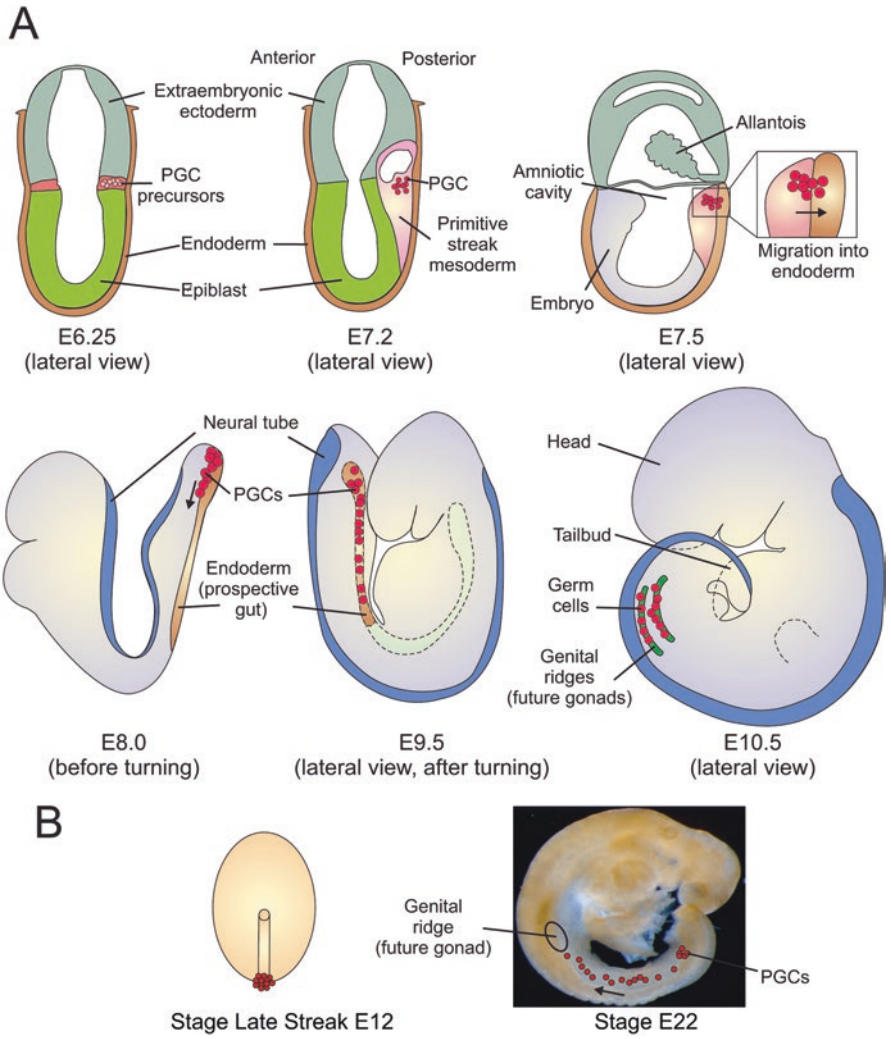

Fig. 8.11 Development of the germ line in mouse and pig. (**a**) Mouse PGC precursors are specified from proximal epiblast cells at E6.25. Between E7.25 and 7.5, the pool of PGCs expands forming a cluster of ~40 cells (*red dots*) located at the base of the allantois. By E8, PGCs start their migration towards the gonadal ridges reaching their destination by E9.5–E10. (**b**) Pig primordial germ cells (*red dots*) are first identified as a cluster in the posterior primitive streak at day 12 (E12). Later, they migrate as single cells through the gut mesentery reaching the gonad by day 22 (E22)

expressing *Blimp1* become lineage restricted PGC precursors (Ohinata et al. 2005). A subsequent analysis using multicolored chimeric embryos estimated that the number of germ cell founders is ~4 cells in the mouse (Ueno et al. 2009). *Blimp1* is thought to act as a transcriptional repressor of genes that promote somatic differentiation in PGCs (Kurimoto et al. 2008). More recently, it has been shown that *Hox*

a/b1, *T* and *Eomes* regulatory regions are bound by Blimp1 in PGCs at a time when mRNA transcripts of these targets are reduced (Magnusdottir et al. 2013). Since Blimp1 regulates gene expression in association with the histone modifier *Prmt5* (Ancelin et al. 2006), and *Prmt5* in *Drosophila* associates with Tudor proteins, a component of germ plasm proteins (Anne and Mechler 2005), it suggests that some of the Blimp1 functions in mice resemble that of molecules that safeguard unipotency of early PGCs in basal organisms (Hayashi et al. 2007; Ancelin et al. 2006).

Following lineage restriction after *Blimp1* activation, these precursors initiate germ cell specification by activating *Prdm14* and *Tcfap2c/AP2g*. *Prdm14* has been identified as the first germ cell gene to be expressed in mice. It plays critical roles in two key processes characterizing the germ cell program, namely, reacquisition of potential pluripotency and epigenetic reprogramming (Yamaji et al. 2008). *Prdm14-KO* mice lack mature germ cells due to a major loss of PGC during specification. *Prdm14-KO* PGCs also fail to upregulate *Sox2* and to erase H3K9me2, which is consistent with the role of Prdm14 in reestablishing pluripotent gene expression and initiation of epigenetic reprogramming of PGCs.

Tcfap2c/AP2g is also a critical early PGC gene, causing germ cell loss at E8 when mutated in mouse (Weber et al. 2010). Both Prdm14 and AP2g work together to activate PGC genes *nanos3* and *dnd1*, and cooperate with Blimp1 in repressing somatic genes and epigenetic modifiers. Indeed, this tripartite network of *Blimp1*, *Tcfap2c/AP2g*, and *Prdm14* seems sufficient for mouse PGC specification, as demonstrated by the induction of PGC-like cells without cytokines when they are overexpressed (Magnusdottir et al. 2013).

How well conserved is the role of Blimp1 during germ cell specification in other mammalian species has not yet been fully elucidated. However a recent report shows that BLIMP1 is activated in human PGC-like cells (hPGCL) after specification by SOX17. The results suggest that the role of BLIMP1 is to prevent the somatic differentiation potential of these SOX17 expressing cells (Irie et al. 2015). Furthermore, hPGCL cells arise from precursors expressing high levels of *BRACHYURY* and low levels of *SOX2*, resembling posterior primitive streak derived progenitors. These observations suggest that human germ cell precursors arise from a population of posterior primitive streak cells that activate BLIMP1 in response to paracrine inductive signals. Information on whether this mechanism also occurs in vivo is still lacking.

8.6.2 Germ Cell Induction Domains in Early Embryos

BMP4 and *BMP8b* homozygous mutants are deprived of PGCs, and heterozygous animals have reduced number of them (Lawson et al. 1999; Ying et al. 2000). By E5.5 both molecules are secreted by extraembryonic ectoderm cells, which are in close contact with proximal epiblast cells (Coucouvanis and Martin 1999). *BMP2* is produced a little later, from ~E6.5, by visceral endoderm cells, in an area next to the proximal epiblast (Coucouvanis and Martin 1999). The role of these molecules in germ cell induction was further demonstrated using isolated mouse epiblasts (Ying

and Zhao 2001; Ohinata et al. 2009), and it was shown that their signals are transduced via phosphorylated Smads (Chang and Matzuk 2001; Tremblay et al. 2001). Using an in vitro culture system it was also established that BMP4 is a more potent inducer than BMP2, and that BMP8b indirectly potentiates germ cell induction by inhibiting signals produced by the visceral endoderm (Ohinata et al. 2009). In other mammalian species less is known about the roles of BMPs in germ cell induction. However, it is worth noting the important anatomical difference between mice and other mammals at this stage of development. After the formation of the ICM, the mouse embryo undergoes cavitation of the epiblast which results in the formation of the egg cylinder. The mechanisms directing this process are mediated by BMP4 secreted by the extraembryonic ectoderm, which promotes cavitation via apoptosis (Coucouvanis and Martin 1999). In species in which cavitation is not apparent, like in human and other mammalian embryos, the polar trophoblast is lost during the formation of the epiblast (or embryonic disc). What is the relationship between the differences in embryological configurations for germ line development?

Previous studies showed that BMP4 can induce the activation of germ cell markers from (Fig. 8.11b) stem cells (Kee and Reijo Pera 2008; Alberio et al. 2010), and BMP2 and -4 have been shown to efficiently induce hPGCL cells from hESC (Irie et al. 2015). The role of BMPs as inductive signals in embryonic disc forming species is consistent with evidence from studies in rabit and pig embryos showing that BMPs are highly expressed in the primitive endoderm as well as in early mesodermal cells at this stage of early gastrulation (Valdez Magana et al. 2014; Hopf et al. 2011). Interestingly, in both species, BMP2 expression in the primitive endoderm precedes BMP4 expression, which is enriched in the mesodermal layer in early gastrulating embryos (Hopf et al. 2011; Valdez Magana et al. 2014). Furthermore, BMP expression in the posterior streak region is mediated via phosphorylated Smad1/5/8 (Valdez Magana et al. 2014), similar to what is known in mice. Thus, these observations suggest that the activation of BMP4 in mice extraembryonic ectoderm had consequences not only for the formation of the egg cylinder, but it may also have resulted in the precocious activation of germ line specification genes from proximal epiblast cells.

8.6.3 Pluripotent Gene Expression During PGC Specification

Intriguingly, during germ cell specification pluripotent gene expression is restored following the reactivation of *Nanog* and *Sox2* in *Oct-4* expressing PGC precursors. Expression of *Nanog*, *Oct-4* and *Sox2* is essential for PGC development, as shown by the loss of PGCs as a result of reduced proliferation (*Sox2* mutants) and death by apoptosis (*Nanog* and *Oct-4* mutants) after conditional repression of these genes (Campolo et al. 2013; Kehler et al. 2004; Chambers et al. 2007). It is not clear however, what are the specific roles of these factors during germ cell development, but it is thought that their expression confers latent pluripotency to the germ line. This latent pluripotency was demonstrated by the derivation of embryonic germ cells from PGCs (Labosky et al. 1994; Matsui et al. 1992). Embryonic germ cells can

also be derived as naïve pluripotent cells in the presence of a specific MAPK inhibitor, a GSK3β inhibitor and LIF (also known as 2i +LIF) conditions, resulting in cells with similar signaling requirements, imprinting status and DNA methylation profiles as naïve mouse ESCs (Leitch et al. 2010, 2013). The link between naïve embryonic germ cells and PGCs suggest that these cell types have a common molecular regulation. In PGCs, this regulatory network helps protect these cells from somatic inducing signals during epigenetic reprogramming (Leitch and Smith 2013).

In the pig, however, both OCT-4 and NANOG are maintained throughout the period of germ cell specification, migration and gonadal colonization (Wolf et al. 2011; Hyldig et al. 2011a), suggesting that during the specification of the germ line these genes play different roles as in mice. No data is currently available for other pluripotency genes, such as SOX2, although in humans it was shown that SOX2 is absent from gonadal PGCs (Perrett et al. 2008). This suggests that the absence of an intact pluripotency network in PGCs of non-rodent mammals may be an underlying reason to explain the impossibility to establish embryonic germ cells using similar culture conditions for mouse embryonic cells. In fact the embryonic germ cell lines reported in other animals fail to form teratomas or to contribute to the germ line in chimeric animals (Alberio and Perez 2012; Turnpenny et al. 2006; Petkov et al. 2011; Shamblott et al. 1998), suggesting that they are captured in a different state of pluripotency.

8.6.4 Epigenetic Reprogramming of the Mammalian Germ Line

Shortly after specification mouse PGC embark on their migration through the hindgut and the colonization of the genital ridges. An important feature of this period is the reprogramming of epigenetic marks in these precursors. Genome wide changes in histone marks and DNA demethylation are thought to be an essential process of PGC development that enables the elimination of epimutations acquired by the previous generation. DNA methylation was reported to decline from E8 as measured by 5-methylcytosine (5-mC) immunostaining and by whole genome sequencing (Seki et al. 2005; Seisenberger et al. 2012). The mechanism of DNA demethylation at this stage seems to be driven primarily by the reduction in DNA methyltransferase activity in PGCs compared to their somatic neighbors, as demonstrated by the reduction in Dnmt3a/b expression. At this stage, 5-hydroxymethylcytosine (5-hmC) staining is not increased, which indicates that conversion into 5-hmC intermediates is not involved in methylation reprogramming in pre-migratory mouse PGCs (Hackett et al. 2013; Yamaguchi et al. 2013). Indeed, it is from E9.5–10.5 that the peak of tet1/2 (Ten–eleven translocation methylcytosine dioxygenase 1 and 2) enzymes is detected in mouse PGCs and this is concurrent with an increase in 5-hmC levels (Hackett et al. 2013; Yamaguchi et al. 2013). In the pig, changes in DNA methylation were investigated in early migratory PGCs from day 15 (equivalent to day E8.5 in the mouse) until day 28. The study showed that genome-wide levels of 5-mC are lower in PGCs from day 15 and remain low until day 21,

increasing again by day 28 (Hyldig et al. 2011b). Another study investigating imprinted gene methylation reprogramming in pig PGCs showed that this process is asynchronous and takes place over several days (Hyldig et al. 2011a). For instance, *IGF2R* (Insulin Growth Factor Receptor 2) is demethylated in early gonadal PGCs (by day 22), whereas the *IGF2-H19* cluster is demethylated later at around day 25 (Hyldig et al. 2011a). In the case of the imprinted gene *PEG10*, complete demethylation was determined by day 27 (Wen et al. 2010). Furthermore, the methylation status of repetitive sequences was also shown to be erased in PGCs between days 28 and 31 (Petkov et al. 2009; Hyldig et al. 2011a), suggesting that at this stage pig PGCs have the lowest levels of methylation. Similar asynchrony in imprinted gene demethylation has been shown in the human germ line (Gkountela et al. 2013).

Concomitant with changes in DNA methylation, reduction in histone H3K9me2 was shown in mouse PGCs from around E7.5 to E8.75, followed by an increase in H3K27me3 from E9.25 until E13.5 (Seki et al. 2005). These changes occur during a very narrow window of time in mice. H3K9me2 and H3K27me3 reprogramming have also been described in pig PGCs; however, these changes are detected over a protracted period between days 15 and 21, consistent with the longer developmental period of pigs (Hyldig et al. 2011a). By E13.5 mouse PGCs have high levels of repressive chromatin marks H3K9me3 and H3K27me3, whereas the permissive marks H3K9ac and H2A.Z are very low (Seki et al. 2005). This is in contrast to findings in human gonadal germ cells that show high levels of the active chromatin marks H3K9ac and H2A.Z and low levels of repressive chromatin marks (Gkountela et al. 2013; Almstrup et al. 2010). In contrast, HP1 (Heterochromatin Protein 1) levels are low in gonadal PGCs of both species (Seki et al. 2005; Bartkova et al. 2011). The differences in histone profiles need to be studied in other animal models to determine whether they may reflect differences in reproductive features between species.

8.6.5 Conclusions

The brief overview presented here highlights the great progress made over the past century and how the new knowledge has contributed to a better understanding of how germ cells form. Importantly, a better knowledge of the developmental characteristics of the germ line of other mammals may enable us to model human disease in relevant species.

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Chapter 9
Cell Cycle Remodeling and Zygotic Gene Activation at the Midblastula Transition

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Abstract Following fertilization, vertebrate embryos delay large-scale activation of the zygotic genome from several hours in fish and amphibians to several days in mammals. Externally developing embryos also undergo synchronous and extraordinarily rapid cell divisions that are accelerated by promiscuous licensing of DNA replication origins, absence of gap phases and cell cycle checkpoints, and preloading of the egg with maternal RNAs and proteins needed to drive early development. After a species-specific number of cell divisions, the cell cycle slows and becomes asynchronous, gap phases appear, checkpoint functions are acquired, and large-scale zygotic gene

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activation begins. These events, along with clearance of maternal RNAs and proteins, define the maternal to zygotic transition and are coordinated at a developmental milestone termed the midblastula transition (MBT). Despite the relative quiescence of the zygotic genome in vertebrate embryos, genes required for clearance of maternal RNAs and for the initial steps in mesoderm induction are robustly transcribed before MBT. The coordination and timing of the MBT depends on a mechanism that senses the ratio of nuclear to cytoplasmic content as well as mechanisms that are independent of the nuclear–cytoplasm ratio. Changes in chromatin architecture anticipate zygotic gene activation, and maternal transcription factors identified as regulators of pluripotency play critical roles in kick-starting the transition from the proliferative, pluripotent state of the early embryo to the more lineage-committed phase of development after the MBT. This chapter describes the regulation of the cell cycle and the activation of zygotic gene expression before and after the MBT in vertebrate embryos.

Keywords Midblastula transition • Maternal zygotic transition • Zygotic transcription • Cell cycle checkpoint • Embryo • DNA damage • Nuclear cytoplasmic ratio • Cell cycle • Pluripotency

9.1 Introduction

Many metazoan embryos initiate development with a series of rapid, synchronous divisions that lack G1 and G2 phases, leading to reduction in cell size with each subsequent division (Bachvarova et al. 1966; Graham and Morgan 1966; Newport and Kirschner 1982a; Tadros and Lipshitz 2009; Gerhart 1980; Davidson 1986; Baroux et al. 2008). Rapid cleavage divisions are typically observed in embryos of vertebrates with larger eggs, such as amphibians, fish, birds, and reptiles. Although mammalian embryos undergo slower, asynchronous cell cycles from the outset, both mammalian and nonmammalian vertebrate embryos delay the onset of zygotic transcription and depend initially on maternal mRNAs and proteins to direct the earliest stages of development.

Rapid cell divisions and global suppression of zygotic transcription during cleavage stages require unique adaptations for the control of DNA replication, mitosis, and early gene expression. During the transition from maternal to zygotic control of development, maternal mRNAs are degraded, the cell cycle lengthens, cell divisions become asynchronous, cells become motile, and large-scale zygotic transcription begins. In amphibians and fish, as well as invertebrates such as *Drosophila*, the appearance of gap phases (G1 and G2) in the cell cycle and the onset of large-scale zygotic transcription occur predictably after a species-specific number of cell/nuclear divisions, termed the midblastula transition (MBT). While not all organisms coordinate the cell cycle changes with the onset of zygotic transcription (mammalian cell cycles are initially slow, whereas sea urchins undergo rapid cleavages but initiate zygotic transcription at fertilization), there are nevertheless conserved features in the regulation of cell cycle and transcription that are reviewed in this chapter.

The terms MBT and MZT (maternal to zygotic transition) have been used variously. We follow the clear definitions in Tadros and Lipshitz (2009) wherein MZT refers to the gradual transition from maternal to zygotic control and can span a broad developmental window in the pregastrula embryo. The MBT, in contrast, refers to a discrete milestone occurring in many species after a species-specific number of cell divisions and is marked by the coordinated acquisition of cell cycle checkpoints, cell cycle asynchrony, and the onset of large-scale zygotic transcription (Tadros and Lipshitz 2009; Gerhart 1980; Signoret and Lefresne 1971; Newport and Kirschner 1982a, b; Audic et al. 1997; Langley et al. 2014). We use the qualified term "large-scale zygotic transcription" because a subset of zygotic genes is transcribed before the MBT in *Xenopus*, zebrafish, and *Drosophila* (Tadros and Lipshitz 2009; Baroux et al. 2008; Nakakura et al. 1987; Yang et al. 2002; Skirkanich et al. 2011; Lindeman et al. 2011; Liang et al. 2008; Harrison et al. 2010; Edgar and Schubiger 1986; Yasuda et al. 1991; Heyn et al. 2014; Collart et al. 2014; Tan et al. 2013; Blythe et al. 2010; Lee et al. 2014), and this early wave of transcription is essential for development at the late blastula stage in *Xenopus* and *Drosophila* (Skirkanich et al. 2011; Liang et al. 2008; Harrison et al. 2010). In this chapter, we focus on the regulation of the cell cycle and zygotic transcription in vertebrate embryos and briefly touch on information from invertebrate species where it directly informs our understanding of vertebrate mechanisms. The MBT in nonvertebrate species has been discussed in more detail in several informative reviews (Yasuda and Schubiger 1992; Tadros and Lipshitz 2009; Baroux et al. 2008; Blythe and Wieschaus 2015a; Lee et al. 2014; Farrell and O'Farrell 2014). Degradation of maternal mRNAs is addressed in detail elsewhere in this volume (see Chapter 10).

9.2 Embryonic Cleavage Divisions

9.2.1 Unique Features of Embryonic Cleavage Cycles

Most actively proliferating cells progress through four distinct phases: the first gap phase (G1), DNA replication (S-phase), a second gap phase (G2), and mitosis (M). Newly fertilized embryos from many vertebrate and invertebrate species, including nonmammalian vertebrates such as fish and frogs, have highly specialized cell cycles that differ significantly from cells of later development and adult organisms (Oppenheimer 1936; Graham and Morgan 1966). These embryonic cell cycles, known as cleavage divisions, are extremely short and lack the gap phases typical of growing cells. Without cell growth, these cleavage cycles are reductive divisions that progressively subdivide a constant volume of cytoplasm from a single large cell into many smaller cells, increasing the nuclear to cytoplasmic (N:C) ratio with each round of DNA replication. Rapid cleavage cycles, which establish a large cell population necessary for gastrulation, continue until the embryo undergoes the midblastula transition (MBT).

Table 9.1 Zygotic gene activation and MBT in vertebrate embryos

Organism	Duration of cleavage divisions[a]	# divisions to earliest ZGA[b]	# divisions to MBT[c]	Time to large-scale ZGA
Xenopus	20–25 min	6	12	6–7 h
Zebrafish	15 min	6	10	3.5 h
Medaka	35 min	6	11–12	7–8 h
Mouse	<12 h	0–1	–	24 h
Human	<12 h	2	–	24–48 h
Chick[d]	–	6–7	–	–

[a]Not including the first cell division, which is longer than subsequent cleavage divisions
[b]ZGA: zygotic gene activation
[c]MBT: midblastula transition, a discrete transition associated with the co-appearance of asynchronous divisions, cell cycle checkpoints, and large-scale zygotic gene activation
[d]Chick divisions are asynchronous after the fourth division (Lee et al. 2013a)

Many metazoans, including nonmammalian vertebrates, undergo a cleavage stage, though cell cycle timing varies (Table 9.1). In zebrafish embryos, the first cell division is initiated approximately 45 min after fertilization, followed by nine cell cycles, each lasting ~15 min. After ten cleavage divisions, embryos initiate the MBT and cell cycle remodeling commences (Kane and Kimmel 1993). Amphibian embryonic cycles have similar dynamics though cleavage cycles are longer. In *Xenopus laevis*, the first cleavage occurs ~90 min after fertilization and is followed by 11 additional ~25 min cleavage cycles before the MBT is triggered (Newport and Kirschner 1982a; Wu and Gerhart 1980). In contrast, mammals delay first cleavage, with mice undergoing the first cell division 12 h after fertilization. Mammalian embryos then enter a period of slower, asynchronous cell divisions to reach the morula stage and do not typically undergo a phase analogous to the rapid cleavage divisions seen in nonmammalian vertebrates.

9.2.2 Molecular Basis of Rapid Cleavages

Cleavage cycles employ many of the same regulators found in somatic cell cycles (for an in-depth description of cell cycle regulation in somatic cells and the early embryo, please see Chapter 3). However, early embryos have adapted their functions significantly in order to drive rapid cell proliferation, particularly the regulation of Cyclin-dependent kinase (Cdk) activity. The following section describes the specialized Cdk regulation in cleavage cycles, including cyclin availability, synthesis and proteolysis, and Cdk phosphorylation.

Cdks are a family of highly conserved serine–threonine kinases that regulate cell cycle transitions by targeting hundreds of substrates to promote cell cycle progression (Ubersax et al. 2003). While Cdk protein levels remain relatively constant for the duration of the cell cycle, Cdk activity is highly oscillatory and relies on several independent mechanisms to ensure stringent control. Cdk activity depends on both

association with cyclin proteins and phosphorylation state. Furthermore, subcellular localization and degradation of cyclin and cyclin/Cdk complexes adds an additional level of regulation.

Many cyclins and Cdks have been identified in somatic cells. Cyclin A/Cdk2, cyclin D/Cdk4, cyclin D/Cdk6, and cyclin E/Cdk2 regulate cell cycle progression and replication during G1 and S phases. Cyclin B/Cdk1, on the other hand, is important for progression from G2 to M (Evans et al. 1983; Murray 2004). However, embryonic cleavage cycles are regulated by only three cyclins, A, B, and E, and two Cdks, Cdk1 and Cdk2 (Hartley et al. 1996). Cdk2 binds to cyclins A and E to mediate DNA replication and centrosome duplication while Cdk1 binds to cyclins A and B to drive mitotic progression (Murray and Kirschner 1989; Rempel et al. 1995; Strausfeld et al. 1996).

9.2.2.1 Phospho-regulation of Cdk1

During somatic cell cycles, Cdk1 activity is regulated by phosphorylation on key residues. Cyclin binding requires phosphorylation of a threonine adjacent to the active site (Ducommun et al. 1991). Additionally, the Wee1 and Myt1 kinases inhibit Cdk1 and Cdk2 by phosphorylating threonine 14 and tyrosine 15. Cyclin-bound Cdk remains in an inactive state until mitosis, when these inhibitory phosphorylations are removed by Cdc25 phosphatases, which triggers mitotic entry (Atherton-Fessler et al. 1994; Krek and Nigg 1991).

In *Xenopus* embryos, Wee1 kinase phosphorylates Tyr15 on Cdk1 and Cdk2 in each pre-MBT cell cycle (Kim et al. 1999; Murakami and Vande Woude 1998). However, this inhibitory phosphorylation occurs at relatively low levels (Kim et al. 1999). Furthermore, although Cdc25A protein is not detected in *Xenopus* oocytes, maternally deposited cdc25 mRNA is translated upon fertilization, and Cdc25 protein steadily increases during the cleavage stages (Kim et al. 1999; Pomerening et al. 2003; Sha et al. 2003; Bouldin and Kimelman 2014; Yang and Ferrell 2013). The low level of Cdk1 inhibitory phosphorylation in cleavage-stage embryos keeps Cdk1 in a "primed" state, ready for activation upon cyclin binding.

9.2.2.2 Regulation of Mitotic Cyclin Protein Levels

Unlike somatic cell cycles, phospho-regulation of Cdk1 activity plays a minor role during early embryogenesis. Instead, Cdk in cleavage-stage vertebrate embryos is predominately regulated by cyclin protein synthesis and degradation. In *Xenopus*, protein levels of cyclin A and B oscillate once per cell cycle, with a nearly identical pattern of expression. Cdk1 activity closely parallels cyclin expression and also oscillates with each cell cycle (Hartley et al. 1996). In somatic cell cycles, cyclin protein expression is regulated by cell cycle phase-specific transcription (Pines 2011). In contrast, cyclin transcripts are preloaded maternally in embryos during

oogenesis, and cyclin protein accumulation is posttranscriptionally regulated. Cell cycle phase-specific translation of cyclins relies mainly on the polyadenylation of mRNAs, which changes in a cell cycle-dependent manner (Groisman et al. 2002).

As in somatic cells, mitotic exit during the cleavage divisions is regulated by cyclin B degradation, mediated by the highly conserved E3 ubiquitin ligase Anaphase Promoting Complex/Cyclosome (APC/C) (Skaar and Pagano 2009). In *Xenopus* embryos, XErp1/Emi2, a homolog of early mitotic inhibitor 1 (Emi1) (Tung et al. 2005), inhibits APC/C activity during mitosis. However, when Cdk1 becomes maximally activated, it antagonizes XErp1/Emi2 function leading to APC/C activation (Tischer et al. 2012). Subsequently, activated APC/C polyubiquinates cyclin B, tagging it for proteasomal degradation. As cyclin B levels fall, Cdk1 activity diminishes and cells exit mitosis (King et al. 1996).

Cdk1 also regulates cyclin B protein expression: inhibiting Cdk1 leads to increased cyclin B protein levels while prematurely activating Cdk1 decreases cyclin B accumulation. These results demonstrate that Cdk1 participates in a negative-feedback loop that attenuates the production of cyclins before mitosis. Limiting cyclin production increases the efficiency and sensitivity of the Cdk1-APC/C negative feedback loop by decreasing the burden of cyclin B degradation at anaphase (Kang and Pomerening 2012). These negative feedback loops between Cdk1, APC/C activation, and cyclin degradation support the rapid cyclin oscillations observed in cleavage-stage embryos.

9.2.2.3 Regulation of Cyclin E/Cdk2

In somatic cells, cyclin E/Cdk2 activity mediates the transition from G1 to S (Elledge et al. 1992). Similarly, cyclin E/Cdk2 regulates the progression of S-phase in cleavage-stage embryos. Inhibition of CyclinE/Cdk2 activity moderately increases cell cycle lengths in pre-MBT *Xenopus* embryos, showing that the activity of Cdk2 also contributes to rapid cell cycle progression (Hartley et al. 1996, 1997). Though cyclins A and B protein levels oscillate during the cleavage cycles, cyclin E protein levels steadily increase following fertilization (Hartley et al. 1996). Despite this, cyclin E/Cdk2 activity oscillates twice per cleavage cycle independently of protein synthesis. While more studies are required to elucidate the regulation of Cdk2 oscillations in cleavage-stage embryos, pre-MBT Cdk2 activity is likely regulated by phosphorylation state (Ciliberto et al. 2003). Indeed, Cdk2 activity is regulated by inhibitory phosphorylation by the Wee1 kinase in *Xenopus* egg extracts and embryos (D'Angiolella et al. 2001; Wroble et al. 2007).

9.2.2.4 Influence of Replication on Cleavage Cycles

Although accumulation and degradation of cyclins is certainly important for mitotic entry, RNAi knockdown of two *Drosophila* mitotic cyclins and reduction of gene dosage for the third cyclin did not prolong interphase but rather led to a

partial activation of mitotic events. These data suggest that cyclin accumulation alone is not enough to mediate all aspects of rapid cell cycle progression (McCleland et al. 2009a).

A second mechanism for maintaining short cell cycles could rely on DNA replication itself. Replication occupies the majority of interphase during cleavage divisions and proceeds quickly due to the close proximity of origins of replication (Harland and Laskey 1980; Spradling 1999). Inhibiting replication in syncytial embryos by injecting Geminin, which blocks the licensing of origins (McGarry and Kirschner 1998; Quinn et al. 2001), abolished S-phase and led to premature mitotic entry, demonstrating that replication defines interphase length in cleavage-stage embryos (McCleland et al. 2009b). This idea was corroborated in *Xenopus*, where replication factors were recently identified that can directly modify cleavage cycle lengths. Highly expressed during the cleavage stages, Cut5, Treslin, Drf5, and RecQ4 become limiting at the MBT, coinciding with cell cycle elongation. Importantly, overexpression of these factors abolished cell cycle lengthening at the MBT (Collart et al. 2013).

In conclusion, pre-MBT cells are preloaded with many of the same cell cycle regulators as seen in most somatic cells. However, it is their specialized regulation that leads to rapid cell proliferation.

9.2.3 Cell Cycle Checkpoints in Early Embryogenesis

Cell cycle checkpoints are present in almost all nonpathologic somatic cells to maintain genome integrity. The DNA damage checkpoint induces cell cycle arrest during interphase in response to DNA damage or stalled replication. The spindle assembly checkpoint (SAC) causes metaphase arrest in response to kinetochores that are not attached to microtubules during mitosis. However, cleavage-stage embryos forgo checkpoint function in their commitment to rapid cell proliferation. Little is known about how checkpoints are suppressed during cleavage stages or how they are acquired at the MBT. The following section reviews our current knowledge of SAC and DNA damage checkpoint signaling prior to the MBT.

9.2.3.1 The DNA Damage Checkpoint in Cleavage Cycles

In somatic cells, DNA damage activates two phosphoinositide 3-kinase-related protein kinases (PIKKs): ataxia-telangiectasia mutated (ATM) and ATM and RAD3-related (ATR). ATM and ATR are similar in structure and share many of the same substrates, but are activated by distinct triggers. ATM is activated by DNA double-strand breaks (DSBs) and ATR is activated by single-strand DNA (ssDNA) or ssDNA–dsDNA junctions. One of the earliest consequences of DNA damage is phosphorylation of Serine 139 on the histone variant H2AX (γH2AX) by ATM and ATR. The formation of γH2AX foci surrounding the damage site creates a docking site that recruits

DNA damage response proteins, promoting DNA repair and checkpoint signal amplification (Sirbu and Cortez 2013). γH2AX is an important read-out for DNA damage checkpoint initiation and the successful sensing of DNA damage (Dickey et al. 2009). ATM and ATR also activate the serine–threonine kinases Chk1 and Chk2, which play a central role in facilitating cell cycle arrest by phosphorylating multiple substrates to ultimately inhibit the activity of Cdks (Bartek and Lukas 2003). For example, Chk1 and Chk2 phosphorylate Cdc25 phosphatases, targeting them for degradation. Chk1 can also activate the Wee1 kinase via phosphorylation (Patil et al. 2013).

Post-MBT embryos have a robust DNA damage response and induce cell cycle arrest efficiently after DNA damage (Hensey and Gautier 1997; Maller et al. 2001). However, when zebrafish or *Xenopus* pre-MBT embryos are treated with ionizing radiation, cleavage cycles continue without arrest or cell cycle delay (Hensey and Gautier 1997; Zhang et al. 2014). Furthermore, DNA polymerase inhibitors or mutations that disrupt replication cause replication stalling and trigger S-phase arrest in somatic cells and post-MBT embryos, but not in pre-MBT *Xenopus*, zebrafish, or *Drosophila* embryos (Dasso and Newport 1990; Freeman and Glover 1987; Freeman et al. 1986; Kimelman et al. 1987; Shamanski and Orr-Weaver 1991; Ikegami et al. 1997).

Rapid DNA repair to explain the lack of cell cycle arrest after damage seems implausible, as irradiated embryos have high levels of DNA fragmentation (Anderson et al. 1997; Finkielstein et al. 2001; Hensey and Gautier 1997). Instead, checkpoint signaling is defective. In zebrafish, the DNA damage checkpoint is properly initiated by ionizing radiation, as irradiated pre-MBT embryos can phosphorylate histone H2AX and activate the effector kinase Chk2. Chk1 is not activated, however, leaving cleavage-stage embryos unable to arrest the cell cycle after DNA damage (Zhang et al. 2014).

DNA damage sustained during the cleavage stages results in embryonic lethality. Without checkpoints to resolve DNA lesions prior to the MBT, damaged DNA accumulates to irreparable levels by the MBT. When the checkpoint program finally becomes functional at the MBT, apoptosis is the only course of action; pre-MBT *Xenopus* embryos treated with ionizing radiation accumulate dense, small nuclei that are typical of apoptosis beginning at the onset of the MBT (Anderson et al. 1997). Further, TdT-mediated dUTP digoxigenin nick end labeling (TUNEL) detected apoptosis after the MBT, but not before (Anderson et al. 1997; Hensey and Gautier 1997; Stack and Newport 1997; Sible et al. 1997). Hensey and Gautier, for example, reported that apoptosis is first detectable at the gastrula stage (Hensey and Gautier 1997).

9.2.3.2 The SAC in Cleavage Cycles

In most cells, the SAC delays anaphase onset and mitotic exit until all kinetochores are attached to microtubules, in order to prevent chromosome missegregation. Each unattached kinetochore recruits SAC proteins to form the mitotic checkpoint complex (MCC), which prevents APC/C activation by targeting its co-activator, Cdc20. SAC proteins are removed from each kinetochore as it binds microtubules. After all kinetochores are attached, MCC disassembly frees Cdc20 to activate the APC/C, which polyubiquinates cyclin B and securin, leading to

their destruction, which is required for anaphase onset and mitotic exit (Amon et al. 1994; Lara-Gonzalez et al. 2012).

Despite robust function after the cleavage stage, the SAC is not active in pre-MBT embryos. Nuclei in *Xenopus* cleavage-stage embryos or egg extracts treated with microtubule poisons have a dramatically different morphology than their post-MBT counterparts, forming irregularly shaped, fragmented micronuclei thought to arise from inappropriate anaphase onset (Clute and Masui 1992; Newport and Kirschner 1984). Furthermore, time spent in mitosis does not change in pre-MBT embryos after microtubule depolymerization (Clute and Masui 1992; Ikegami et al. 1997; Zhang et al. 2015).

9.2.4 Cell Cycle Remodeling at the MBT

Cell cycle remodeling, in which cells elongate their cell cycles, add gap phases, and gain functional cell cycle checkpoints, is a hallmark of the MBT. This section reviews our current understanding of cell cycle elongation and checkpoint acquisition at the MBT.

9.2.4.1 Timing the Onset of Cell Cycle Lengthening

Cell cycle remodeling occurs after a fixed number of cleavages that is characteristic of the species. This observation led to the hypothesis that cell cycle elongation at the MBT is triggered by a mechanism that can measure the number of cell divisions or elapsed time after fertilization. However, a series of elegant experiments (Newport and Kirschner 1982a, b) demonstrated that the onset of cell cycle lengthening and asynchrony associated with the MBT is determined by a threshold N:C ratio (Fig. 9.1). Using a method adapted from Spemann (Sander and Faessler 2001), a strand of hair tied around the embryo partially constricted it at the single-cell stage to trap the nucleus on one side of the embryo. This manipulation effectively halved the cytoplasmic volume carrying the nucleus. The section with the nucleus cleaved 11 times before cell cycles became asynchronous. However, after two divisions, a daughter nucleus migrated through the narrow channel of the constriction to the side that originally had no nucleus. This side of the embryo now underwent 11 more cleavage cycles before becoming asynchronous, even though the nucleus had undergone two mitoses prior to migration (Newport and Kirschner 1982a). Similarly, a fourfold reduction in the cytoplasm of eggs of the Japanese newt accelerated the onset of mitotic asynchrony by two divisions (Kobayakawa and Kubota 1981).

Further experiments showed that the end of the cleavage stage is not determined by a mechanism that counts cell divisions. When cell division was blocked with cytochalasin B or by gently centrifuging fertilized eggs, the embryos continued to synthesize DNA at an exponential rate for 6 h and then abruptly slowed DNA synthesis, similar to control embryos at the MBT. Furthermore, increasing DNA content in cleavage-stage embryos, for example in polyspermic eggs or eggs injected

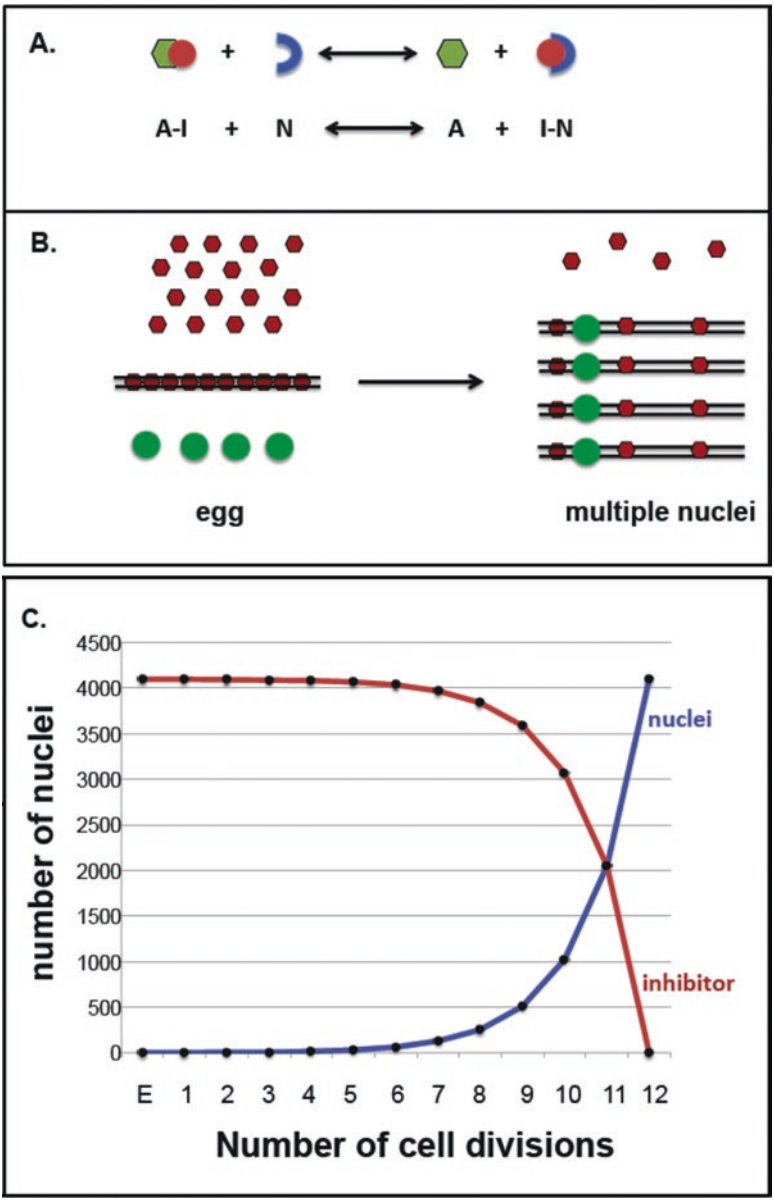

Fig. 9.1 N:C Ratio Model. (**a**) The N:C ratio model argues for a maternally provided inhibitor (I) of an activity (A), for example cell cycle checkpoints or zygotic transcription, and that the inhibitor is titrated by a nuclear component (N). As the number of nuclei increases, the equation shifts to the right, freeing the activator. (**b**) A variation on this model is that the inhibitor competes with the activator for a limited number of binding sites. As the number of binding sites (for example DNA) increases with each cell cycle, the inhibitor (for example histones) becomes limiting and is no longer sufficient to impede the activator (for example a transcription factor). (**c**) The exponential increase in the number of nuclei (in *blue*) leads to a sharp increase in the nuclear factor and a sharp decline in the inhibitor as embryos approach the MBT. The inhibitor falls below a threshold and events at MBT commence. The scale for the inhibitor is arbitrary

with exogenous DNA, advances the timing of the MBT (Dasso and Newport 1990; Newport and Kirschner 1982a, b; Mita and Obata 1984). Similarly in zebrafish, tetraploid embryos begin cell cycle lengthening about a cycle early and haploid embryos begin cell cycle lengthening about a cycle later than diploid embryos (Kane and Kimmel 1993). Partial enucleation of multicellular embryos, an approach analogous to the ligature experiment adapted from Spemann, also has a similar effect. At the early stages zebrafish blastomeres maintain intercellular bridges, allowing migration of a single nucleus into an enucleated cell. As the nucleus now occupies a larger cytoplasm, the daughter cells undergo additional cell divisions before cell cycles become asynchronous (Kane and Kimmel 1993). Further, haploid *Drosophila* embryos undergo one extra syncytial division, presumably because the N:C ratio associated with the MBT is achieved one cell cycle later than in diploid embryos (Di Talia et al. 2013; Edgar et al. 1986).

Taken together, work in *Xenopus* and other amphibians, zebrafish, and *Drosophila* indicate that cell cycle elongation and loss of cell cycle synchrony at the MBT is not determined by the chronological time after fertilization, the number of divisions, or a progressive change in chromatin state with each cell cycle. Rather, these MBT events are initiated when embryos reach a threshold N:C ratio resulting from rounds of nuclear replication without cell growth. A proposed mechanism of regulation is that a cytoplasmic factor that inhibits the MBT during the cleavage cycles is titrated out by binding DNA (Fig. 9.1) (Newport and Kirschner 1982b).

In addition to the ratio of DNA content to cytoplasm, as discussed above, the ratio of nuclear volume to cytoplasmic volume may contribute to the onset of the MBT. Injection of nuclear scaling factors, including import proteins, lamins, and reticulons, into cleavage-stage *Xenopus* embryos showed that increasing nuclear volume causes premature cell cycle remodeling, whereas a reduced N:C volume ratio increases the number of rapid cell divisions and delays cell cycle remodeling (Jevtic and Levy 2015). These experiments are discussed in more detail in the section on transcription.

9.2.4.2 Molecular Mechanisms of Cell Cycle Elongation

Cell cycle elongation at the MBT is achieved by the restraint and modification of cyclin/Cdk activities found in the cleavage stage. Embryos use several mechanisms to downregulate Cdc25 phosphatase, which allows Cdk1 to accumulate inhibitory phosphorylation and become inactivated after the last cleavage-stage mitosis. At the first asynchronous cycle after the MBT, lower Cdk1 activity results in an extended replication phase (Farrell et al. 2012). After replication is complete, cells must wait until zygotic Cdc25 is synthesized to restore Cdk1 activity for mitotic entry. In effect, these delays represent cell cycle elongation via extension of S-phase and the acquisition of G2 phase.

Regulation of Cdc25 at the MBT has been studied extensively in flies. *Drosophila* embryos express two maternally supplied Cdc25 homologs, String and Twine (Edgar et al. 1994). Altering the number of maternal copies of these Cdc25 homologs

showed that mutant embryos with increased maternal supplies of Cdc25 have one extra rapid, synchronous mitotic cycle. Conversely, mutant embryos with reduced maternal Cdc25 elongate cell cycles prematurely. These findings provided strong evidence that maternally loaded Cdc25 phosphatases are dosage-sensitive regulators that determine when cell cycles elongate (Edgar and Datar 1996). Furthermore, cell cycle elongation depends on rapid degradation of Twine protein at the MBT (Di Talia et al. 2013; Farrell and O'Farrell 2013).

9.2.4.3 Cell Cycle Elongation via Zygotic Transcription

Cdc25 stability at the MBT is sensitive to the N:C ratio, as Twine protein in *Drosophila* haploid embryos is degraded one cell cycle later in haploids compared to diploids (Farrell and O'Farrell 2013). This finding may be a result of N:C ratio-regulated transcription of specific genes that control Cdc25 degradation. Embryos injected with α-amanitin, an RNA Polymerase II inhibitor, undergo an extra round of rapid division and exhibit extended Twine stabilization (Farrell and O'Farrell 2013). Furthermore, flies with the RNA polymerase II mutation RPII215, which prematurely activates zygotic transcription, have a reduced number of nuclear divisions before cellularization. These data independently corroborate that zygotic transcription affects the timing of cell cycle remodeling at the MBT (Sung et al. 2013).

The genes transcribed at the MBT that regulate Cdc25 destruction are just beginning to be elucidated. One candidate in *Drosophila* is tribbles, which mediates Cdc25 destruction via proteolysis (Mata et al. 2000). Precocious tribbles expression via mRNA injection can arrest embryos in cycle 13 due to significant reduction in Twine levels (Farrell and O'Farrell 2013; Grosshans and Wieschaus 2000). Additionally, RNA sequencing of staged embryos revealed that tribbles expression increases dramatically at the MBT. Importantly, gene expression profiling of haploid embryos demonstrated that tribbles expression is sensitive to the N:C ratio (Lott et al. 2011; Lu et al. 2009). The Cdk1 inhibitor (CKI) fruhstart is another *Drosophila* zygotic gene important for cell cycle elongation at the MBT. Also sensitive to the N:C ratio, fruhstart appears immediately after the last cleavage division at the beginning of the 14th cell cycle (Lu et al. 2009). Moreover, when precociously expressed via mRNA injection, fruhstart can arrest the cell cycle during cleavage divisions (Grosshans et al. 2003). To inhibit Cdk1, Fruhstart binds tightly to mitotic cyclins, sequestering them from Cdk1 (Gawlinski et al. 2007).

Work in zebrafish has provided additional insight into the influence of zygotic genome activation on cell cycle remodeling in vertebrate systems. Similar to the results in *Drosophila*, inhibiting zygotic transcription in zebrafish embryos hinders the acquisition of G1 phase at the MBT (Zamir et al. 1997). However, the relationship between cell cycle lengthening and zygotic transcription seems more complex, since acquisition of G2 is independent of zygotic transcription (Dalle Nogare et al. 2009).

9.2.4.4 Developmental Use of the Replication Checkpoint for Cell Cycle Elongation

Proteins involved in the cellular response to DNA damage or replication stress also contribute to cell cycle remodeling at the MBT. Most checkpoint proteins are dispensable in somatic cells; knocking out individual components leads to mutations and aneuploidy but rarely inviability. However, several checkpoint proteins are essential for viability in a variety of model systems, have non-checkpoint related cell cycle functions, and have important roles in early development (Brown and Baltimore 2000; Liu et al. 2000).

The Chk1 serine–threonine kinase is an important checkpoint component that mediates cell cycle arrest in response to DNA single-strand breaks caused by stalled replication forks and fork collapse. Cleavage stage embryos are sensitive to Chk1 activity, and exogenous expression of wild type Chk1 or constitutively active Chk1 in *Xenopus* and zebrafish embryos induces a dose-dependent delay of cleavage cycles (Kappas et al. 2000; Zhang et al. 2014, 2015). Chk1 is also transiently activated at the MBT and required for cell cycle lengthening, suggesting that embryos co-opt this checkpoint protein to remodel the cell cycle (Shimuta et al. 2002).

Developmental activation of Chk1 causes cell cycle lengthening by promoting inhibitory phosphorylation of Cdk1, through regulation of the Cdc25 phosphatases and Wee1 kinase. In *Xenopus*, Chk1 phosphorylates Cdc25A, targeting it for proteasomal degradation. Dominant-negative forms of Chk1 injected into *Xenopus* embryos stabilize Cdc25A protein, while wild-type Chk1 overexpression leads to precocious Cdc25A destruction (Shimuta et al. 2002). Chk1 can also phosphorylate Cdc25 to inhibit its interaction with cyclin/Cdk complexes (Petrus et al. 2004; Uto et al. 2004). Furthermore, Chk1 enhances Wee1 kinase activity, thereby using a two-pronged approach for maintaining inhibitory phosphorylation of Cdk1 (Lukas and Bartek 2009).

Chk1 is also essential for cell cycle remodeling at the MBT in *Drosophila*: the Chk1 mutant, *grapes*, undergoes extra syncytial divisions and dies at gastrulation (Sibon et al. 1997, 1999). Chk1 enhances Wee1 kinase activity, as in *Xenopus* (Campbell et al. 1995), but does not play a significant role in Cdc25 destruction at MBT. Although String destruction is Chk1 dependent, Chk1 does not affect the stability of Twine, the Cdc25 homolog that is primarily associated with MBT cell cycle elongation (Farrell and O'Farrell 2013; Di Talia et al. 2013). As an additional mechanism for Cdk1 inhibition, Chk1 can inhibit nuclear accumulation of cyclin B, which prevents its interaction with Cdk1 in the nucleus (Royou et al. 2008). Together, these findings in *Xenopus* and *Drosophila* demonstrate that Chk1 is a potent regulator of cell cycle length during early embryogenesis and is necessary for early embryonic development.

While the molecular mechanisms of Chk1 activation at the MBT have yet to be fully elucidated, several hypotheses have been proposed that implicate a role for the N:C ratio (Edgar et al. 1994; Edgar and O'Farrell 1989; Sibon et al. 1999). In one model, a maternally loaded replication factor is titrated by increasing chromatin concentrations

in cleavage-stage embryos. Eventually, a limiting amount of this replication factor leads to a delay in replication. While this is not DNA damage per se, it may be effectively recognized as replication stress: ssDNA and ssDNA-dsDNA-binding proteins like RPA, Rad17-RPC and 9-1-1 may have an opportunity to bind, leading to Chk1 activation. Supporting this possibility, a recent study identified replication factors that could regulate cell cycle length during *Xenopus* cleavage divisions (Collart et al. 2013). When these replication factors are abundant in the cleavage-stage environment, the rapid origin firing may not allow binding of ATR and Chk1 activating factors to chromatin. However, the replication factors may become limiting at the MBT as the N:C ratio increases, slowing replication and permitting ATR-Chk1 activation.

A second model is that the Chk1 adaptor protein Claspin is regulated by the N:C ratio. Chk1 activation in somatic cells and *Xenopus* egg extracts depends on Claspin, which recruits Chk1 to ATR for phosphorylation (Kumagai and Dunphy 2000, 2003; Chini and Chen 2003). Claspin is typically phosphorylated by ATR after replication stress, but *Xenopus* embryos phosphorylate Claspin at low levels even in the absence of ATR activity. However, Claspin phosphorylation is responsive to the N:C ratio: addition of sperm nuclei to *Xenopus* egg extracts to increase N:C ratios to MBT levels resulted in Claspin phosphorylation in an ATR-independent manner. These data suggest that a threshold N:C ratio may either activate a novel kinase or titrate a kinase inhibitor to allow Claspin phosphorylation (Gotoh et al. 2011), in addition to the canonical phosphorylation of Claspin by ATR after MBT activation of the replication checkpoint.

Finally, a third hypothesis suggests that transcriptional activity itself can lead to replication checkpoint activation, regardless of the products of transcription. In *Drosophila*, binding of the replication protein RPA70 to DNA is tightly correlated to DNA-binding of RNA polymerase II, which increases gradually throughout the cleavage stages in preparation for zygotic transcription at the MBT (Blythe and Wieschaus 2015b). These data support the hypothesis that sites of transcriptionally engaged DNA can be sources of replication stress that activate the replication checkpoint and confer cell cycle remodeling.

9.2.5 Checkpoint Acquisition at the MBT

The molecular mechanisms that underlie checkpoint acquisition at the MBT are poorly understood. The following section reviews what is known about DNA damage checkpoint and SAC acquisition at the MBT, with an emphasis on the role of the N:C ratio.

9.2.5.1 DNA Damage Checkpoint Acquisition

The first hints towards elucidating checkpoint regulation came from studies using *Xenopus* egg extracts. As mentioned above, addition of DNA replication inhibitors, DNA damaging agents, or microtubule poisons had no effect on cell cycle progression in control extracts (Dasso and Newport 1990; Kumagai et al. 1998).

However, when additional DNA was supplied via the addition of sperm chromatin, the extracts became sensitive to replication stress and DNA damage and arrested their cell cycles, indicative of restored checkpoint function.

Several models have been proposed to explain the influence of the N:C ratio on DNA damage checkpoint acquisition. One hypothesis suggests that pre-MBT embryos cannot efficiently amplify the DNA damage signaling response in the unusually large cytoplasm associated with low N:C ratios. Injecting embryos with varying amounts of dsDNA to mimic DNA double-strand breaks (DSBs), together with plasmid DNA to increase the DNA content (and thus N:C ratio), induced a precocious DNA damage response that activated Chk1, increased phosphorylation of Cdk1, and caused subsequent cell cycle delay (Conn et al. 2004). This activation of the checkpoint only occurs at a critical DNA to cytoplasm ratio, demonstrating the importance of N:C ratio in checkpoint acquisition (Conn et al. 2004; Peng et al. 2008).

Checkpoint acquisition at the MBT likely centers around Chk1 gain of function. In zebrafish pre-MBT embryos, checkpoint proteins are maternally supplied, and the ATM-Chk2 axis responds robustly to DNA damage, but Chk1 is not activated (Zhang et al. 2014). These results suggest that lack of Chk1 activity limits checkpoint function prior to the MBT. Given these findings and the transient Chk1 activation at the MBT observed in *Xenopus* (Shimuta et al. 2002), we propose that Chk1 activation is sensitive to the N:C ratio and that Chk1 is a master regulator of cell cycle remodeling at the MBT, contributing to both cell cycle elongation and checkpoint acquisition.

9.2.5.2 SAC Acquisition

To examine SAC acquisition, "mini-embryos" with reduced cytoplasmic volume were created using a modified version of the Spemann method. A loop of baby's hair was placed around the animal pole of a newly fertilized *Xenopus* embryo to constrict a portion of the nucleus-containing cytoplasm, effectively increasing the N:C ratio. These mini-embryos had a cytoplasmic volume of about 1/8–1/12 the size of a normal embryo and continued to cycle like their unperturbed counterparts, but with a much higher N:C ratio (Clute and Masui 1995). The cell cycles of these mini-zygotes became asynchronous two cycles before the usual time of the MBT (cleavage 10). At this point, the N:C ratio of the mini-embryos corresponds to the N:C ratio of unperturbed embryos at MBT (cleavage 12). This finding suggests that cell cycle remodeling is controlled by the N:C ratio. Surprisingly, however, mitotic delay after microtubule depolymerization occurred at the same time as in control embryos, despite the disparity in N:C ratio and precocious cell cycle elongation in the mini-zygotes. Similar findings were demonstrated with zebrafish embryos: when pre-MBT cell cycles were artificially lengthened with the addition of activated Chk1, thereby slowing the increase of the N:C ratio, embryos still acquired a functional SAC at 3 h post fertilization (hpf), the normal time of the MBT, despite not having reached the usual MBT N:C ratio (Zhang et al. 2015).

These studies suggest that while the onset of cell cycle asynchrony depends on the N:C ratio, the SAC is acquired at an absolute time that is independent of N:C ratio. However, they differ from earlier findings in egg extracts, which can activate

the SAC and arrest in metaphase if enough sperm chromatin is added (Minshull et al. 1994), implying that SAC acquisition is coupled to the N:C ratio. One explanation for this result is that a threshold N:C ratio could trigger zygotic transcription, leading to SAC acquisition indirectly by transcription of checkpoint components that are not maternally supplied. However, work in zebrafish and *Xenopus* shows that blocking transcription with α-amanitin in pre-MBT embryos does not abrogate SAC acquisition at the MBT (Zhang et al. 2015; Newport and Kirschner 1982b). Instead, the seemingly contradictory results may indicate that both a developmental timer and increases in the N:C ratio contribute to SAC acquisition. Full activation of the SAC relies on the generation of MCCs at kinetochores and diffusion throughout the cytoplasm to inhibit the APC/C. Cleavage stage embryos may express as-yet unidentified SAC inhibitors, or may not generate enough MCCs to overcome the large cytoplasmic volume. A threshold concentration of DNA (and also kinetochores) may be required to generate enough active MCCs or to titrate a SAC inhibitor. During normal development, when the number of kinetochores is fixed, a set time may be required for the synthesis and accumulation of SAC proteins that amplify signaling downstream from initial SAC activation at kinetochores. However, the need for accumulation of these proteins could be bypassed if exogenous DNA is added, providing large numbers of kinetochores to amplify SAC signaling.

9.2.6 Summary

A principal goal of the early embryo is to accumulate a large cell population in preparation for gastrulation and the later stages of development. This feature is especially important in oviparous organisms where embryonic development occurs outside the mother and rapid embryonic development is a survival advantage. To do so, cleavage-stage embryos have modified several facets of cell cycle regulation to encourage rapid proliferation: cyclin/Cdk activities oscillate to achieve efficient cell cycling and checkpoint function is sacrificed. It is not until the MBT that cell cycles are remodeled to behave more like their somatic counterparts: cell cycles lengthen dramatically, acquire checkpoints, and become asynchronous. Though these features occur simultaneously, they are driven by various triggers, be it the N:C ratio, absolute time post fertilization, or zygotic transcription.

9.3 Regulation of Zygotic Transcription in the Early Embryo

9.3.1 Changes in Transcription from Oocyte Maturation Until Zygotic Gene Activation

During oogenesis, robust transcriptional activity generates maternal transcripts essential for post-fertilization development. However, the maternal genome becomes quiescent upon meiotic maturation and remains inactive after fertilization until

zygotic genome activation (Table 9.1) (Wormington and Brown 1983; De La Fuente and Eppig 2001; Bachvarova and Davidson 1966; Woodland and Gurdon 1969; Newport and Kirschner 1982a). In *Xenopus*, large-scale zygotic transcription is not activated until the 12th embryonic cleavage, approximately 6–7 h after fertilization (Newport and Kirschner 1982a). In zebrafish, large-scale zygotic gene activation is not observed until the tenth cleavage division (Kane and Kimmel 1993). In medaka, large-scale zygotic transcription was reported to begin around division 11–12 (Aizawa et al. 2003). The major wave of zygotic transcription in mammals is delayed from one to several days, depending on the species, and occurs after fewer cell divisions than observed in nonmammalian vertebrates. In mice and rabbits, the major wave of zygotic gene activation is established by the 2-cell stage, whereas in cows, sheep, pigs, and humans, major zygotic transcription begins at the 4- to 16-cell stage (Telford et al. 1990; Li et al. 2013; Hamatani et al. 2004; Wang et al. 2004; Zeng and Schultz 2005; Flach et al. 1982; Tesarik et al. 1988; Sawicki et al. 1981; Park et al. 2013; Xue et al. 2013; Aoki et al. 1997).

While the time until zygotic gene activation varies between organisms, it is highly reproducible in a given species (Table 9.1). Furthermore, most species that delay large-scale ZGA nevertheless demonstrate an earlier, minor wave of zygotic transcription (reviewed by Tadros and Lipshitz 2009; Baroux et al. 2008; Lee et al. 2014), consistent with mechanisms that both suppress early transcription and activate zygotic genes at the appropriate stage. The following sections review the evidence that early embryos have the capacity for transcription despite global repression of most zygotic genes and discuss the regulation of zygotic gene activation, including potential mechanisms to regulate the timing of zygotic gene activation.

9.3.2 Transcriptional Repression in Pre-MBT Embryos

Despite the limited zygotic transcription after fertilization in vertebrates, the basal transcriptional machinery is present in oocytes and early embryos of *Xenopus* and other amphibians, zebrafish, mouse, and other mammals (Brown 2004; Veenstra 2002; Wiekowski et al. 1993; Li et al. 2013; Lee et al. 2014), as are multiple gene specific transcription factors. These findings suggest that the low level of transcription is due to a repressive mechanism that functions until the MBT/ZGA, as discussed further in Sect. 9.3.5.

For example, RNA polymerases (RNAP) I, II, and III are present in *Xenopus* oocytes and eggs, which have served as an abundant source for purification of polymerases and preparation of extracts competent for transcription of exogenous templates (Roeder 1974a, b). Importantly, RNAPII is present in an active form in early embryos. RNAPII is phosphorylated in a repeat sequence within the C-terminal domain in manner that correlates with its initiating (serine-5 phosphorylated) and elongating (serine-2 phosphorylated) state. In vivo, RNAPII phosphorylated at serine-2 is detectable in cleavage-stage embryos, consistent with a low level of transcription before the MBT. Although several studies reported that serine-2 phosphorylation

decreases after fertilization and increases after the MBT, each of these studies nevertheless identify elongating RNAPII with a serine-2 phosphorylation specific antibody (H5) throughout pre-MBT stages (Palancade et al. 2001; Collart et al. 2009; Blythe et al. 2010). Phosphorylated RNAPII is also detectable before the MBT in zebrafish and increases steadily through the MBT (Zhang et al. 2014). This level of serine-2 phosphorylated RNAPII is actually high relative to the amount of DNA template in cleavage-stage embryos; while genome wide ChIP has not detected elongating RNAPII associated with chromatin before the MBT, the sensitivity of ChIP has so far been limited by the low N:C ratio in early embryos (Lindeman et al. 2011; Akkers et al. 2009; Vastenhouw et al. 2010).

Furthermore, oocyte and egg extracts are transcriptionally competent. *Xenopus* egg extracts arrested in interphase will transcribe exogenous type III genes (Hartl et al. 1993; Wolffe and Brown 1987; Almouzni et al. 1990, 1991; Toyoda and Wolffe 1992; Wolffe 1989; Amodeo et al. 2015). In *Xenopus* oocytes and eggs, injected plasmids with type III promoters are transcribed transiently (Mertz and Gurdon 1977; Brown and Gurdon 1977; Newport and Kirschner 1982a; Prioleau et al. 1994; Almouzni and Wolffe 1995). Furthermore, a type II promoter-reporter is active throughout pre-MBT stages if an appropriate activator (GAL4-VP16) is present at sufficient levels (Almouzni and Wolffe 1995). A plasmid reporter with an EF1α promoter injected into zebrafish is also transcribed before the MBT (Harvey et al. 2013). The regulation of type II and type III promoters before and after the MBT is described in more detail below.

Mouse embryos are able to initiate transcription of exogenous templates—either injected plasmids or paternal transgenes—during S phase of the first cell cycle in the male pronucleus (Martinez-Salas et al. 1989; Ram and Schultz 1993; Wiekowski et al. 1993). Endogenous RNAPII dependent transcription has also been detected at the 1-cell stage (Bouniol et al. 1995; Aoki et al. 1997; Matsumoto et al. 1994), including transcription of specific mRNAs Hsp70.1 and MuERV-L (Christians et al. 1995; Kigami et al. 2003; Latham et al. 1992). The presence of ß2-microglobulin protein expressed from the paternal allele by the 2-cell stage in mice also indicates earlier transcription of this zygotic gene (Sawicki et al. 1981) (also see Hamatani et al. 2004; Xue et al. 2013; Park et al. 2013).

9.3.3 Detection of Pre-MBT Transcription

One of the strongest arguments that pre-MBT embryos are competent for transcription is the observation of RNAPII dependent transcription before the MBT. Although large-scale zygotic transcription is delayed in embryos of most commonly studied model organisms, a minor wave of pre-MBT zygotic transcription has been documented in several model organisms (Edgar and Schubiger 1986; Kimelman et al. 1987; Nakakura et al. 1987; Shiokawa et al. 1989; Yasuda and Schubiger 1992; Yang et al. 2002; Heyn et al. 2014; Aoki et al. 1997; Liang et al. 2008; ten Bosch et al. 2006; De Renzis et al. 2007; Harrison et al. 2011; Tani et al. 2010; Kraeussling et al.

2011). The finding of pre-MBT transcription was for many years only a footnote to the study of ZGA, but over the past decade has been reinforced by functional studies, advances in gene profiling methods, and the discovery of mechanisms that regulate pre-MBT transcription in *Drosophila*, *Xenopus*, and zebrafish.

9.3.3.1 Metabolic Labeling

The initial work describing pre-MBT transcription of endogenous genes in *Xenopus* used metabolic labeling of anonymous transcripts in dissociated blastomeres (Nakakura et al. 1987) or in cleavage-arrested (coenocytic) embryos (Kimelman et al. 1987), perturbations that may disrupt the normal regulation of transcription. Indeed, Kimelman et al. noted "a generalized inhibition of pol II transcription in coenocytic embryos" (Kimelman et al. 1987). Furthermore, Lund et al. showed that perturbations that cause cleavage arrest reduce endogenous DNA content and impair RNAP II dependent transcription of 4-8S RNAs (Lund and Dahlberg 1992). Thus, measurements of RNAPII-dependent transcription in pre-MBT embryos may be confounded by cleavage arrest. However, injection of [^{32}P]-UTP into otherwise unperturbed, cleaving embryos demonstrated readily detectable new transcription of heterogeneous, polyadenylated RNAs as early as the 128-cell stage, six divisions before the canonical MBT (Yang et al. 2002; Nakakura et al. 1987). While some of these early transcripts may have been mitochondrial RNAs, as observed in Lund et al., they also included specific RNAP II dependent transcripts as described in the next section.

Similarly, metabolic labeling in zebrafish embryos detects transcripts as early as the 64-cell stage (see Fig. 9.2 for selected pre-MBT genes) (Heyn et al. 2014), which is in contrast to the findings of Kane and Kimmel, who reported incorporation of labeled nucleotides at the tenth division (Kane and Kimmel 1993); these differences may simply reflect the sensitivity of the respective detection methods. Metabolic labeling in mouse was used to identify a minor wave of zygotic transcription within the first 2 h of S-phase in 1-cell embryos (Aoki et al. 1997). Considerably less is known about the onset of zygotic transcription or the presence of an MBT in avians and reptiles, but recent staining for phosphorylated RNAPII in the chick suggested that transcription begins during cleavage divisions at the seventh to eighth division (64–128 cell stage) (Nagai et al. 2015). Similarly, zygotic mRNA synthesis begins in quail during cleavage stages, while new rRNA transcription is delayed until blastula stage (5000 cells) (Olszanska et al. 1984).

9.3.3.2 Identification of Specific Pre-MBT mRNAs

The first specific RNAPII dependent pre-MBT transcripts identified in vertebrate embryos were the *Xenopus* nodal related genes *Xnr5* and *Xnr6*, which were detected as early as the 256-cell stage (Yang et al. 2002). *Xnr5* and *Xnr6* are multicopy genes regulated by Wnt signaling and by the maternal T box transcription factor VegT

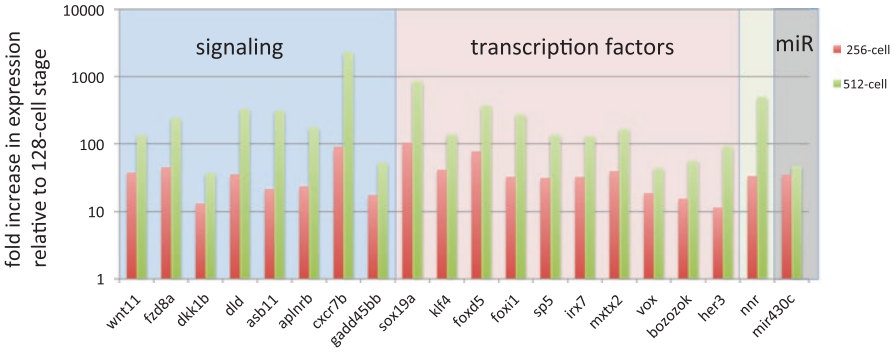

Fig. 9.2 Selected zygotic genes expressed before the MBT in zebrafish. A subset of zygotic genes transcribed before the MBT in zebrafish (Heyn et al. 2014), supported by at least one other study, is shown. The fold-increase in expression at 256-cell (two cycles before MBT) and 512-cell stage relative to 128-cell was calculated from RNA-seq data (Supplemental Table S1) in Heyn et al. (Note that Heyn et al. presented expression data as $\log_2$[FPKM] whereas here we are showing fold-change in expression (FPKM) *relative* to FPKM at 128-cell (not absolute expression) to indicate the rapid increase in expression from the 128-cell stage onward). All genes were present at >10 FPKM by 256 or 512-cell stage (Heyn et al. 2014) and an increase in the detection of each of these genes at the 256-cell and 512-cell stages was also evident in supplemental data reported by Harvey et al. (2013). Within this set of genes, pre-MBT histone H3 lysine-4 trimethylation, generally indicative of "active" promoters, was reported for *dld, gadd45bb*, *klf4*, *foxd5*, and *irx7* and pre-MBT zygotic transcription of *irx7* was also demonstrated by RT-PCR (Lindeman et al. 2011). Pre-MBT expression of *bozozok* (also known as *dharma*) was first identified by in situ hybridization (Leung et al. 2003)

(Takahashi et al. 2000; Hilton et al. 2003). The *pre-MBT* expression of *Xnr5*/*Xnr6* also requires Wnt signaling and VegT, is localized to early dorsal-vegetal blastomeres, and is inhibited by α-amanitin (Yang et al. 2002; Skirkanich et al. 2011). In situ hybridization confirmed zygotic expression of *Xnr5* in dorsal-vegetal blastomeres before the MBT (Takahashi et al. 2006). Furthermore, Yanai et al. using microarrays reported a parallel increase in *Xnr5* and *Xnr6* expression between the 2-cell stage and the early to mid-blastula stage (stage 8) in *X. laevis* and *X. tropicalis* (Yanai et al. 2011), and Collart et al. also identified *Xnr5* and *Xnr6* as pre-MBT transcripts in *X. tropicalis* (Collart et al. 2014). Thus, *Xnr5* and *Xnr6* are transcribed in a highly regulated manner before the MBT.

As discussed above, type II transcription can be sustained before the MBT if a strong gene-specific activator is present. Thus, the zygotic expression of *Xnr5/6* before the MBT may depend on maternal transcription factors that may in turn activate additional zygotic genes before the MBT. *Xnr5/6* are direct targets of VegT (Takahashi et al. 2000; Hilton et al. 2003), and other direct targets of VegT (Xanthos et al. 2001), including *mixer*, *bix4*, *derriere*, and *sox17α* are also newly transcribed between the seventh and eighth cleavage divisions (Skirkanich et al. 2011). Each of these zygotic transcripts was detected at the 256-cell stage and rose exponentially, with an increase of approximately 2 orders of magnitude by the onset of the MBT. The pre-MBT expression of these mesendodermal genes required RNAPII and maternal

VegT (Skirkanich et al. 2011). Hence, multiple direct targets of VegT are initially transcribed before the MBT in *Xenopus laevis*, consistent with gene-specific transcription factor dependent expression before the MBT, as seen with exogenous Gal4-VP16 (Almouzni and Wolffe 1995) in *Xenopus* and for endogenous genes in *Drosophila* (e.g., Liang et al. 2008; ten Bosch et al. 2006; Harrison et al. 2011).

The microRNA miR-427 (Watanabe et al. 2005) is a homologue of zebrafish miR-430 (Giraldez et al. 2006), and both of these miRNAs regulate maternal mRNA clearance and nodal signaling (Choi et al. 2007; Rosa et al. 2009). The miR-427 cluster is highly expressed before the MBT in *X. laevis* (Lund et al. 2009). The precursor form, pre-miR-427, and the primary transcript pri-miR-427 are detectable by Northern blot several hours before MBT (Lund et al. 2009) and an exponential increase in expression begins at the 64-cell stage based on qRT-PCT (Fig. 9.3a, Jing Yang, personal communication). Pre-MBT transcription of miR-427 is RNAPII dependent, based on α-amanitin sensitivity. Interestingly, mIR-427 targets *Xnr5* and *Xnr6b* and regulates mesendoderm development (Rosa et al. 2009). Inhibition of miR-427 with an antimir reduces expression of nodal inducible genes and mesoderm development, mimicking other inhibitors of nodal signaling. Although these effects on mesoderm development were associated with miR-427 regulation of *lefty*, another TGF-ß family member (Rosa et al. 2009), the coincident pre-MBT expression of both miR-427, *Xnr5/6*, and other regulators of mesendoderm induction is intriguing.

In zebrafish, miR-430 is also transcribed at a high level before the MBT, as early as the 64-cell stage (Fig. 9.3b) (Heyn et al. 2014), strongly supporting a conserved requirement for pre-MBT expression of this microRNA family. miR-430 transcription is activated by the maternal transcription factors Nanog, Pou5f3 (closely related to mammalian Oct4/Pou5f1 (Frankenberg et al. 2014)) and SoxB1, which also contribute to zygotic genome activation at the MBT (Lee et al. 2013b; Leichsenring et al. 2013). The regulation of miR-430 by these factors is analogous to miR-309 in *Drosophila*, which also regulates maternal mRNA clearance and is activated (by Zelda) before the MBT (Blythe and Wieschaus 2015b; Biemar et al. 2005). Similar to miR-427 in *Xenopus*, miR-430 interacts with nodal1/squint and lefty to regulate nodal signaling (Choi et al. 2007). miR-430 is also detectable by Northern blot before the proposed MBT in medaka (Tani et al. 2010). In addition, expression of the dorsal Wnt target gene *bozozok* (*dharma*) is detected in zebrafish before the MBT by in situ hybridization (Leung et al. 2003) and by RNA-Seq (Heyn et al. 2014; Harvey et al. 2013; Lee et al. 2013b).

9.3.3.3 Pre-MBT Transcription Identified by Gene Profiling

Multiple gene profiling studies have been reported on early developmental stages in *Xenopus tropicalis*, zebrafish, mouse, and other mammals. Most of the profiling studies in *Xenopus* and zebrafish have confirmed pre-MBT transcription of multiple protein coding genes and microRNAs. Interesting biological patterns emerge from interspecies comparisons, including the robust early expression of microRNAs that regulate degradation of maternal RNAs and early expression of nodal signaling components.

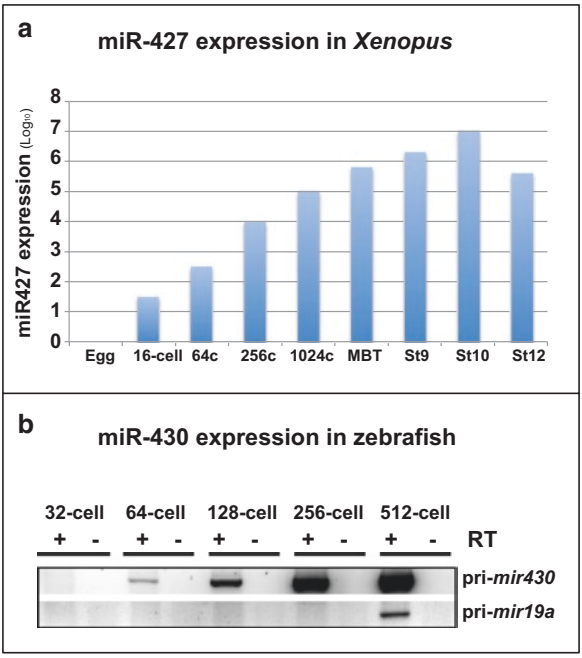

Fig. 9.3 Zygotic expression of *miR-427/430* during cleavage stage in *Xenopus* and zebrafish. (**a**) Real-time RT-PCR analysis of pri-*mir-427* at the indicated stages in *Xenopus* laevis. Data courtesy of Dr. Jing Yang, University of Illinois, Urbana-Champagne. Pre-MBT expression of mir-427 was first detected by Northern blot as shown by Lund et al. (2009). (**b**) RT-PCR analysis of pri-*mir-430* and pri-*mir-19a* at the indicated stages in zebrafish, from Heyn et al. (2014) Supplemental data (Fig. S4C), reprinted with permission from the authors and from Cell Press. "+" and "−" indicate reaction products with and without reverse transcriptase (RT), respectively

Xenopus

Yanai et al. used custom microarrays to compare the temporal profiles for gene expression in *X. laevis* and *X. tropicalis* (Yanai et al. 2011). While they were not focused on gene expression changes during cleavage stages, they found multiple genes that increase in abundance from the 2-cell stage to stage 8 (MBT is at stage 8.5), which likely includes pre-MBT transcripts. Although a caveat of this analysis is that differences in signal could reflect changes in polyadenylation rather than zygotic transcription (see below), they observe in both *X. tropicalis* and *X. laevis* an increase at stage 8 in the VegT-regulated pre-MBT genes identified by Skirkanich et al., consistent with a conserved mode of regulation for these pre-MBT genes.

Subsequent RNA-Seq analyses using more detailed temporal analysis of multiple stages throughout cleavage stage development revealed additional type II zygotic genes transcribed before the MBT in *X. tropicalis*. Collart et al. used both oligo-dT primed and ribosome depleted RNA for libraries, and collected samples at 30 min intervals. They identified 960 RNAs that increased in apparent abundance

during pre-MBT stages. The earliest increase in detectable mRNAs was due to increased polyadenylation of 551 maternal RNAs. A second pool of 409 transcripts appeared around the 128-cell stage and these were confirmed as newly transcribed mRNAs. These included 113 transcription factors and 20 signaling molecules, including *nodal-related 5* and *6* (*Xnr5* and *Xnr6*). They also assessed changes in a subset of mRNAs independently of polyadenylation using a "nanostring counter" (see Collart et al. 2014; Geiss et al. 2008) to confirm that the first wave of increased transcript detection correlated with polyadenylation of maternal transcripts whereas the second pre-MBT wave correlated strongly with new transcription. They interpreted their data as indicating that there is a gradual increase in zygotic transcription as opposed to a distinct pre-MBT wave of transcription.

Paranjpe et al. also compared polyadenylated RNAs and ribosome depleted RNAs, and found that, between stage 6 (32-cell) and late blastula, some genes are activated early. However, pre-MBT transcription was not a focus of that work, which only contained one pre-MBT time point (Paranjpe et al. 2013).

Tan et al. collected multiple cleavage stages through the 64-cell stage (stage 6), stage 8, and post-MBT stages of *X. tropicalis* (Tan et al. 2013). They identified 150 genes that were upregulated by stage 6 (including the Oct4 ortholog Oct25); 24 % of these genes were orthologous to genes whose detection also increased before the MBT in zebrafish (Aanes et al. 2011). They used oligo-dT priming for cDNA synthesis and library preparation, which does not distinguish changes in polyadenylation from new transcription. To address this issue, they performed qRT-PCR on cDNA synthesized with random primers and validated pre-MBT expression of 13 of the 20 genes sampled, indicating that 50–80 % of these (75–90 genes) are newly transcribed before the MBT (Tan et al. 2013). The fact that they could detect zygotic transcripts at the 64-cell stage suggests that these genes are robustly transcribed. They did not examine embryos between the 64-cell stage and the MBT (a window of six cell divisions), and therefore did not detect *Xnr5*, *Xnr6*, and other genes known to be expressed during this pre-MBT window in *X. tropicalis* (Collart et al. 2014) and *X. laevis* (Skirkanich et al. 2011; Takahashi et al. 2006).

Zebrafish

An RNA-Seq analysis of staged zebrafish embryos detected 847 transcripts that appeared before the MBT and were not present in oocyte RNA (Aanes et al. 2011; Lindeman et al. 2011). This analysis was based on detection of polyadenylated transcripts, making it difficult to distinguish new zygotic transcription from cytoplasmic polyadenylation of maternal transcripts. The authors concluded that the increase in pre-MBT reads reflected increased cytoplasmic polyadenylation of maternal RNAs based on direct measurement of polyadenylation of 11 of these 847 mRNAs (Aanes et al. 2011). However, this leaves open the possibility that some of the other 836 mRNAs are newly transcribed before the MBT. Indeed, in a follow-up publication, one of nine genes selected for further analysis, *irx7*, was transcribed before the MBT (Lindeman et al. 2011), as independently confirmed in two other RNA-Seq studies

(Heyn et al. 2014; Harvey et al. 2013). Thus the RNAs detected during pre-MBT stages in this study likely include both newly transcribed RNAs and maternal RNAs that undergo cytoplasmic polyadenylation.

Metabolic labeling of nascent RNAs bypasses the concerns of changes in polyadenylation and also makes it possible to distinguish newly transcribed from maternal RNAs. In an elegant analysis, Heyn et al. injected 4-thio-UTP into zebrafish eggs and collected visually staged embryos at the 64-, 128-, and 512-cell stages, all prior to MBT, followed by biotinylation and affinity purification of nascent RNAs. They then performed RNA-Seq and found new zygotic transcription of 592 genes between the 128 and 512-cell stages (a subset of these genes is shown in Fig. 9.2). These were primarily type II genes encoding proteins (including *klf4*, the myc family member *mycl1a*, the mix related factor *mxtx2* (a regulator of nodal signaling), *one-eyed pinhead* (*oep*), and, at a lower level, *nodal1/squint* and *FoxH1*) and miRNAs (miR-430 and miR-19a). As described above zygotic transcription of miR-430 was detected by the 64-cell stage. They found that RNAs transcribed before MBT tended to be short and/or with limited introns and suggested this population is enriched for evolutionarily young genes. Although they found little similarity in the population of early-transcribed genes when compared to *Drosophila* or mouse, the pre-MBT expression of miR-430 and nodal signaling components provides striking parallels to findings in *Xenopus* (discussed below).

As an alternative approach to distinguish new zygotic transcripts from maternal RNAs in zebrafish, Harvey et al. used RNA-Seq to identify polymorphisms between paternal and maternal alleles (Harvey et al. 2013), as described previously in mouse (Sawicki et al. 1981; Xue et al. 2013) and medaka (Aizawa et al. 2003). Harvey et al. collected embryos at 2-cell, 64-cell, and MBT, as well as post-MBT stages. New zygotic transcripts, based on appearance of SNPs in paternal genes, were reported as first detectable at the tenth division, the MBT in zebrafish as established by Kane and Kimmel (1993) and analogous to the similar findings in medaka (Aizawa et al. 2003). At first glance, this conclusion is at odds with Heyn et al. (2014), Vesterlund et al. (2011), and Leung et al. (2003). However, the low frequency (~25 %) of genes with distinguishing SNPs limits the sensitivity of their approach: Heyn et al. (2014) detected 350 zygotically transcribed genes that lack informative SNPs. Furthermore, Harvey et al. did not examine embryos between the 64-cell stage and MBT for paternal SNPs, when most pre-MBT genes are first transcribed, and therefore cannot rule out zygotic transcription in this developmental window. In fact, a separate analysis in that paper revealed multiple genes that increase from the 128-cell stage through MBT. They did not apply SNP analysis or other means to distinguish polyadenylation from new transcription for this group of genes, but at least 20 genes that increase in detection before the MBT were identified as pre-MBT transcripts by Heyn et al. (Fig. 9.2), including miR-430 (Figs. 9.2 and 9.3b), which appears to increase dramatically before the MBT in both datasets (as well as in Lee et al. 2013b). Thus, while the SNP analysis does not detect new transcription at the 64-cell stage in zebrafish, compelling parallels between Heyn et al. and Harvey et al. provide strong support for zygotic transcription between the 64-cell stage and the MBT.

As another approach to identify zygotic transcripts in zebrafish, Lee et al. performed RNA-Seq for intronic sequences, which also distinguishes new transcripts from maternal RNAs. That comprehensive study focused on gene expression after the canonical MBT (4 hpf) and did not examine stages between the 64-cell and MBT (Lee et al. 2013b); their approach nevertheless identified a large number of zygotic transcripts. A subset designated as "first wave" zygotic transcripts (still transcribed when zygotic gene function is blocked), including miR-430, *klf4b*, *nnr*, *oep*, *blf*, *vent*, *her3*, *foxi1*, *mxtx2*, were also identified as pre-MBT transcripts by Heyn et al. and some of these were also found to increase in detection before MBT by Harvey et al. Lee et al. further showed that loss of *Nanog*, *SoxB1*, and/or *Pou5f3* (which has also been referred to as *Pouf5/1* or *Oct4*, see Frankenberg et al. 2014) reduced the expression of "first-wave" genes, including multiple genes identified before the MBT in the Heyn et al. study (e.g., *miR-430*, *klf4*, *blf*, *vent*, *her3*, *foxi1*, *mxtx2*, *vox*, *foxa3*, *foxd3*, and *sox32*) (Lee et al. 2013b; Leichsenring et al. 2013). Consistent with this, Heyn et al. (2014) observed that 50 % of the pre-MBT genes that they identified contained Pou-Sox binding sites. Thus the transcription of a subset of "first zygotic wave" genes identified by Lee et al. may well begin between the 64-cell stage and the MBT.

Mammals

Gene profiling in mouse has revealed multiple mRNAs that increase in abundance in 1-cell mouse embryos (Xue et al. 2013; Park et al. 2013). Park et al. (2013) used ribosome depleted RNA in their studies and identified ~600 genes that increased in apparent expression in the 1-cell embryo. However, Abe have also found marked promiscuous transcription of RNAs in the 1-cell mouse embryo, with transcription of intergenic regions lacking clear promoters; these RNA species are low abundance and of as yet uncertain significance (Abe et al. 2015). Gene profiling studies of the maternal to zygotic transition have also been reported for various mammalian species (as cited in Li et al. 2013: Hamatani et al. 2004; Zeng and Schultz 2005; Misirlioglu et al. 2006; Sirard et al. 2005; Vallee et al. 2008; Wang et al. 2004; Whitworth et al. 2005; Zeng et al. 2004).

9.3.3.4 Limitations in Detecting New Transcription Before the MBT

These studies provide informative resources on early gene expression and also illustrate some of the challenges inherent in analyzing new transcription in the early embryo. Most importantly the sensitivity of detection methods is a critical issue, especially when looking for new transcription over the background of a maternal store of RNAs that is orders of magnitude higher than the RNA in a typical somatic cell. This is especially true and underappreciated in RNA profiling approaches. Two recent studies on the sensitivity and reproducibility of next generation sequencing across multiple platforms (Li et al. 2014; SEQC/MAQC-III 2014) demonstrated that

a substantial number of transcripts will be missed in somatic cell RNA at less than 1 billion mapped reads. Because of cost limitations, most RNA-Seq studies on staged early embryos are based on 10–100 times fewer mapped reads per stage; these studies are informative about large scale changes in gene expression but not sufficiently powered to rule out the expression of low abundance or undetected transcripts. As a practical example, increasing sequencing depth to 1.5 billion reads identified numerous new lncRNAs in early zebrafish not detected in earlier studies (Pauli et al. 2012).

Detecting new gene expression in early embryos is also complicated by highly dynamic cytoplasmic polyadenylation and deadenylation of up to ~25 % of mRNAs during early development (Dworkin et al. 1985; McGrew et al. 1989; Graindorge et al. 2006; Paranjpe et al. 2013). Detection methods that depend on oligo-dT priming alone cannot distinguish between changes in polyadenylation of maternal RNAs and changes in RNA abundance. This concern can be addressed using ribosome depleted RNAs, metabolic labeling of nascent RNAs, sequencing of introns, identification of paternal polymorphisms, and/or validating by qRT-PCR using random-primed cDNA, as in the above studies. However, in general, distinct transcriptomes are represented in libraries prepared by polyA enrichment versus ribo-depletion, with more low abundance genes detected using ribosome depletion compared to oligo-dT priming (Li et al. 2014; SEQC/MAQC-III 2014).

Accurate staging of multicellular embryos is a potential concern, especially when comparing stages close to the MBT and when comparing different clutches, different rearing temperatures, or related but genetically distinct species (see Yanai et al. 2011; Harvey et al. 2013). For example, the first cell cycle in zebrafish is 45 min, compared with 15 min cleavage cycles subsequently; therefore the use of natural matings can introduce significant variation if staged samples are collected based on time alone (Langley et al. 2014; Steven Harvey, personal communication). This concern can be readily alleviated by using small numbers of embryos and visually staging embryos during cleavage stages, as clearly described (Heyn et al. 2014; Karla Neugebauer, personal communication). In addition, several of the studies cited above did not examine pre-MBT stages later than the 64-cell stage of zebrafish or *X. tropicalis*, and therefore likely missed pre-MBT transcription detected by others between early cleavage stages and the MBT.

9.3.4 Function of Pre-MBT Gene Expression

The above expression analyses demonstrate that new transcription before the MBT is a conserved phenomenon in vertebrates (as in invertebrates) and suggest specific groups of zygotic genes that regulate early developmental events may be similarly regulated. However, many of these pre-MBT genes are initially present at low levels of expression, and the concern that this represents transcriptional noise has been raised. The evidence that early transcription is specifically regulated includes: (1) A limited set of genes is reproducibly transcribed before the MBT; (2) pre-MBT transcription is restricted to specific blastomeres for some genes (e.g., *bozozok*, *Xnr5*, *Xnr6*); (3)

maternal transcription factors are required for transcription of type II genes before the MBT; and (4) Pre-MBT genes regulate at least two fundamental biological processes that occur near the MBT—turnover of maternal RNAs and mesendoderm induction.

The Nodal genes *derriere*, *Xnr5*, and *Xnr6* are expressed well before the MBT in *Xenopus laevis* (Yang et al. 2002; Blythe et al. 2010; Skirkanich et al. 2011; Takahashi et al. 2006; Collart et al. 2014). Nodal signaling is essential for mesoderm formation and, in coordination with dorsally localized Wnt/ß-catenin activity, establishes the dorsal-ventral axis of the embryo (Luxardi et al. 2010; Agius et al. 2000; Schier 2001; Xu et al. 2012; Schier and Talbot 2005). Nodal signaling is active at or before the MBT, as determined by the presence of phosphorylated Smad2, and pre-MBT expression of *Xnr5* and *Xnr6* are required for this activation (Skirkanich et al. 2011; Schohl and Fagotto 2002; Faure et al. 2000; Lee et al. 2001). Inhibition of Nodal signaling before the MBT abrogates mesoderm induction, whereas inhibition after the MBT no longer blocks induction of mesodermal markers. Furthermore, activation of Nodal/Smad signaling before the MBT induces post-MBT expression of mesodermal markers, whereas activation of Smad2 after MBT no longer induces mesoderm markers. Taken together, these data demonstrate that pre-MBT Nodal signaling, initiated by pre-MBT transcription of *Xnr5* and *Xnr6*, is required for patterning of the *Xenopus* embryo (Skirkanich et al. 2011).

MiR-430 in zebrafish and medaka and miR-427 in *Xenopus* are robustly expressed before the MBT and are essential for the degradation of maternal mRNAs during the maternal to zygotic transition. In *Drosophila*, the miR-309 cluster, which is activated by Zelda and is also essential for clearance of maternal mRNAs, strongly recruits RNAPII and is abundantly expressed at least two cycles before the MBT (Blythe and Wieschaus 2015b; Biemar et al. 2005). Whether the expression of these microRNAs specifically *before* the MBT is essential has not yet been tested, but their conserved pre-MBT expression in widely divergent species is consistent with an important role for their pre-MBT transcription in the maternal to zygotic transition. Furthermore, miR-430 and miR-427 regulate mesendoderm development in zebrafish and *Xenopus* (Choi et al. 2007; Rosa et al. 2009) and their early transcription is therefore consistent with the early transcription of other mesendoderm regulators, such as *Xnr5*/6 in *Xenopus* and *nodal1/squint*, *mxtx2*, and other nodal pathway genes in zebrafish.

These findings reinforce the developmental significance of pre-MBT transcription in *Xenopus* and zebrafish, as also reported in *Drosophila* (Liang et al. 2008; Harrison et al. 2010, 2011; Karr et al. 1985; Ali-Murthy et al. 2013; Biemar et al. 2005), and specifically underline the critical importance of early transcription of microRNAs that regulate maternal mRNA clearance and of early transcriptional activation of nodal signaling.

9.3.5 Large Scale Genome Activation at the MBT

The large-scale increase in zygotic gene expression associated with the MBT is firmly established in *Xenopus* and zebrafish. This has been documented by metabolic labeling (Newport and Kirschner 1982a; Kane and Kimmel 1993; Kimelman et al.

1987; Heyn et al. 2014; Yang et al. 2002) and gene profiling (Lee et al. 2014; Heyn et al. 2014; Harvey et al. 2013; Aanes et al. 2011; Collart et al. 2014; Paranjpe et al. 2013; Tan et al. 2013). Lee identified >7000 genes that are transcribed at or near the MBT in zebrafish, a majority of which were maternal genes not previously identified as transcribed again at the onset of large-scale zygotic transcription. A major wave of transcription in the first few days of development has also been documented in diverse mammalian species (reviewed in Li et al. 2013; Latham and Schultz 2001). The repression of transcription before MBT and activation at the MBT is also illustrated by the activity of injected RNAPIII dependent reporters.

Tadros and Lipschitz have nicely summarized the onset of zygotic transcription in diverse model organisms, and describe a minor wave of transcription that precedes the major wave, even in organisms that begin zygotic transcription early in the developmental program, suggesting a requirement for limited transcription in early development followed by large-scale changes required for the maternal to zygotic transition (Tadros and Lipshitz 2005). While it has been suggested that the minor wave might simply be a shoulder to, and part of, the major wave of transcription at the MBT, the fact remains that genes dedicated to specific functions, e.g., microRNAs involved in maternal RNA degradation and signaling molecules involved in mesendoderm development, are consistently transcribed 4–6 cell cycles before the major wave of zygotic transcription in multiple organisms. This regulation suggests that the minor and major waves of transcription are distinct in both their regulation and their functions, as also seen in *Drosophila* (Liang et al. 2008; ten Bosch et al. 2006; De Renzis et al. 2007).

How then is the major wave of transcription regulated? Specifically, how is the onset of zygotic transcription coordinated with other events of the MBT and what are the mechanisms of repression before pre-MBT or activation of large-scale expression at the MBT? The timing of zygotic gene activation and the MBT in general has been proposed to be coupled to the nucleus–cytoplasm (N:C) ratio or alternatively dependent on a timer that is uncoupled from the N:C ratio. There is evidence for both mechanisms in vertebrates as in *Drosophila*. The mechanisms for transcriptional regulation before and after the MBT may reflect the absence of an activator that accumulates or the presence of an inhibitor that is disengaged at the MBT. Again, there is evidence for both mechanisms (Tadros and Lipshitz 2005; Blythe and Wieschaus 2015a; Lee et al. 2014; Langley et al. 2014; Farrell and O'Farrell 2014; Lu et al. 2009).

9.3.5.1 Mechanisms of Repression and the N:C Ratio

The evidence for a repressive factor that must be titrated out by the exponential increase in zygotic DNA was elegantly and persuasively presented in the classical work of Newport and Kirschner and supported by subsequent work in zebrafish (Kane and Kimmel 1993) and *Drosophila* (Di Talia et al. 2013; Blythe and Wieschaus 2015a; Lu et al. 2009; Edgar and Schubiger 1986). Those experiments are summarized in Sect. 9.2, and here we only address the titration model with respect to the control of zygotic transcription.

At the time of Newport and Kirschner's experiments, it was clear that *Xenopus* embryos contain a large store of RNA polymerase that is active in extracts, suggesting that the lack of transcription before the MBT is due to repression. To test this hypothesis, Newport and Kirschner injected a yeast tRNA plasmid into cleavage-arrested *Xenopus* eggs. The tRNA was initially transcribed but was silenced within 2–3 h and remained silent until the MBT. This suppression correlated with assembly of the injected DNA into chromatin. As described for the onset of cell cycle asynchrony, injection of nonspecific DNA at approximately the amount found in the embryo at MBT caused premature activation of the yeast tRNA plasmid, as well as some endogenous, small RNAs, consistent with a repressive mechanism, at least for type III genes (Newport and Kirschner 1982b). In addition, Newport and Kirschner showed that increasing DNA content by generating polyspermic embryos led to transcription activation (of small RNAs) two cycles earlier than MBT in controls, also consistent with the timing of transcription depending on the N:C or more precisely the DNA–cytoplasm ratio.

Prioleau found that the c-myc promoter could also be transiently activated if preloaded with TATA binding protein (TBP) before injection (Prioleau et al. 1994). Similar to type III genes described by Newport and Kirschner, this plasmid was also assembled into chromatin and repressed before MBT, and coinjecting nonspecific DNA blocked repression. TBP had to be preloaded on the plasmid; overexpression by injection of TBP mRNA was insufficient to allow early expression of the reporter. These authors proposed that assembly of exogenous DNA into chromatin prevents binding of activating factors and thereby restrains transcription until the MBT (Almouzni et al. 1990; Newport and Kirschner 1982b; Prioleau et al. 1994; Amodeo et al. 2015).

Amodeo et al. (2015) further showed that histone H3 and H4 are titratable inhibitors of transcription in the pre-MBT embryo. They used *Xenopus* egg extracts to identify a threshold level of sperm chromatin needed to overcome an inhibitor present in extracts and in embryos. As nonspecific DNA will titrate the inhibitor, they assayed for removal of the inhibitor by DNA coated beads, then performed biochemical fractionation to isolate inhibitory factors identified by mass spec as histones H3 and H4. Purified H3/H4 inhibited transcription of sperm chromatin in vitro; furthermore, overexpression of H3 suppressed and knockdown of H3 accelerated the onset of RNAPIII dependent transcription in vivo. While this biochemical tour-de-force provides strong support for the findings of Newport and Kirschner, Prioleau et al., and Almouzni et al., who showed that addition of H2A/H2B dimers also inhibited transcription in *Xenopus* extracts (Almouzni et al. 1990, 1991), it also raises new questions. It remains unclear how the reduced ratio of core histones to DNA at the MBT results in gene specific activation; additional regulation, such as nucleosome remodeling, changes in chromatin architecture or attachment to the nuclear matrix, and posttranslational modification of core histones, likely contribute to the control of zygotic transcription (comprehensively reviewed in Bogdanovic et al. 2012; Veenstra 2002), as discussed further below.

The above work is consistent with the Newport and Kirschner hypothesis that zygotic transcription at the MBT is determined by the N:C ratio, similar also to findings in *Drosophila*. However, much of the work on transcription in early

Xenopus and *Drosophila* has focused on type III transcription (Newport and Kirschner 1982b; Almouzni et al. 1990; Amodeo et al. 2015; Brown 2004; Brown and Gurdon 1977, 1978), and although Prioleau showed that the c-myc promoter is also repressed before MBT, a different story emerged for the regulation of other type II genes in cleavage stage *Xenopus* embryos. Increasing nonspecific DNA content did not cause premature activation of exogenous U1 genes, which are RNAPII dependent (Lund and Dahlberg 1992). Similarly, Almouzni and Wolffe found that injection of nonspecific DNA does not activate a CMV promoter or other type II genes (Almouzni and Wolffe 1995). Furthermore, an exogenous type II promoter can be activated if the transactivator is also present: Expression of GAL4-VP16 at sufficient levels drives a GAL4 dependent reporter throughout pre-MBT stages, showing that the type II transcription apparatus is functional (Almouzni and Wolffe 1995). Almouzni and Wolffe proposed that the suppression of type II transcription is through a mechanism distinct from type III regulation, and, in addition to regulation by nucleosome assembly, depends on the availability of gene-specific transcription factors (as discussed in detail above for endogenous genes in *Xenopus* and zebrafish).

Transcription During Short Cell Cycles

The notion that transcription is not compatible with the rapid cell cycles of cleavage divisions has been proposed. According to this model, the high density of replication origins in cleavage stage embryos and the progress of the replication apparatus disrupt transcription complexes and furthermore the short duration of S-phase limits transcription to short RNAs. In this model, slowing of the cell cycle at the MBT allows transcription.

In support of this model, treatment of *Xenopus* embryos with cycloheximide two cycles before the MBT blocks progression into mitosis and causes premature activation of type III zygotic gene expression, despite the DNA content being fourfold lower than MBT. Similar observations were made in *Drosophila* (Edgar and Schubiger 1986). However, cycloheximide is a blunt instrument, and treatment at earlier stages in either *Xenopus* or *Drosophila* has the opposite effect to block zygotic transcription at MBT (Edgar and Schubiger 1986; Lund and Dahlberg 1992; Blythe and Wieschaus 2015a; Lee et al. 2014; Sible et al. 1997).

Using a more targeted approach, Collart et al. showed that the replication factors Cut5, RecQ4, Treslin, and Drf1 become limiting for DNA replication as the N:C ratio increases in *Xenopus* embryos (Collart et al. 2013), as also discussed above. They proposed that the limited availability of these replication factors slows the cell cycle and permits transcription at the MBT. Overexpression of the four factors delayed expression for a subset of zygotic genes, consistent with these factors acting as repressors that are titrated out as the N:C ratio approaches a critical value. While it remains possible that overexpression of these factors impairs transcription of this subset of genes independently of the effects on replication, these data nevertheless show a compelling correlation between availability of the four replication factors and multiple events at the MBT.

However, a simple model involving constraint from rapid DNA replication does not take into account the following observations: (1) Sea urchins initiate large scale zygotic transcription during early cleavage stages despite cleavage divisions as rapid as *Xenopus* or zebrafish (Davidson 1986), and *C. elegans* similarly initiates zygotic transcription during a period of rapid cell division, arguing that rapid DNA replication alone is not sufficient to delay zygotic transcription. (2) Mammalian embryos delay zygotic transcription for up to several days, despite their slow cell cycles, implying the need for a distinct mechanism to delay zygotic gene activation in mammals. (3) Transcription of endogenous genes is readily detectable during early cleavage stages in *Xenopus*, zebrafish, medaka (Tani et al. 2010; Kraeussling et al. 2011), and *Drosophila* and is essential for post-MBT development in *Xenopus* and *Drosophila*. (4) Prolonging the cell cycle by activation of Chk1 before the MBT in zebrafish embryos does not cause premature zygotic transcription (Zhang et al. 2014). Similarly, prolonging the cell cycle before the MBT does not enhance transcription of exogenous type II genes in *Xenopus* (Almouzni and Wolffe 1995), 1-cell mouse embryos (Aoki et al. 1997), or *Drosophila* (inferred from normal timing of cellularization; McCleland and O'Farrell 2008). (5) *Xenopus* embryos deficient for Wee-1 continue to undergo rapid cell cycles after the MBT, yet activate zygotic transcription at the 12th division, similar to control embryos, demonstrating (as in sea urchins) that slowing of the cell cycle is not required to activate transcription (Murakami et al. 2004), and (6) Overexpression of the four DNA replication factors described above extends rapid cell cycles beyond the 12th division (MBT), and while the onset of transcription of some genes is delayed, a "large number" of zygotic genes is still activated at the canonical MBT (Collart et al. 2013). These observations argue that the restriction of zygotic transcription is not explained solely by the constraints of rapid DNA replication. In fact, the converse appears to be true in *Drosophila*, where recruitment of RNAPII drives DNA replication stalling in early *Drosophila* embryos (Blythe and Wieschaus 2015b).

In contrast, transcription of most loci is suppressed during mitosis, although some transcription factors remain associated with chromatin throughout mitosis (Kadauke and Blobel 2013). If RNA polymerase moves at a rate of ~50–100 nt/s, then a 10 min S-phase may limit synthesis of unprocessed RNAs to <60 kilobases. Empirically, a bias toward short transcripts has been reported for pre-MBT genes in zebrafish (Heyn et al. 2014) and *Drosophila* (Rothe et al. 1992; Shermoen and O'Farrell 1991; De Renzis et al. 2007; Swinburne and Silver 2008).

DNA Methylation

Differential DNA methylation was at one time proposed as a mechanism to control zygotic gene activation at the MBT (Stancheva and Meehan 2000). Knockdown of the maintenance DNA methyltransferase, *Dnmt1*, reduces global 5-methylcytosine levels and leads to precocious activation of a subset of zygotic genes in *Xenopus* (Ruzov et al. 2004, 2009; Dunican et al. 2008). However, knockdown does not alter methylation patterns at the promoters of precociously expressed genes and a catalytically inactive

form of Dnmt1 restores normal timing of expression, indicating that transcriptional repression by Dnmt1 is not mediated by DNA methylation (Dunican et al. 2008). These observations are consistent with the finding that global DNA methylation does not change from pre-MBT to post-MBT stages (Veenstra 2002).

Zebrafish and mammalian embryos undergo global demethylation and remethylation after fertilization (Andersen et al. 2012, 2013; Potok et al. 2013). The interplay of DNA methylation and regulation of early gene expression in mouse embryos has been thoroughly reviewed elsewhere (Plasschaert and Bartolomei 2014; Weaver et al. 2009; Paranjpe and Veenstra 2015; Rivera and Ross 2013; Li et al. 2010, 2013). In zebrafish, DNA methylation reaches a nadir at the 64-cell stage and then increases through the MBT. DNA methylation status mostly correlates with the time of gene expression, with genes expressed at MBT typically being hypomethylated and genes involved in later development being hypermethylated. Most promoters maintain their respective states of methylation from pre-MBT to post-MBT stages, although a minority undergoes dynamic changes in methylation (Andersen et al. 2012, 2013; Potok et al. 2013). Remarkably, the DNA methylation pattern in sperm is highly similar to embryonic DNA at MBT, whereas the maternal DNA methylation pattern is reprogrammed during embryogenesis, suggesting that the methylation pattern in sperm could inform later patterns of gene expression in the embryo (Potok et al. 2013).

N:C Volume Ratio

As discussed above, the N:C *volume* ratio also contributes to the timing of MBT, including the onset of zygotic transcription (Jevtic and Levy 2015). Jevtic and Levy increased nuclear volume by overexpressing importin-α and lamin B3 in one half of *Xenopus* embryos and examined the onset of expression of GS17, an established MBT marker (Krieg and Melton 1985; Harvey et al. 1986). In the injected halves, GS17 was expressed well before the MBT in ~80 % of embryos, but not until the MBT on the control side. Conversely, reducing nuclear volume by overexpression of the reticulon family protein Rtn4a delayed the onset of GS17 expression. They also observed increased pre-MBT expression of *Xnr5*, a known pre-MBT zygotic transcript (Yang et al. 2002), when they increased nuclear volume, suggesting that pre-MBT transcription may also be sensitive to the N:C volume ratio (Jevtic and Levy 2015).

9.3.5.2 Activating Factors and an N:C Independent Timer

In addition to taking a foot off the brakes, the embryo may need to step on the accelerator. Accumulation of activating factors, for example by translation of maternal mRNAs, may contribute to a developmental timer that functions independently of the N:C ratio. Howe et al. described a developmental timer that coordinates cyclin E1 degradation with the MBT (Howe and Newport 1996). Evidence for a translation

coupled timer for the MBT is further supported by the observations in *Xenopus* and zebrafish that cycloheximide exposure during early cleavage stages (as opposed to treatment one or two cycles before MBT) blocks zygotic transcription, even when nonspecific DNA is injected to match the expected concentration of DNA at the MBT (Lund et al. 2009; Lee et al. 2014).

As discussed above, *Nanog*, *Pou5f3*, and *SoxB1* are required for zygotic gene activation in zebrafish, and are in this sense analogous to *Zelda* in *Drosophila*. Knockdown of all three genes causes lethality similar to treatment with α-amanitin (Lee et al. 2013b). As these three factors are present as maternal mRNAs and are translated prior to the MBT, their progressive accumulation could function as an N:C independent timer. Notably, these factors are required for very early zygotic expression of miR-430, and therefore for the regulation of maternal mRNA stability as well as global gene activation at the MBT. *Oct4* (*Pou5f1*) and *Nanog* have also been implicated in the activation of zygotic gene expression at the two-cell stage in mouse (Foygel et al. 2008; Tan et al. 2013). A similar role for pluripotency factors has not yet been reported in the regulation of zygotic transcription in *Xenopus*.

TBP

As described above, TBP can facilitate the expression of exogenous reporter plasmids in cleavage stage *Xenopus* embryos, especially type III promoters. More generally, TBP is required for basal and active transcription and is considered an essential component of the transcription complex (Hernandez 1993). TBP and closely related factors are essential for embryonic development in *Xenopus*, zebrafish, and *C. elegans* (reviewed in Lee et al. 2014; Bogdanovic et al. 2012; Veenstra 2002). TBP mRNA is present maternally but the protein is barely detectable in the egg. Translation during cleavage stages leads to accumulation of TBP protein that correlates with large-scale zygotic gene activation in frogs and mice, suggesting new translation of TBP contributes to transcriptional activation at the MBT (Veenstra et al. 1999; Bell and Scheer 1999; Bogdanovic et al. 2012; Veenstra 2002). Regulated translation of maternal TBP mRNA during early development could therefore be a component of an N:C independent timer for zygotic transcription at the MBT.

Smicl and RNAPII

Collart et al. reported an increase in C-terminal phosphorylation of RNAPII (at sites associated with elongating RNAPII) at the MBT that was mediated by the Smad-interacting CPSF 30-like factor (Smicl), which translocates from the cytoplasm to the nucleus at the MBT in *Xenopus*. Knockdown of Smicl expression reduced the expression of multiple genes at the MBT and delayed the degradation of several maternal mRNAs (Collart et al. 2009), supporting the hypothesis that enhanced nuclear translocation of Smicl and increased CTD phosphorylation of RNAPII facilitates zygotic transcription at the MBT.

Geminin

Geminin is an unstable protein that inhibits the replication factor Cdt1 to prevent a second round of DNA replication during S-phase. Knockdown of Geminin or overexpression of Cdt1 in *Xenopus* embryos causes G2 arrest one cycle after the MBT and markedly reduces expression of multiple genes normally expressed at the MBT, but not expression of the pre-MBT zygotic transcripts for *Xnr5* (Kerns et al. 2012). These observations suggest that Geminin is essential specifically for zygotic transcription at the MBT, but not before. However, Lim et al. (2011) found that Geminin overexpression represses expression of lineage specific markers after the MBT and knockdown (with a different morpholino than that used by Kerns et al.) enhanced their expression. These two studies appear incompatible, and the reasons for these differences are not clear.

9.3.6 Histone Modifications During Early Development

In addition to assembly of nucleosomes and DNA methylation, discussed above, posttranslational modifications of histones that correlate with both activation and repression are dynamic across development, from the gametes through zygotic gene activation. The chromatin landscape through development has been comprehensively covered in authoritative reviews (Bogdanovic et al. 2012; Lee et al. 2014; Blythe and Wieschaus 2015a; Andersen et al. 2013), and is only touched on briefly here.

Surveys of histone modifications during development in *Xenopus* and zebrafish have revealed some shared general features and interesting differences. In general, chromatin modifications precede gene expression in many cases, consistent with a chromatin prepattern or poised chromatin state (Lindeman et al. 2011; Blythe et al. 2010; Potok et al. 2013). In general, the detection of both activating and repressive marks increases during the blastula stage, although the time that these marks can first be detected relative to the MBT depends on the sensitivity of the ChIP methods, a critical issue in an early embryo that contains a limiting number of nuclei/chromosomal DNA within a large cytoplasmic volume, filled with abundant yolk proteins, maternal RNAs, and free histones that may also carry posttranslational modifications (Shechter et al. 2009; Toyama et al. 2008).

Akkers et al. assessed genome-wide H3K4me3, H3K27me3, and RNAPII occupancy in gastrula stage *Xenopus tropicalis* by ChIP-Seq and showed that presence of both H3K4me3 and H3K27me was associated with genes that are transcribed in a localized manner (Akkers et al. 2009); these marks were present on distinct nucleosome populations, distinguishing them from bivalent marks described for example in mouse embryonic stem cells. Using ChIP-PCR for a selected set of developmental genes, they first detected H3K4me3 in post-MBT blastulae either coincident with or preceding expression. H3K27me appeared later, at the gastrula stage, and was predictive of localized expression. Examining a distinct set of

genes, Blythe et al. detected H3K4me3, RNAPII recruitment, H3R8me2a, and specific transcription factor binding in *X. laevis* two cell cycles before the MBT at both transcribed genes and nontranscribed, poised promoters (Blythe et al. 2010). The establishment of poised chromatin architecture at dorsal specifying genes before the MBT was required for localized expression and dorsal development after the MBT, demonstrating an essential developmental function for pre-MBT chromatin modifications.

An initial study in zebrafish used a microarray-based approach (ChIP-chip) to survey chromatin marks in 31 Mb of the zebrafish genome; in contrast to findings in *X. tropicalis*, they found that H3K4me3 and H3K27me3 were present within the same population of nucleosomes, consistent with bivalent chromatin marks (Vastenhouw et al. 2010). They were first able to detect H3K4me3 and H3K27me3 after the MBT. However, more sensitive methods in zebrafish subsequently detected >1000 promoters with H3K4me3 before the MBT (256-cell), and fewer genes marked with H3K27me3 (200) and H3K9me3 (500) (Lindeman et al. 2011). Lindeman et al. detected chromatin marks as early as three cell cycles before the MBT by western blot and immunofluorescence. Many of the genes containing H3K4me3 lacked evidence of elongating RNAPII and were presumed to be poised, non-expressed genes, although direct measurements demonstrated that a subset of genes with pre-MBT H3K4me3 marks are in fact expressed before the MBT (see above). Nevertheless, the prevalence of modified histones prior to the MBT at genes that are mostly inactive suggested a chromatin prepattern that anticipates later developmental gene expression.

The pattern of histone modifications at the MBT in zebrafish embryos was very similar to the pattern in sperm, as also observed for DNA methylation (Potok et al. 2013), consistent with inheritance of a chromatin prepattern that may lay the transcriptional groundwork for early development.

The regulation of zygotic gene activation by changes in chromatin architecture has also been investigated in mammals, principally mouse, and is more thoroughly reviewed elsewhere (Li et al. 2010, 2013). Maternal loss of Brg1, a core component of Swi/Snf chromatin remodeling complexes, impairs activation of ~30 % of zygotic genes, reduces H3K4 methylation, and causes peri-implantation lethality in the mouse (Bultman et al. 2006). Similarly, overexpression in mouse oocytes of a mutant form of histone H3.3 that cannot be methylated at lysine-4 (or knockdown of the H3K4 methyltransferase Mll3/4) causes developmental arrest after fertilization and impairs the minor wave of zygotic gene expression in the paternal pronucleus (Aoshima et al. 2015). Loss of TIF1a (transcription intermediary factor 1a), which is recruited to sites occupied by Brg1, also causes embryonic lethality at the 2–4 cell stage and disrupts activation of zygotic gene expression (Torres-Padilla and Zernicka-Goetz 2006). Activation of zygotic genes depends on additional factors involved in chromatin or nuclear structure such as the PRC1 components Ring1 and Rnf2 (Posfai et al. 2012), CTCF (Wan et al. 2008), and nucleoplasmin 2 (Npm2) (Burns et al. 2003), the pluripotency factors Oct4 (Pou5f1) (Foygel et al. 2008) and Sox2 (Pan and Schultz 2011), as well as other factors, as cited by Li et al. (2010, 2013).

9.3.7 Summary

Vertebrate embryos delay the first major wave of zygotic transcription from hours to days after fertilization, although the number of cell divisions until zygotic gene activation varies. Large-scale changes in chromatin architecture appear to anticipate and/or accompany these changes, although the timing and strategy for regulation of gene expression may vary between organisms. The capacity for transcription exists already in the egg, indicating that repressive mechanisms are programmed to delay zygotic transcription. In oviparous species a repressive activity linked to chromatin assembly is titrated out as the DNA content and/or nuclear volume increases relative to the cytoplasm, but there is also evidence for N:C independent timing mechanisms that may regulate zygotic gene activation, maternal RNA clearance, and cell cycle remodeling. Recent work has identified specific mRNAs and microRNAs robustly expressed before the MBT that are essential for critical developmental processes that define the maternal to zygotic transition, including clearance of maternal RNAs and activation of the earliest steps in germ layer formation. Whether expression of these early RNAs depends on the N:C ratio or instead is regulated by an N:C independent timer remains to be investigated.

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Chapter 10
Clearance of Parental Products

Petr Svoboda, Helena Fulka, and Radek Malik

Abstract The beginning of development is controlled parentally. For example, early zygotic proteosynthesis produces proteins encoded by the maternal transcriptome. As parental factors become replaced by factors synthesized in the embryo, parental developmental control is gradually passed to the embryo. This chapter focuses on the clearance of parental factors during oocyte-to-embryo transition in vertebrates. Coordinated removal of parental factors erases ancestral oocyte identity of the zygote and facilitates reprogramming of gene expression into a state that will support development of a new organism. Here, we will review functional and mechanistic aspects of clearance of selected parental factors from early embryos, including different types of maternal RNAs, proteins, erasure of chromatin features of maternal and paternal genomes, as well as consumption of yolk and elimination of paternal mitochondria.

Keywords mRNA degradation • Protein degradation • Proteasome • Fertilization • Maternal • Zygotic • Chromatin • Oocyte • Reprogramming

10.1 Introduction

This chapter summarizes various aspects of clearance of parental products during oocyte-to-embryo transition (OET, Fig. 10.1). OET is a process during which a fully grown oocyte undergoes meiotic maturation, fertilization, and zygotic genome activation (ZGA, sometimes also referred to as midblastula transition (MBT)) and becomes transformed into a developing embryo.

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The term OET is closely intertwined with an interchangeably used term maternal-to-zygotic transition (MZT). MZT is defined as a period that begins with the elimination of maternal transcripts, continues through the production of zygotic transcripts, and ends with the first major requirement for zygotic transcripts in embryonic development (Tadros and Lipshitz 2009). As our review on clearance of parental products goes beyond transcriptome changes, we opt for using OET, which is a broader term for transition of a fully grown oocyte to an embryo. This will also simplify reviewing different vertebrate model systems together given differences in stage nomenclature and different usage of terms such as zygote or embryo in different communities studying vertebrate models.

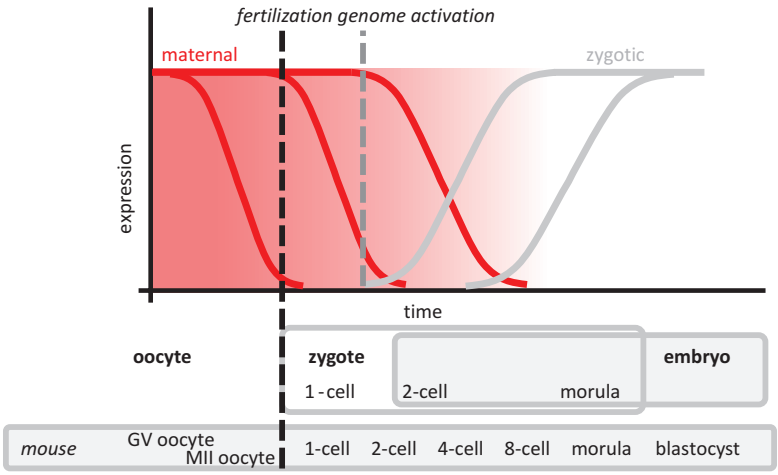

Fig. 10.1 Vertebrate oocyte-to-embryo (OET) transition. Vertebrate OET spans a period from the end of oocyte's growth phase to the formation of an embryo where gene expression control is relieved from maternal factors (represented by the *red gradient*). Maternal factors, as maternal mRNAs (represented by *red curves*), are eliminated differentially, frequently in response to developmental events such as transcription or genome activation. Zygotic gene expression is represented by gray curves representing differential gene expression appearing from the onset of the zygotic genome activation. The scheme corresponds well to the stages in the mouse model depicted at the bottom of the scheme. Above the mouse model is a more generalized labeling of the OET graph where the *rectangles* indicate loosely defined usage of the terms zygote and embryo with regard to embryonic stages. The term zygote can be used for the fertilized egg or for all cleavage stages embryos until the genome activation (or midblastula transition), which takes place at different timepoints/early developmental stage in different species. As a consequence, research on different model systems recognizes slightly different preferences for the terminology

10.1.1 Oocyte-to-Embryo Transition in Brief

While a thorough review of OET in vertebrates is beyond the scope of this chapter, we need to introduce essential landmarks appearing during the journey of a fully grown oocyte toward a "self-sufficient" embryo (Fig. 10.1). OET begins at the end of the oocyte growth phase when transcription ceases and the oocyte that was arrested in the prophase of the first meiotic division resumes meiosis. These two events (transcriptional quiescence and resumption of meiosis) may occur at different timepoints, and each of them is associated with signaling events/cytoplasmic changes with effects on stability of maternal factors. Next, the oocyte progresses through meiosis and typically becomes arrested at the metaphase of the second meiotic division (metaphase II, MII). Fertilization triggers release from the second meiotic arrest and completion of meiosis. After fertilization, maternal and paternal genomes begin to occupy the same nucleus (in mammals, the first true zygotic nucleus appears at the 2-cell stage) and, at some cleavage stage, will become transcriptionally active. Finally, zygotic expression will take over maternal gene products, and embryo's development will become fully controlled by genes expressed from its own genome.

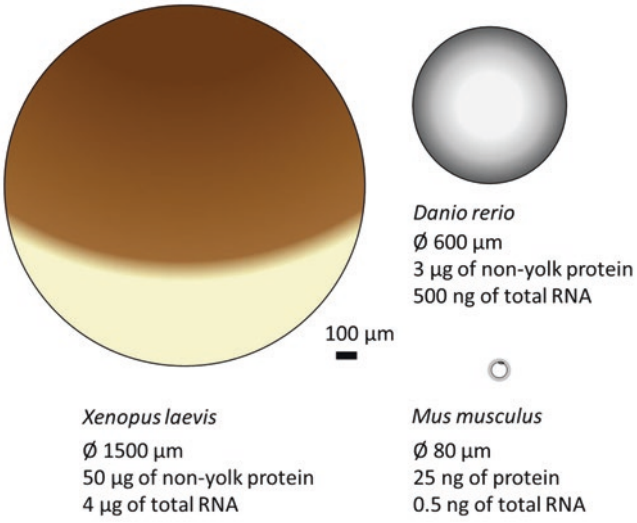

Fig. 10.2 Vertebrate oocyte diversity. Shown are schematic drawings of *Xenopus*, zebrafish, and mouse oocytes. Oocytes are drawn to the same scale indicated by the scale bar 100 µm

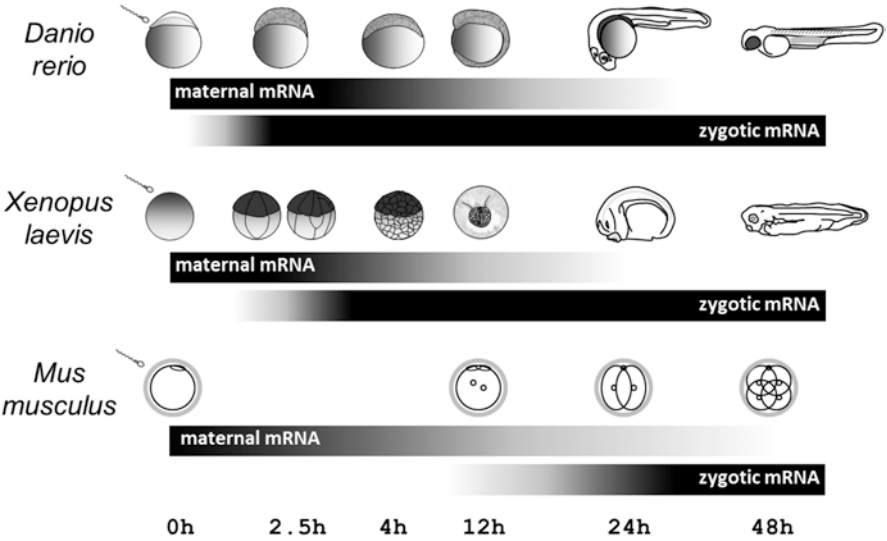

Fig. 10.3 Variable timescales of vertebrate OETs. Shown is early development of the three common vertebrate models for studying early development. Maternal mRNA decay and zygotic genome activation (ZGA) are drawn according to published data. In a strictly temporal timescale, the earliest zygotic genome activation is observed in the zebrafish followed by the *Xenopus* model. Zygotic transcription in mouse embryos is observed during the 1-cell stage; however, this transcription does not seem to produce functional mRNAs replacing maternal mRNAs. ZGA in the mouse embryo takes place at the 2-cell stage; in other mammals (e.g., cows, humans), ZGA takes place later

This chapter is centered around the mouse model, which is compared and contrasted with other mammals, *Danio rerio* and *Xenopus laevis*/*tropicalis*. There are plenty of aspects important for a comparative summary of vertebrate OET, which deserve attention. First, it must be appreciated that vertebrates are a highly diverse group, which evolved over 500 million years. Vertebrates include aquatic, amphibian, or terrestrial animals having external or internal fertilization. Accordingly, embryonic development exists in three forms differentiated by parental involvement—embryos develop externally or internally with or without connection to the maternal cardiovascular system. Adaptations to different habitats and reproductive strategies can explain extreme diversity of vertebrate egg morphology (Fig. 10.2), different amounts of deposited nutrients and information, as well as pace of initial development (Fig. 10.3). For example, external development of zebrafish and *Xenopus* embryos is very fast relative to the embryonic development of placentals. Zebrafish and *Xenopus* embryos undergo intense organogenesis 1 day after fertilization. The 24-h-old mouse zygote has two cells and just initiated its own mRNA synthesis; it will take it two more days to initiate separa-

tion of extraembryonic and embryonic lineages and over another day to implant and gastrulate.

10.1.2 Accumulation of Maternal Factors

Maternal factors, which accumulate in vertebrate oocytes, can be divided into (1) energetic/synthetic resources (e.g., yolk, mitochondria, ribosomes) and (2) control factors (e.g., mRNAs, RNA-binding proteins, transcription factors). The yolk content in some vertebrate oocytes buttresses the notion of a warehouse stockpiled with material and fuel for the embryo—as exemplified by the ostrich egg, the largest egg, which is ~15 × 13 cm in diameter and weights 1.4 kg (Hyde 2004). It is astonishing that this entire structure filled with gigantic amount of yolk evolved to support embryonic development from a single diploid nucleus. *Xenopus* eggs, the largest of the three vertebrate models discussed in detail here, are ~1.5 mm in diameter and contain ~4 μg of total RNA (Hough and Davidson 1975) and ~50 μg of non-yolk proteins (Smits et al. 2014). Zebrafish eggs are smaller (~0.7 mm) and contain ~500 ng of total RNA (de Jong et al. 2010) and ~3 μg of non-yolk proteins (Link et al. 2006). An important characteristic of *Xenopus* and zebrafish oocytes is that they are polarized. This polarization is accompanied by differential distribution of factors, which constitute the maternal preprogramming of the elementary body plan and the future germline (reviewed in Pelegri 2003).

Mammalian oocytes offer a different picture. Apart from the relatively low amount of yolk and lack of evidence for polarization that would determine embryonic axis formation, there does not seem to be such extensive stockpiling as observed in zebrafish and *Xenopus*, which have external fertilization and development. Mammalian oocytes are small relative to the examples above (80–120 μm in diameter); it was estimated that they contain 0.3–2 ng of total RNA (Gilbert et al. 2009; Lequarre et al. 2004; Piko and Clegg 1982) and 25–450 ng of total protein (Gilbert et al. 2009; Schultz and Wassarman 1977).

Taken together, zebrafish and *Xenopus* zygotes are truly stockpiled with maternal factors and develop at a fast pace. In contrast, mammalian zygotes develop relatively slowly, and maternal stockpiling and parental governance of embryonic development are rather moderate.

10.1.3 Aims and Scope of the Chapter

This chapter will explore elimination of parental factors. Among reviewed maternal factors will be RNAs, proteins, and epigenetic marks. These factors can be divided into oocyte-determining factors, which need to be removed to erase oocyte's

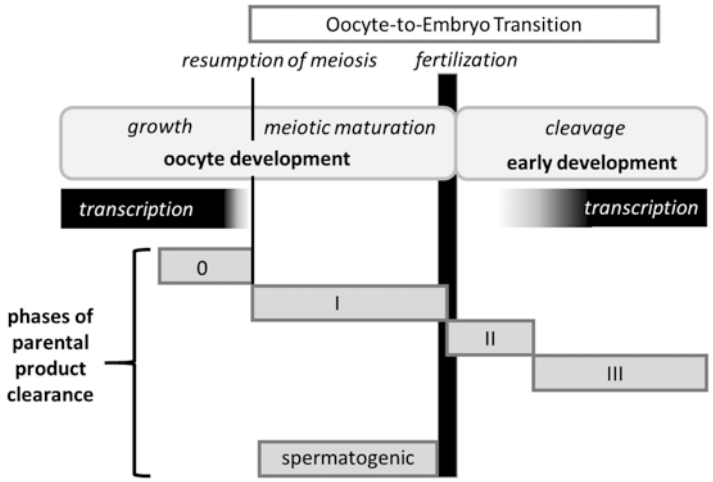

Fig. 10.4 Schematic depiction of different phases of parental product clearance during vertebrate OET. Phase I is positioned at resumption of meiosis, which is traditionally taken as the first transition occurring during OET

identity, and reprogramming factors, which are provided by the oocyte to reprogram gene expression in the zygote into a totipotent state. Although the majority of paternal gene products defining male germs cell are eliminated during spermatogenesis, including most of the cytoplasm and nucleosomal chromatin, some paternal factors are still transmitted to the zygote. To acknowledge that OET also includes elimination of paternal factors, we will review two contributions from the father to the zygote, which are eliminated during early development: paternal DNA methylation and paternal mitochondria.

The process of clearance of parental products will be divided into phases whose initiation or termination is defined by one of the major developmental transitions during OET (Fig. 10.4). We designate clearance of maternal products initiated upon resumption of meiosis as Phase I since resumption of meiosis is conventionally seen as OET initiation. Phase II is framed by fertilization and initiation of the first major wave of zygotic transcription. It includes clearance of maternal products (e.g., mRNAs, proteins) as well as some paternal factors (e.g., paternal DNA methylation in mammals). Phase III is initiated by ZGA. In addition, we introduce two phases for events that occur outside the three phases above. Phase 0 includes clearance of maternal products occurring already before resumption of meiosis. Finally, we introduce a phase designated spermatogenic to highlight the fact that the process of spermatogenesis is characterized by extensive elimination of paternal factors (e.g., paternal mRNAs and nucleosomal chromatin).

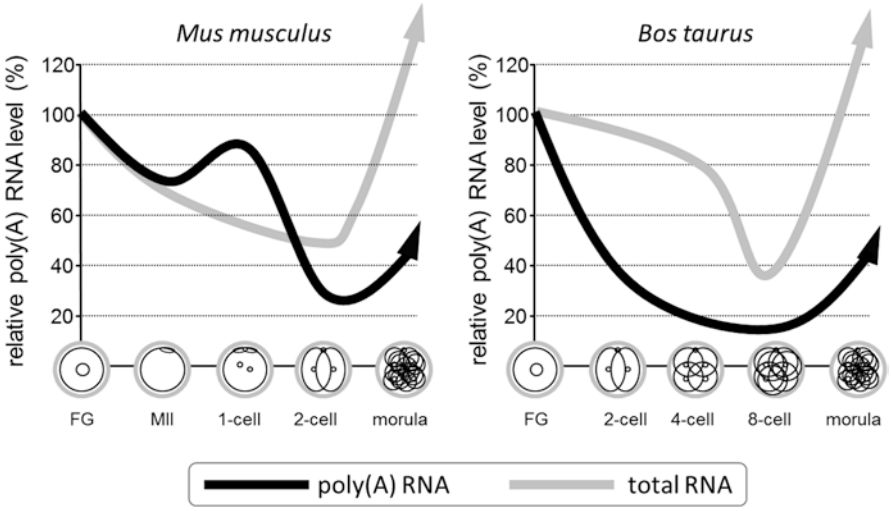

Fig. 10.5 RNA degradation during mammalian OET. Total RNA and poly(A) RNA changes during OET in mice (*left graph*) and cows (*right graph*). Graphs are based on published RNA analyses (Gilbert et al. 2009; Piko and Clegg 1982; Bachvarova and De Leon 1980)

10.2 Maternal RNA Clearance

This section will review the fate of maternal RNA with a particular focus on degradation of three specific types of RNAs: small RNAs, mRNAs, and ribosomal RNAs. As mentioned above, vertebrate oocytes differ in the amount of deposited RNA that will support the newly developing organism. Analyses of mammalian (mouse, cow, rabbit, buffalo) and nonmammalian models (zebrafish, *Xenopus*, chicken) suggest two distinct scenarios of maternal RNA clearance in vertebrate early development. In mammals, total RNA is severely reduced (by 50 % or more) between oocyte's release from the ovary and the full zygotic genome activation in the cleaving embryo (Fig. 10.5). This indicates that dependence on maternal RNAs in mammals does not extend far into development. At the same time, it should be kept in mind that early mammalian development is relatively slow; hence, maternal RNA degradation per unit of time is perhaps not necessarily faster than in zebrafish or *Xenopus* whose embryonic development runs at much faster pace (Fig. 10.3). Analysis of total RNA content during the first 12 h of zebrafish development (unfertilized egg to 8-somite stage) showed that RNA yield does not significantly differ among stages (de Jong et al. 2010). Thus, it appears that fast-developing zebrafish embryos, which activate their genome within a few hours after fertilization, efficiently replenish the maternal RNA pool without a robust drop in RNA content. A similar situation is observed in *Xenopus* where the total RNA content is relatively stable from fertilization to gastrulation (Brown and Littna 1964).

10.2.1 Elimination of Maternal Small RNAs

Small RNA pathways utilize small noncoding RNAs as sequence-specific guides for mRNA degradation and/or translational repression (Ghildiyal and Zamore 2009). Vertebrates utilize three distinct small RNA pathways: RNA interference (RNAi), microRNA (miRNA), and PIWI-associated RNA (piRNA) pathways (Fig. 10.6). Molecular mechanisms of RNAi, miRNA, and piRNA pathways have been described in detail elsewhere (Jinek and Doudna 2009; Kim et al. 2009; Nejepinska et al. 2012a; Siomi et al. 2011; Chekulaeva and Filipowicz 2009). Below, we provide only basic characterization of these pathways while focusing on maternal small RNA functionality and turnover during OET.

10.2.1.1 microRNAs

miRNAs are genome-encoded posttranscriptional regulators of gene expression. Nuclear miRNA precursor transcripts are preprocessed, transported to the cytoplasm, and cleaved by RNase III Dicer to produce ~22-nucleotide-long miRNAs, which are loaded on Argonaute (AGO) proteins, which mediate silencing effects (reviewed in Kim et al. 2009). Animal miRNAs typically base-pair imperfectly with 3′UTRs of cognate mRNAs and cause their translational repression and/or degradation. The silencing mode depends on the degree of complementarity and

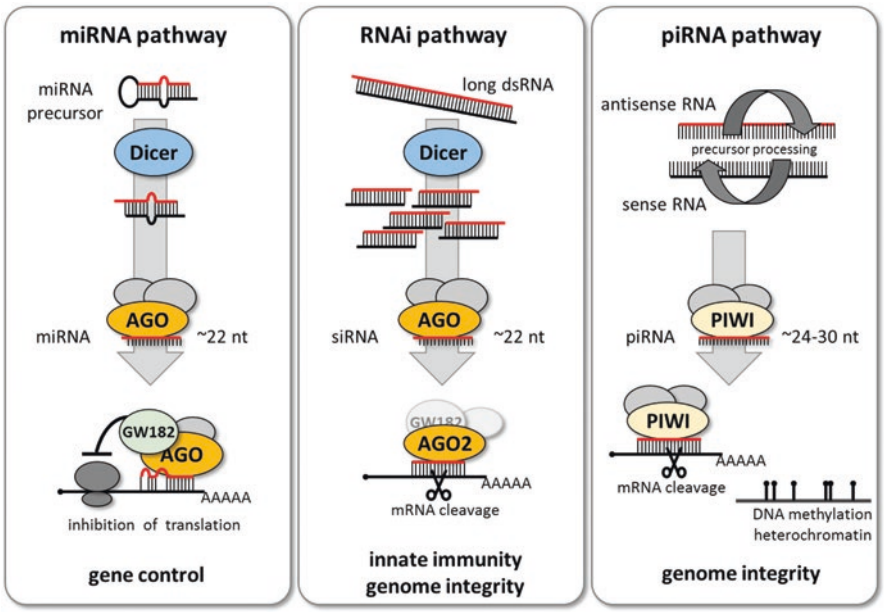

Fig. 10.6 Schematic depiction of vertebrate RNA silencing pathways and their key components

a specific AGO isoform. AGO2 and perfect complementarity result in a direct endonucleolytic cleavage; this effect requires only AGO2 loaded with a small RNA. Other combinations result in translational repression followed by mRNA destabilization and require fully formed effector complex containing translational repressor GW182 (reviewed in Braun et al. 2013). The miRNA pathway is the dominant small RNA pathway in animals—miRNAs are present in all cell types, and there is an ever-growing list of essential roles of miRNAs in a broad variety of cells and biological processes.

Next-generation sequencing of small RNAs during mouse OET revealed that maternal miRNAs are a relatively minor small RNA population in oocytes (Ohnishi et al. 2010; Garcia-Lopez et al. 2014). This notion is also consistent with the zebrafish and *Xenopus* miRNA analyses (Chen et al. 2005; Tang and Maxwell 2008; Yao et al. 2014); it appears that vertebrate oocytes do not accumulate maternal miRNAs during the growth phase. An exception of the rule might be miR-202-5p, which was reported to be enriched in *Xenopus* oocytes (Armisen et al. 2009). Although miRNAs can be detected in the sperm and may end up in the zygote, paternal miRNAs are extremely unlikely to contribute to OET as their levels would be too low to have significant effects (Amanai et al. 2006).

So far, there is no strong evidence for an important miRNA role in growing or fully grown oocytes. Dicer-deficient zebrafish oocytes develop and can be successfully fertilized (Giraldez et al. 2005). The phenotype in maternal/zygotic Dicer mutant fish appears during gastrulation and neural development and can be largely rescued by zygotic miR-430 miRNA (Giraldez et al. 2005). This suggests that maternal Dicer functions in biogenesis of zygotic miRNAs, while maternal miRNAs are functionally nonessential. A similar observation was made in the oocyte-specific conditional mouse knockout of *Dgcr8*, an essential miRNA biogenesis factor (Suh et al. 2010). Mouse oocytes lacking canonical miRNAs can be fertilized and develop to the term. Maternal/zygotic *Dgcr8* mutants can activate the genome and develop until the blastocyst stage (Suh et al. 2010).

Interestingly, canonical maternal miRNAs become nonfunctional in the mouse model in growing oocytes although their biogenesis and loading on AGO2 remain apparently intact (Ma et al. 2010). Uncoupling miRNAs from the repression of translation could be seen as the earliest known elimination of a parental factor during OET in the mouse. According to the proposed model, the global suppression of miRNA activity during OET facilitates the exchange of maternal and zygotic miRNAs in a gear-shift analogy: maternal miRNAs (e.g., Let-7 family) are disengaged from translational repression and replaced by zygotic miRNAs (e.g., miR-290 cluster), which become engaged when they reach significant amounts around the 8-cell/morula stages (Svoboda 2010). The molecular mechanism responsible for suppression of miRNAs in mouse oocytes remains unknown.

At this point, the dynamics of vertebrate maternal miRNA clearance is not clear, as high-throughput datasets from different vertebrate models do not completely cover miRNA changes during meiotic maturation. One wave of maternal miRNA degradation is induced by fertilization as reported for mice (Garcia-Lopez et al. 2014; Tang et al. 2007). This degradation appears global as all tested miRNAs

showed similar downregulation patterns (Tang et al. 2007). This likely reflects the fact that mature miRNAs do not carry any specific regulatory sequences, while their elimination should not interfere with an upcoming accumulation of zygotic miRNAs. Maternal miRNAs are rapidly eliminated after fertilization also in the zebrafish, as shown by small RNA cloning (Chen et al. 2005). However, some data suggest that miRNA degradation after fertilization is the second wave of maternal miRNA elimination. Northern blots of several maternal miRNAs in *Xenopus* show that miRNA degradation is initiated already before fertilization as indicated by lower miRNA levels in ovulated eggs (Watanabe et al. 2005). Furthermore, deep sequencing of fully grown and meiotically mature porcine oocytes also suggested reduction of miRNA levels during meiosis (Yang et al. 2012).

Although the molecular mechanism of maternal miRNA degradation in vertebrates has not been identified yet, it presumably involves RNA-specific ribonucleotidyl transferases (Martin and Keller 2007). First, uridylation or adenylation of RNAs by these enzymes is a well-established regulation of miRNA stability (reviewed in Ha and Kim 2014). Second, a noncanonical poly(A) polymerase Wispy was identified as the factor responsible for maternal miRNA adenylation and degradation in *Drosophila* (Lee et al. 2014).

10.2.1.2 Short Interfering RNAs (siRNAs) from the RNAi Pathway

RNAi is sequence-specific mRNA degradation mediated by small RNAs generated by Dicer from long double-stranded RNA (dsRNA). This pathway was originally identified in *C. elegans* (Fire et al. 1998) and later in vertebrates (Svoboda et al. 2000; Wargelius et al. 1999; Wianny and Zernicka-Goetz 2000; Nakano et al. 2000). Remarkably, oocytes were the first vertebrate cell type where RNAi was identified. The vertebrate RNAi is somewhat enigmatic; the pathway employs the same protein factors as the miRNA pathway, but endo-siRNAs (short interfering RNAs produced by Dicer from dsRNA) are typically found in negligible numbers in vertebrate somatic cells (e.g., Nejepinska et al. 2012b; Wei et al. 2012; Faunes et al. 2011). This is in agreement with functional analyses of human Dicer, which showed that it is efficiently processing miRNA precursors but not long perfect duplexes because of an autoinhibitory function of its N-terminal helicase domain (Ma et al. 2008a; Chakravarthy et al. 2010), which is conserved across vertebrates.

The RNAi pathway is apparently intact in vertebrate oocytes since mRNA knockdown was efficiently induced with long dsRNA in zebrafish (Wargelius et al. 1999) and *Xenopus* oocyte (Nakano et al. 2000). However, strong nonspecific effects of dsRNA were also observed in the zebrafish model (Zhao et al. 2001a). Furthermore, RNAi efficiency in *Xenopus* oocytes and early embryos might have reduced functionality due to limiting amounts of AGO2 protein (Lund et al. 2011). In mammals, RNAi effects can be masked by the interferon (IFN) pathway, which is a sequence-independent innate immunity response to dsRNA (reviewed in Gantier and Williams 2007). However, mouse oocytes lack the IFN response to dsRNA (Stein et al. 2005), and microinjected long dsRNA induces

robust RNAi in mouse (Svoboda et al. 2000; Wianny and Zernicka-Goetz 2000), bovine (Paradis et al. 2005), porcine (Anger et al. 2004), and ovine (Yan et al. 2010) oocytes.

So far, the only evidence for functional importance of endogenous RNAi in vertebrates comes from the mouse oocyte model, which represents a notable exception in terms of siRNA biogenesis. Mouse oocytes express a unique truncated Dicer isoform, which lacks the autoinhibitory N-terminal helicase domain and is efficiently producing endo-siRNAs (Flemr et al. 2013). This Dicer isoform evolved because of a retrotransposon insertion, which functions as an oocyte-specific promoter and is found only in the Muridae family (Flemr et al. 2013). As a consequence, mouse oocytes contain relatively high amounts of endo-siRNAs; most of them are retrotransposon-derived, but many originate from processed pseudogenes. The latter endo-siRNAs appear to functionally substitute for miRNAs in terms of regulation of endogenous genes (Tam et al. 2008; Watanabe et al. 2008). Importantly, endo-siRNAs in mouse oocytes appear essential for normal meiotic progression (Flemr et al. 2013; Tam et al. 2008; Watanabe et al. 2008; Kaneda et al. 2009). This likely reflects endo-siRNA-mediated mRNA degradation during Phase 0 of clearance of maternal mRNAs (see also Sect. 10.2.2.2). At the same time, the unique presence of the truncated Dicer isoform in the Muridae family and rapid evolution of processed pseudogenes suggest that an essential role for endogenous RNAi during meiotic maturation is presumably restricted to mice and closely related species.

Taken together, except in the mouse model, endogenous RNAi in vertebrate oocytes does not appear to be a significant pathway. Endo-siRNAs loaded on AGO proteins do not have any known specific features, which would distinguish them from miRNAs. Therefore, it is most likely that vertebrate maternal endo-siRNAs are degraded together with miRNAs.

10.2.1.3 PIWI-Associated RNAs (piRNAs)

piRNAs are found mainly in germ cells. The piRNA pathway (reviewed in Weick and Miska 2014) differs in several ways from miRNA/RNAi pathways. Briefly, piRNA complex biogenesis involves PIWI proteins (a specific clade of Argonaute protein family, which is expressed during gametogenesis) but not Dicer. piRNAs are longer (24–30 nucleotides) than miRNAs, and they are chemically modified at their 3′ end by 2′O-methylation. piRNAs originate from distinct genomic regions and come in two flavors—one represented by retrotransposon-derived piRNAs, which help to maintain genome integrity. The second flavor is represented by piRNAs derived from non-repetitive sequences; they are overrepresented in a unique piRNA population called pachytene piRNAs, which are essential for spermatogenesis, but their exact function is still being debated (Weick and Miska 2014).

piRNA pathway mutants often have sterile phenotypes, which underscore the functional importance of piRNAs for germline development. In the zebrafish, PIWI proteins are essential for both spermatogenesis and oogenesis (Houwing et al.

2007; Houwing et al. 2008). piRNAs are the most abundant small RNA class in zebrafish oocytes (Yao et al. 2014; Houwing et al. 2007). High piRNA abundance was also observed in *Xenopus* oocytes (Armisen et al. 2009). In mammals, piRNAs are present in fully grown oocytes; however, their abundance is much lower than that observed in zebrafish or *Xenopus* models (Ohnishi et al. 2010; Garcia-Lopez et al. 2014; Yang et al. 2012).

Regarding the clearance of maternal piRNAs, piRNA levels already gradually decrease during *Xenopus* oocyte growth although they remain abundant at the time of ovulation (Armisen et al. 2009). Their decay subsequently continues during early development (Armisen et al. 2009; Harding et al. 2014). Fertilization clearly accelerates piRNA decay. Deep sequencing of developing zebrafish embryos revealed that the population of maternal piRNAs was reduced by about a half within several hours after fertilization and gradually disappeared within 2 days (Yao et al. 2014). Postfertilization clearance of maternal piRNAs was also observed in mouse zygotes (Ohnishi et al. 2010; Garcia-Lopez et al. 2014). The mechanism of maternal piRNA clearance is unknown; maternal piRNAs may be targeted for degradation directly, or their clearance may reflect turnover of maternal PIWI proteins.

10.2.1.4 Maternal Small RNAs and Their Clearance: General Summary

Taken together, the composition of the maternal small RNA population varies among the vertebrate models. In general, miRNAs appear to be a relatively underrepresented small RNA class in vertebrate oocytes. The same appears true for endo-siRNAs, with the notable exception of mouse oocytes. piRNAs are highly abundant in zebrafish and *Xenopus* oocytes, while their levels are relatively low in mammalian oocytes. Deep sequencing of porcine oocytes suggests that piRNAs and endo-siRNAs might be even less abundant than miRNAs (Yang et al. 2012). Clearance of maternal small RNAs in vertebrates starts during oocyte growth when piRNA levels begin to decline. piRNA clearance is subsequently accelerated upon fertilization. Meiotic maturation seems to initiate miRNA degradation, which is also accelerated after fertilization. Thus, the general picture of vertebrate small RNA turnover during OET implies that maternal small RNAs of all types are eliminated after fertilization and become replaced by zygotic microRNAs, which are the dominant small RNA class in the developing embryo.

10.2.2 Degradation of Maternal mRNAs

Regulation of mRNA stability and translation is controlled by *cis*- and *trans*-acting factors. Eukaryotic mRNAs are protected by a 5′ cap structure and a 3′ poly(A) tail that control translation and mRNA stability. mRNA degradation mechanisms (reviewed in detail in Houseley and Tollervey 2009; Schoenberg and Maquat 2012; Balagopal et al. 2012) typically involve deadenylation of the 3′ poly(A) tail and/or decapping. In mammalian somatic cells, deadenylation coupled with decapping is

the main pathway regulating mRNA decay (Yamashita et al. 2005). In specific cases, mRNAs can be cleaved by an endonucleolytic activity (e.g., by RNA interference (RNAi)). The final step in degradation of mRNAs with accessible 5′ or 3′ end is exonucleolytic degradation by XRN1 or the exosome, respectively.

Maternal mRNAs can be divided into three groups with respect to their function during OET:

1. Translated mRNAs encoding proteins needed during oocyte growth but not later (e.g., oocyte-specific transcription factors). Some of these maternal factors might be even detrimental to OET and must be removed.
2. Translated mRNAs encoding proteins functioning during OET, i.e., during meiotic maturation and/or during early embryogenesis. This category includes proteins with OET-specific roles as well as proteins with housekeeping function.
3. Dormant maternal mRNAs—stable untranslated mRNAs, which are recruited for translation during meiotic maturation or early embryogenesis through *cis*-acting cytoplasmic polyadenylation elements (CPEs), which are bound by *trans*-acting CPE-binding proteins (CPEBs) (reviewed in Standart and Minshall 2008; Radford et al. 2008). Uncoupling an mRNA from translation allows for its accumulation and arrival of the encoded protein during transcriptional quiescence. This category includes factors involved in the control of meiosis (e.g., *Mos* or *Cyclin B1* (Sheets et al. 1994)) or regulating maternal mRNA degradation or zygotic genome activation (ZGA) (e.g., *Dcp1a*, *Dcp2*, *Cnot6l*, *Cnot7*, *Pan2*, and *Lin28a* in mice (Flemr et al. 2014; Ma et al. 2013a; Ma et al. 2015)).

Maternal mRNA degradation is driven by the three major OET transitions: resumption of meiosis (the traditionally recognized OET starting point), fertilization, and zygotic genome activation (Fig. 10.4). Transcriptome analyses in the mouse model suggest that each of these three transitions stimulates a wave of mRNA degradation (Fig. 10.7). At the molecular level, each wave ostensibly involves changes in the amount or activity of *trans*-acting factors regulating RNA stability (i.e., RNA-degrading enzymes or RNA-binding proteins), whose combinations determine changes in stabilities of individual mRNAs.

According to the phases of OET introduced in Sect. 10.1.3 (Fig. 10.4), we will divide maternal mRNA degradation (and RNA degradation in general) into three main phases according to developmental transitions initiating mRNA degradation:

Phase I (Sect. 10.2.2.3)—mRNA degradation initiated during meiotic maturation
Phase II (Sect. 10.2.2.4)—mRNA degradation induced by fertilization
Phase III (Sect. 10.2.2.5)—mRNA degradation induced by ZGA

Apart from the three stages mentioned above, we will discuss Phase 0 (Sect. 10.2.2.2), which concerns mRNAs whose degradation occurs before resumption of meiosis. Since a steady-state mRNA level reflects the balance between synthesis and turnover, decay of maternal mRNAs will commence with termination of transcription of at the end of the growth phase (Bouniol-Baly et al. 1999). Thus, maternal transcripts with a high turnover can decay even before resumption of meiosis.

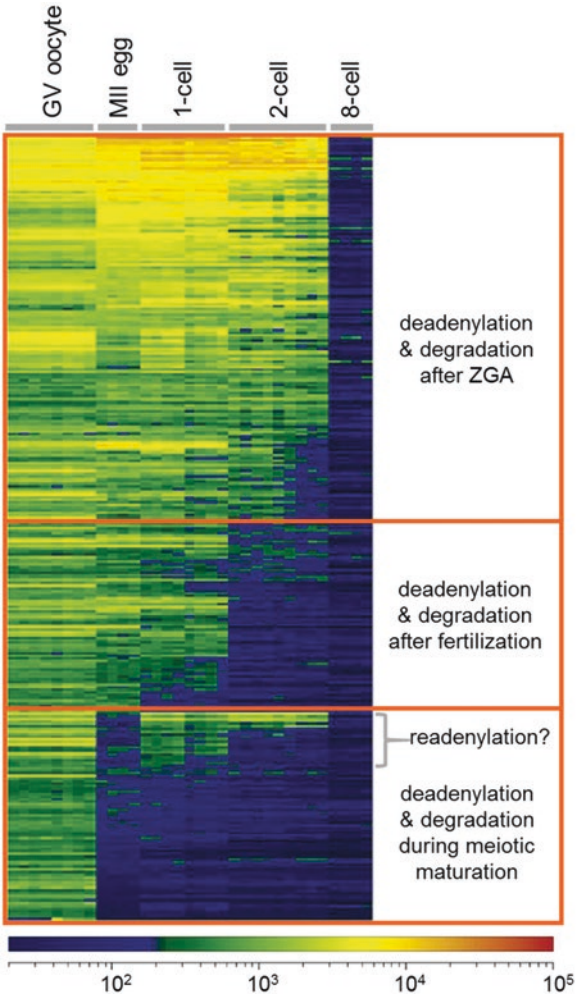

Fig. 10.7 Waves of maternal mRNA degradation during mouse OET. Shown is the normalized microarray hybridization signal on individual MOE430 arrays from different stages (*columns*) for 300 oocyte-specific genes (*rows*). Oocyte-specific genes were extracted from the BioGPS database (Su et al. 2004). OET expression was built from published datasets deposited in the GEO database (Su et al. 2007; Zeng and Schultz 2005; Zeng et al. 2004). The color scale indicates relative transcript abundance

It is important to mention that the four phases above represent a grossly simplified concept. In reality, RNA half-life during OET is a dynamically changing summation of multiple stronger and weaker regulations of *cis*- and *trans*-acting factors, while degradation of a specific transcript usually extends from one phase into another.

10.2.2.1 Dynamics of Maternal Poly(A) RNA During OET

Estimations of poly(A) RNA content during meiotic maturation and early development of mice and cattle provided initial insights into the dynamics of deadenylation and mRNA degradation (Fig. 10.5). Maternal mRNAs in mouse oocytes

are unusually stable during the growth phase, which takes about 2.5 weeks, with an average half-life of ~10–14 days as compared to hours or minutes in somatic cells (Bachvarova et al. 1985; Bachvarova and De Leon 1980; Brower et al. 1981). It is after the resumption of meiosis, when a globally destabilizing environment forms, in which many maternal mRNAs are extensively degraded (Bachvarova and De Leon 1980; Su et al. 2007). While the bulk of maternal mRNA is eliminated by the time the mammalian zygotic genome is fully activated (2-cell mouse embryos or 8-cell bovine embryos (Graf et al. 2014; Zeng and Schultz 2005)), maternal mRNAs are not degraded uniformly. The observed global elimination of maternal RNA during OET is a result differential stabilization/destabilization of populations of maternal mRNAs (Fig. 10.7).

The scale and complexity of differential control of maternal RNA stability in vertebrates have been revealed during the last decade by high-throughput transcriptome analyses using microarrays and next-generation sequencing in fish (Mathavan et al. 2005; Vesterlund et al. 2011; Harvey et al. 2013; Kleppe et al. 2012), frog (Paranjpe et al. 2013; Tan et al. 2013), and mammals (Su et al. 2007; Zeng and Schultz 2005; Wang et al. 2004; Hamatani et al. 2004; Zeng et al. 2004; Abe et al. 2015; Park et al. 2013; Xue et al. 2013; Yan et al. 2013; Deng et al. 2014; Xie et al. 2010).

It must be stressed that poly(A)-based mRNA estimations made for OET are an indirect measure of mRNA degradation or transcription because reversible deadenylation may cause false data interpretation. For example, although a slight increase in the total amount of poly(A) RNA was observed during fertilization in mice (Piko and Clegg 1982) (Fig. 10.5, left graph), it should be attributed to cytoplasmic RNA polyadenylation as there is no transcription at that time. Thus, a conserved core of the initial ZGA identified by single-cell poly(A) RNA sequencing (Xue et al. 2013) rather reflects changes in the cytoplasmic polyadenylation than true zygotic transcription detected by total RNA sequencing (Abe et al. 2015).

10.2.2.2 "Passive" Maternal mRNA Elimination Before Meiotic Maturation

Maternal mRNA degradation before resumption of meiosis (Phase 0) has not received much attention in the literature. However, the mouse transcriptome remodeling during Phase 0 has several interesting features, which justify its specific recognition. Phase 0 occurs in an mRNA-stabilizing environment where an average mRNA half-life is ~10–14 days (Bachvarova et al. 1985; Bachvarova and De Leon 1980; Brower et al. 1981). Thus, mRNA degradation during Phase 0 involves maternal mRNAs with naturally high turnover. Such high-turnover RNAs in fully grown oocytes might be even seen as a countdown clock turned on when transcription ceases.

Genome-wide analysis of transcript stability in mouse fully grown oocytes identified approximately 1000 mRNAs with a half-life of <48 h (Puschendorf et al. 2006). Only tens of mRNAs appeared to have a half-life of <12 h (i.e., were reduced greater

than eightfold during 48 h) (Puschendorf et al. 2006). Analysis of the 3′UTR composition of maternal transcripts showed that transcripts with higher turnover tend to contain U-rich motifs in their 3′UTRs (Svoboda, unpublished observation). However, the molecular mechanism associated with U-rich motifs remains unknown. There are many mechanisms that could cause selective transcript destabilization during Phase 0 such as RNA interference (RNAi, reviewed in Svoboda 2014), Staufen-mediated mRNA decay (reviewed in Park and Maquat 2013), or AU-rich element-mediated mRNA destabilization (summarized in Barreau et al. 2005). Interestingly, unstable transcripts were also derived from retrotransposons suggesting that RNAi, which is highly active in mouse oocytes (Flemr et al. 2013; Tam et al. 2008; Watanabe et al. 2008), might be one of the molecular mechanisms operating during Phase 0.

The functional significance (if any) of Phase 0 of mRNA degradation is unclear. The abovementioned countdown aspect of Phase 0 is not associated with the maintenance of developmental competence. The evidence comes from artificial extension of Phase 0. Since high cAMP levels prevent resumption of meiosis (Eppig et al. 1983; Schorderet-Slatkine et al. 1982), inhibitors of phosphodiesterase III allow to reversibly prevent resumption of meiosis in oocytes. Mammalian oocytes cultured in the presence of such inhibitors for 18–24 h apparently retain developmental competence (Naruse et al. 2012; Somfai et al. 2003). However, Phase 0 could be associated with establishment of developmental competence at the end of the growth phase, when the oocyte undergoes nuclear and cytoplasmic changes manifested as termination of transcription and chromatin remodeling of the non-surrounded nucleolus (NSN into the surrounded nucleolus (SN) chromatin configuration. NSN nuclei show rather diffuse DNA staining with several bright foci (chromocenters) containing constitutive heterochromatin, while SN nuclei are characterized by a bright ring surrounding the nucleolus. Nuclear transplantation experiments suggest that both nuclear and cytoplasmic factors (but not chromatin remodeling itself) determine the meiotic and developmental competence of fully grown SN oocytes in antral follicles (Inoue et al. 2008). Cytoplasmic factors contributing to the acquisition of developmental competence during the NSN/SN transition are poorly understood. Deep sequencing of NSN and SN oocyte transcriptomes revealed differences in several metabolic pathways, and more than 200 transcripts reduced more than twofold during the NSN/SN transition (Ma et al. 2013b). At the same time, there is a minimal overlap between downregulated transcripts identified in two independent studies (Puschendorf et al. 2006; Ma et al. 2013b), which precludes conclusions about transcripts degraded during Phase 0. In any case, the idea that RNA decay during Phase 0 might be contributing to NSN/SN transition and acquisition of developmental competence is worthy of further investigation.

10.2.2.3 mRNA Degradation Induced During Meiotic Maturation

The first major wave of maternal mRNA degradation appears during meiotic maturation. In mammals, 25–50 % reduction in poly(A) RNA has been observed during meiotic maturation (Lequarre et al. 2004; Piko and Clegg 1982; Bachvarova et al.

1985). There is also a 50 % decline in the percentage of polyadenylated RNA during meiotic maturation in *Xenopus* (Sagata et al. 1980). Poly(A) RNA reduction during meiosis is not simply continuous mRNA decay in the absence of transcription but is rather a result of induced and active mRNA degradation. This is evidenced by a minimal overlap between transcripts degraded during meiotic maturation and mRNAs that are intrinsically unstable in fully grown mouse oocytes (Su et al. 2007; Puschendorf et al. 2006). Interestingly, mRNAs degraded during meiotic maturation contradict the concept of an oocyte stockpiling resources for early development because mRNAs degraded during meiotic maturation encode proteins with housekeeping and basic metabolic functions, for example, mRNAs encoding essentially all ribosomal proteins (Su et al. 2007).

One of the *trans*-acting factors facilitating mRNA destabilization is MSY2, an abundant germ cell-specific RNA-binding protein that coats mRNAs and supports global mRNA stability in mouse oocytes (Yu et al. 2004; Medvedev et al. 2008, 2011). MSY2 becomes phosphorylated during meiotic maturation, and, as a consequence, maternal mRNAs become more accessible to the oocyte's mRNA decay machinery (Medvedev et al. 2008).

Another mechanism facilitating destabilization of maternal mRNAs is dormancy of decapping (Ma et al. 2013a) and deadenylation (Ma et al. 2015). *Dcp1a* and *Dcp2* mRNAs (whose protein products form the decapping complex) are maternal transcripts recruited during maturation via *cis*-acting CPE, thus providing an elegant mechanism for raising the oocyte's capacity to degrade maternal mRNA (Ma et al. 2013a). A similar observation was made for components of CCR4 and PAN2/3 deadenylases (Ma et al. 2015). Inhibition of the maturation-associated increase in DCP1A and DCP2 proteins prevents degradation of a large population of maternal mRNAs and affects ZGA (Ma et al. 2013a). A similar observation was made for inhibition of CCR4 deadenylase (Ma et al. 2015). These data suggests that deadenylation and decapping during meiotic mRNA degradation are not redundant and could be uncoupled (Ma et al. 2013a). This would be consistent with *Xenopus* oocytes where deadenylation also appears uncoupled from decapping (Gillian-Daniel et al. 1998).

Taken together, the transition from mRNA stability to instability during mouse oocyte maturation is consistent with the following model: mRNAs are relatively stable during the growth phase because MSY2 binding confers mRNA stability, while the activity of the mRNA degradation machinery is relatively low. Meiotic maturation is associated with phosphorylation of MSY2, which makes mRNAs more susceptible to degradation. In parallel, recruitment of dormant mRNAs encoding components of decapping and deadenylase complexes increases the mRNA degradation capacity of the maturing oocyte. That the increase in DCP1A, CNOT7, and PAN2 occurs after extrusion of the first polar body suggests that degradation of the bulk of maternal mRNAs during Phase I occurs late in meiotic maturation (Ma et al. 2013a, 2015) and presumably extends into Phase II.

One of the most puzzling questions concerning maternal mRNA degradation is as follows: How is selectivity of mRNA degradation achieved? The classical model proposes a combinatorial system of *cis*-acting sequence/structural motifs and

trans-acting factors, which would result in selective mRNA destabilization. However, the mouse model offers a different view. Bioinformatics analysis of mRNAs degraded during meiosis from Su et al. (Su et al. 2007) suggests that degraded transcripts tend to have shorter 3′UTRs and degradation is not associated with specific *cis*-acting 3′UTR motifs. In contrast, stable mRNAs have longer 3′UTRs and are enriched in *cis*-acting AU-rich motifs (Svoboda, unpublished). Interestingly, AREs, which are a class of AU-rich motifs, can direct mRNA deadenylation without triggering ARE-mediated mRNA decay in *Xenopus* oocytes, resulting in stabilization of deadenylated transcript until the midblastula transition (Voeltz and Steitz 1998). The opposing effects of ARE and CPE on maternal mRNA polyadenylation timing in *Xenopus* oocytes (Belloc et al. 2008) suggest that AREs and ARE-binding proteins are key modulators, balancing the CPE-mediated polyadenylation and translational activation. Mouse oocytes express a number of ARE-binding proteins with roles during oocyte development and beyond (Ramos et al. 2004; Chalupnikova et al. 2014).

Taken together, mouse transcriptome changes during meiotic maturation are rather consistent with selective stabilization in an mRNA-destabilizing environment. In addition, increased RNA turnover could be also coupled with translation, which would be consistent with the housekeeping nature of many of the degraded mRNAs. While this model certainly needs further experimental support, it brings an important concept to studies of maternal mRNA degradation—that degradation of mRNAs does not need to be selective per se because selectivity may concern mRNA stabilization. It remains to be examined to what extent is the selectivity of maternal mRNA degradation during oocyte maturation driven by sequence-specific recruitment of the degradation machinery and to what extent is it driven by the loss of protection of a large portion of the transcriptome against degradation, e.g., protection conferred by RNA-binding proteins such as MSY2.

10.2.2.4 mRNA Degradation Induced by Fertilization

The mechanism of how fertilization induces degradation of mRNAs, which were relatively stable during meiotic maturation, is poorly understood. One of the known modifications of the mouse transcriptome after fertilization is an extensive cytoplasmic polyadenylation. It is manifested as an apparent increase in the poly(A) RNA content in the 1-cell embryo (Fig. 10.5). It is possible that this process is linked not only to recruitment of dormant mRNAs after fertilization for translation but also to increased turnover of maternal mRNAs. This idea is actually supported by the latest deep sequencing datasets from unfertilized eggs and 1-cell embryos (Abe et al. 2015; Xue et al. 2013). If the fertilization-induced increase in mRNA polyadenylation correlates with increased mRNA decay, one should simultaneously observe an apparent increase in mRNA abundance after fertilization in deep-sequenced poly(A) RNA (Xue et al. 2013), while total RNA deep sequencing would reveal reduced abundance of the same mRNA (Abe et al. 2015). We examined how frequent this scenario might be, and we have found that

over a half of mRNAs degraded after fertilization show behavior consistent with polyadenylation-accelerated mRNA decay (Svoboda et al., unpublished). This suggests a potential role for noncanonical poly(A) polymerases (reviewed in Martin and Keller 2007) in maternal mRNA degradation. In fact, robust expression of some of the poly(A) polymerases occurs in mouse oocytes (Su et al. 2002). It is off-note that degradation of miRNAs is also triggered by fertilization, while the noncanonical poly(A) polymerase Wispy was implicated in maternal miRNA adenylation and degradation in *Drosophila* (Lee et al. 2014).

10.2.2.5 mRNA Degradation Induced by ZGA

Mechanistically, mRNA degradation induced by ZGA fits the classical model of mRNA degradation during developmental transitions where a *trans*-acting regulatory factor is produced through transcriptional activation in the later developmental state (Alonso 2012). A well-studied example of a zygotic *trans*-acting factor inducing degradation of maternal mRNAs after fertilization is microRNA (miRNA)-mediated mRNA degradation in zebrafish embryos where miRNAs of the miR-430 family become strongly upregulated a few hours after fertilization and target maternal mRNAs. The zygotic miRNA-mediated maternal mRNA degradation is rather extensive—it was estimated that the miR-430 family may target up to 40 % of maternal mRNAs (Giraldez et al. 2006).

A similar role was proposed for miR-427, a *Xenopus* orthologue of miR-430 (Lund et al. 2009). After fertilization, miR-427 is synthesized as multimeric primary transcripts, and the mature miR-427 accumulates to a high level (~10^9 molecules) by the midblastula transition when the zygotic mRNA expression is initiated. Presence of miR-427 cognate sequences in selected maternal mRNAs was shown to be necessary and sufficient for their deadenylation. Conversely, inactivation of miR-427 resulted in stabilization of those mRNAs (Lund et al. 2009).

The two examples above come from models in which development is very fast and zygotic miRNAs appear when maternal mRNAs are still abundant (Fig. 10.3). Fast development likely elicits higher pressure on removing maternal mRNAs (so that their products cannot interfere with differentiation). In this sense, zygotic miRNAs offer a simple solution.

Mammals have orthologues of the miR-430 family, such as the miR-290 family (reviewed in Svoboda and Flemr 2010). Transcription of the murine miR-290 family initiates during ZGA at the 2-cell stage, but accumulation of mature miRNAs is first observed from the 4-cell stage to the blastocyst (Tang et al. 2007; Zeng and Schultz 2005). Thus, the miR-290 family has a minimal if any role in maternal mRNA degradation since the majority of maternal mRNAs is eliminated before miR-290 family miRNAs reach functionally relevant levels. Furthermore, as mentioned above (Sect. 10.2.1.1), genetic studies and functional analysis of the miRNA pathway in the mouse OET model showed that miRNA activity is globally suppressed during and nonessential for the entire OET (Suh et al. 2010; Ma et al. 2010). At this point, there is no evidence that the global miRNA suppression and functional irrelevance

for OET would apply to all mammals in general. However, the minimal contribution of zygotic miRNAs to maternal mRNA degradation could be a general rule in mammals as massive maternal mRNA degradation precedes ZGA in bovine embryos as well (Gilbert et al. 2009) (Fig. 10.5).

Taken together, maternal mRNA degradation occurs in phases initiated by termination of transcription, resumption of meiosis, fertilization, and ZGA. The length and intensity of each phase may differ among vertebrates. What also differs is the stage of development until which maternal mRNAs contribute to gene expression. In mammals, maternal mRNAs are essentially gone between 8-cell and blastocyst stages, and maternal mRNAs do not control germ cell development. In contrast, maternal mRNAs are still present during organogenesis in zebrafish and *Xenopus*, and they control germline development in the embryo.

10.2.3 Elimination of Maternal Ribosomal RNA and Maternal Ribosomes

Ribosomal RNA (rRNA) forms the RNA component of the ribosome. Three rRNA molecules are present in the larger ribosomal subunit (5S, 5.8S, and 28S) and one in the smaller subunit (18S). 5S rRNAs are encoded by clusters of tandem repeats transcribed by RNA polymerase III (reviewed in Ciganda and Williams 2011). 18S, 5.8S, and 28S rRNAs are transcribed by RNA polymerase I as a single transcript (47S rRNA), which is subsequently endonucleolytically processed to give a rise to 18S, 5.8S, and 28S rRNAs (reviewed in Henras et al. 2008). Genes encoding 47S rRNAs are tandemly arrayed in specific genomic locations and exhibit unique control of gene expression (reviewed in Grummt and Langst 2013).

rRNA forms the bulk of maternal RNA (63–95 % of total RNA) (Piko and Clegg 1982; Davidson 1976; Phillips 1982). In *Xenopus laevis* embryos, rRNA synthesis is detected during gastrulation (Brown and Littna 1964), but maternal ribosomes can support development much further, as demonstrated in *nu* mutants, which are defective in ribosomal synthesis. Homozygous *nu* mutants, which carry only ribosomes provided by their heterozygous mothers, can develop until the swimming tadpole stage (Brown and Gurdon 1964). It was estimated that *Xenopus* oocyte contains 3.6 μg rRNA, which corresponds to 10^{12} ribosomes (Davidson 1976). Interestingly, the total RNA content is relatively stable from fertilization to gastrulation (Brown and Littna 1964), which suggests a minimal rRNA turnover in the absence of its transcription and, subsequently, balanced embryonic rRNA transcription and turnover in *Xenopus* embryos.

A different situation exists in mammals. Although the mammalian oocyte is relatively small and rRNA expression starts early in development (Abe et al. 2015; Knowland and Graham 1972; Zatsepina et al. 2003), biochemical data suggest that majority of mouse ribosomes are not in polysomes during ovulation and fertilization, presumably being stored for later use (Bachvarova and De Leon 1977;

Bachvarova et al. 1981). rRNA in growing mouse oocytes is highly stable until ovulation (Bachvarova 1981) where the estimates for rRNA content range from 0.2 to 0.5 ng (Bachvarova 1974; Sternlicht and Schultz 1981)—this would translate into 50–100 million ribosomes, which would be consistent with data from electron microscopic morphometry, which estimated that the zygote contains ~55 million ribosomes (Piko and Clegg 1982). However, several lines of evidence suggest that proteosynthetic machinery is not stockpiled for mammalian OET but rather undergoes rapid turnover and is quickly replaced by the embryonic one. Maternal ribosomes are destabilized upon resumption of meiosis (Bachvarova et al. 1985) and are eliminated during OET at a much faster rate than zygotic rRNA synthesis occurs (Gilbert et al. 2009; Piko and Clegg 1982). In mice, approximately 20 % of total RNA is lost during meiotic maturation (Bachvarova et al. 1985), and the total RNA is reduced approximately twofold between the fully grown oocyte and the late 2-cell stage (Piko and Clegg 1982). Both RNA polymerase I transcription and pre-rRNA processing machineries that produce pre-rRNA transcripts and mature rRNAs are extensively eliminated by MII; another wave of degradation occurs after fertilization (Zatsepina et al. 2000; Fulka and Langerova 2014). New transcripts for proteins supporting rRNA production are made by the mouse embryo during ZGA as shown by single-nucleotide polymorphism (SNP) analysis (Fulka and Langerova 2014). Although zygotic rRNA expression is initiated at the 2-cell stage, the main upregulation of rRNA synthesis takes place at the 4-cell stage (Abe et al. 2015; Zatsepina et al. 2003). Thus, when the mouse 2-cell embryo initiates synthesis of translational apparatus, at least a half of the maternal ribosomal pool is already eliminated, and it will take an additional 24 h to have highly active rRNA synthesis (Abe et al. 2015). A similar pattern is observed during bovine OET where the major ZGA occurs at the 8-cell stage (Graf et al. 2014). rRNA amount in 8-cell embryos (estimated by capillary electrophoresis) is more than threefold lower than that in the fully grown oocyte (Gilbert et al. 2009) (Fig. 10.5). The notion of active elimination of the maternal translational apparatus is supported also by microarray data, which suggest active degradation of mRNAs encoding ribosomal protein-encoding genes during meiotic maturation and after fertilization (Su et al. 2007; Zeng et al. 2004).

Taken together, if the maternal support would be compared to fueling a car for a journey, mammalian oocytes would be reminiscent of a car with a tank having just enough fuel to get to the nearest gas station. In zebrafish or *Xenopus* cars, the tank would be full, and by the time this car would reach the gas station, the fuel gage would not even move. A "full tank" concept observed in oviparous species sounds self-evident. However, why would viviparous species adopt the other strategy? One could speculate that the chosen "fueling strategy" is a cost-efficient adaption of early development to the environment. In other words, in oviparous species, there is presumably a high pressure to fuel the tank as much as possible as the embryo developing in an external environment is highly dependent on maternally deposited resources. Thus, more fuel likely translates into better fitness. In contrast, mammalian embryos do not need to carry that much maternal supply as they develop in a stable, nutrition-rich environment and, in fact, continue to receive maternal support throughout pregnancy.

10.3 Maternal Protein Degradation

Almost every discussion of maternal factors regulating OET puts into the spotlight maternal mRNAs, while much less attention is paid to proteins. This is understandable because analysis of maternal mRNAs is much easier in terms of experimental approaches and the amount of required material. Undeniably, maternal mRNA control is an important aspect of OET; however, it must be recognized that OET is executed by proteins, not by mRNAs per se. Accordingly, this section is devoted to maternal proteins and their degradation.

Numerous studies suggest that protein degradation by the ubiquitin-proteasome pathway is essential for OET (reviewed in DeRenzo and Seydoux 2004). There is also a growing amount of high-throughput data concerning protein dynamics during OET. Here, we will review the current knowledge of maternal proteome elimination after fertilization, including degradation mechanisms, model examples, and recent proteome-wide data.

Maternal protein degradation has two roles: (1) nourishing the early embryo by releasing amino acids from maternal protein stores, prominent in oocytes with large yolk deposits (yolk is discussed in the Sect. 10.5.2), and (2) transforming the oocyte into a totipotent zygote by removing proteins that confer oocyte identity. There are two major pathways for protein degradation in vertebrate cells: (1) autophagy-mediated lysosomal degradation and (2) ubiquitin-proteasome pathway

10.3.1 Autophagy-Mediated Lysosomal Degradation During OET

Autophagy (reviewed in Mizushima 2007) is a bulk degradation system. During autophagy, a portion of cytoplasm is sequestered into an autophagosome, which fuses with a lysosome, resulting in degradation of the engulfed material. As such, the role of autophagy during OET would lay in general protein turnover, amino acid recycling, and structural rearrangements in the zygote rather than in specific protein targeting.

Autophagy is important in many cellular processes as well as for early development in mice. Formation of autophagosomes is upregulated immediately after fertilization, and early embryos contain a large number of lysosomes (Tsukamoto et al. 2008). Increased autophagy in zygotes is induced by fertilization; it is not induced by starvation in unfertilized ovulated eggs (Tsukamoto et al. 2008). Functional significance of autophagy in mouse development was shown in mice lacking *Atg5*, an essential factor for autophagosome formation (Mizushima et al. 2001). Remarkably, $Atg5^{-/-}$ mice obtained by crossing heterozygotes develop until birth (Mizushima et al. 2001), but $Atg5^{-/-}$ embryos lacking the maternal pool of ATG5 exhibit an early embryonic arrest (4-/8-cell stage), while $Atg5^{-/-}$ oocytes fertilized with a wild-type sperm develop to term (Tsukamoto et al. 2008). These

results revealed that autophagy is essential for a specific phase of OET but not for mammalian postimplantation development. As the authors note, mammalian preimplantation development progresses slowly without extracellular nutrient stores in contrast to birds, fish, and amphibians; thus, autophagy may be a unique strategy to support mammalian development (Tsukamoto et al. 2008). The importance of autophagy for OET in zebrafish or *Xenopus* remains unknown.

10.3.2 Ubiquitin-Proteasome Pathway During OET

The ubiquitin-proteasome pathway is the main pathway for regulated protein degradation in eukaryotic cells (reviewed in Yao and Ndoja 2012). It is initiated by covalent attachment of ubiquitin chain to a targeted protein, which is followed by recognition of ubiquitinated protein and its degradation by the proteasome. Proteasome assembly requires a set of dedicated chaperones (reviewed in Murata et al. 2009). Mouse oocytes and zygotes express a zygote-specific proteasome assembly chaperone, whose expression peaks at the 2-cell stage and enhances the biogenesis of 20S proteasome, thus presumably augmenting degradation of maternal proteins (Shin et al. 2013).

The ubiquitin proteolytic system is selective. Selectivity is mediated by a multimeric ubiquitin-ligating protein (E3) complex, which contains a variable substrate recognition factor targeting a specific protein for ubiquitination. A common substrate recognition factor is a member of the F-box family of proteins (reviewed in Jin et al. 2004). GNF SymAtlas (BioGPS, Su et al. 2004) contains expression data for approximately 100 mouse genes encoding F-box proteins. Of them, approximately three quarters show detectable expression in the oocyte, and 21 of them show at least four times higher expression in the oocyte than a mean expression value in the GNF1M set of somatic tissues (Su et al. 2004). In agreement with transcriptome profiling, proteome profiling of mouse oocytes identified a large group of maternally expressed F-box proteins (Wang et al. 2010), which match F-box protein-encoding genes found in the GNF SymAtlas.

A common tool to specifically and reversibly inhibit the ubiquitin-proteasome pathway is the proteasomal inhibitor MG132. Treatment of oocytes and embryos with MG132 shows that ubiquitin-proteasome pathway-mediated protein degradation has numerous roles during OET. MG132-treated (75 μM) zebrafish eggs showed delayed completion of MII and extrusion of the second polar body. An embryotoxic effect was found when matured eggs were held in 100 μM MG132 (Siripattarapravat et al. 2009). Inhibition of the ubiquitin-proteasome pathway with MG132 (up to 20 μM) in mouse embryos delayed the onset of ZGA (Shin et al. 2010).

Taken together, the ubiquitin-proteasome pathway is active in mammalian oocytes and early embryos and plays multiple roles by selectively targeting proteins for degradation. The number of F-box proteins expressed during OET suggests that a complex system of protein degradation exists, which would provide another layer of control over OET.

10.3.2.1 Examples of Selective Protein Degradation During OET

ELAVL2 Elimination During NSN/SN Transition in Fully Grown Mouse Oocytes

Elavl2 encodes one of the ARE-binding proteins, which act in the ARE-mediated mRNA decay or translation control (reviewed in Garneau et al. 2007; see also Chap. 2). Mouse oocytes express a short ELAVL2 isoform that acts as a pleiotropic translational repressor. *Elavl2* mRNA is stable during meiotic maturation and is downregulated after fertilization. In contrast, ELAVL2 protein rapidly decreases already at the end of the oocyte growth phase, during the NSN/SN transition, which makes it a rare example of a protein eliminated during Phase 0. Functional analyses of ELAVL2 suggested that it is important for efficient production of fully grown oocytes, while its removal during NSN/SN transition might contribute to the acquisition of the developmental competence (Chalupnikova et al. 2014). The molecular mechanism of ELAVL2 degradation remains unclear, but the selectivity of its destabilization suggests that the ubiquitin-proteasome system is at play.

CPEB Degradation During Meiotic Maturation in *Xenopus* Oocytes

CPEB is a cytoplasmic polyadenylation element-binding protein, which plays an important role in coordinated translational control of dormant maternal mRNAs in *Xenopus* oocytes (Pique et al. 2008) and elsewhere (reviewed in Richter 2007; see also Chap. 2). In maturing *Xenopus* oocytes, CPEB is phosphorylated and degraded by the ubiquitin-proteasome system, and this degradation facilitates entry into the second meiotic division (Reverte et al. 2001; Mendez et al. 2002). CPEB degradation during meiotic maturation was observed also in mammalian oocytes (Uzbekova et al. 2008; Hodgman et al. 2001).

Selectivity of CPEB degradation is mediated by a specific F-box protein β-TrCP in the $SCF^{\beta\text{-}TrCP}$ E3 ubiquitin ligase complex, which recognizes a specific CPEB sequence (Setoyama et al. 2007). $SCF^{\beta\text{-}TrCP}$ binding depends on a specific phosphorylation in the recognition motif. According to the model, CDC2 and PLX sequentially phosphorylate CPEB during meiotic maturation and target it for degradation via $SCF^{\beta\text{-}TrCP}$ (Setoyama et al. 2007).

10.3.2.2 Proteome Dynamics During OET

For decades, proteome analysis during OET had to rely on two-dimensional (2D) electrophoresis (e.g., Link et al. 2006; Tay et al. 2006), often combined with metabolic labeling. Compared to microarray analyses and next-generation sequencing, proteome studies offer somewhat lower-resolution, but nonetheless highly interesting, insights into proteome changes during OET. Given the amount of material required for extensive proteome analysis, the most

accessible OET system for proteomic studies has been that of *Xenopus*. In 2014, three proteomic studies provided insights into *Xenopus* oocyte proteome composition and its dynamics during OET.

Sun et al. implemented isobaric tags for relative and absolute quantitation (iTRAQ) analysis of four *Xenopus* early development stages from egg to neurula and provided protein dynamics for nearly 4000 proteins classified in six clusters according to their expression pattern (Sun et al. 2014). Two clusters harbored maternal proteins degraded after fertilization where one protein cluster became destabilized upon fertilization (179 proteins) and one protein cluster remained stable until MBT/ZGA and then was degraded (190 proteins). In addition, they identified two additional clusters of proteins, which were encoded by maternal mRNAs and became synthesized upon fertilization either in a transient manner (88 proteins) or as a complement to sustain levels of embryonic expression (Sun et al. 2014).

Smits et al. performed single-cell proteomic analysis of *Xenopus* eggs using mass spectrometry (Smits et al. 2014). They quantified over 5800 proteins and provided a proteomic comparison of oocytes and gastrula. It showed that proteomes of oocytes and early embryos highly correlate ($r=0.90$) and that less than 10 % proteins were significantly regulated (including 313 significantly downregulated maternal proteins) (Smits et al. 2014). Remarkably, authors observed minimal if any correlation between transcript and protein levels suggesting that posttranscriptional control is the major control layer determining protein abundance.

Wuhr et al. provided the most extensive proteome catalogue of *Xenopus* oocytes with more than 11,000 identified proteins, many of which were found even in the absence of corresponding mRNAs, being possibly acquired from blood plasma together with yolk (Wuhr et al. 2014). Thus, uptake of maternal non-yolk proteins might form another layer of developmental control executed in the embryo by maternal proteins not expressed from oocyte's genome (Wuhr et al. 2014).

In mammals, proteomic analyses of oocytes and early embryos are limited by the amount of available material, which is two to three orders of magnitude lower than in zebrafish or *Xenopus* oocytes. For example, a proteomic analysis of ~3000 mouse MII oocytes using unlabeled proteins resolved by 2D electrophoresis revealed several hundred proteins, but only dozens of them were identified by tandem mass spectrometry (Calvert et al. 2003). However, the catalogue of the maternal proteome continued to grow. A similar later study used 80 μg of total protein isolated from MII oocytes and identified 869 spots resolved by 2D electrophoresis corresponding to 380 unique proteins (Ma et al. 2008b). Zhang et al. used 2700 oocytes and identified 627 different proteins in MII oocytes (Zhang et al. 2009), and Pfeiffer et al. reported a catalogue of 3699 proteins (Pfeiffer et al. 2011).

Several studies also addressed proteome dynamics during OET. Vitale et al. analyzed proteome dynamics during meiotic maturation (Vitale et al. 2007). Using 500 fully grown and MII oocytes profiled by 2D electrophoresis, they identified 12 differentially expressed proteins, which were identified by mass spectrometry. Of the 12 proteins, seven were downregulated during meiosis. A similar later study by Cao et al. comparing fully grown and MII oocytes recognized 1114 protein spots, of which ~10 % were differentially expressed (fold change >1.5). In

the end, they identified 63 differentially expressed proteins, 21 of which had decreased expression (Cao et al. 2012). The overlap between the studies was rather unimpressive. Cao et al. identified six (50 %) of the differentially regulated proteins reported by Vitale et al.; tens of identified differentially expressed proteins are in a stark contrast with transcriptome profiling, which typically identifies ten to hundred times more transcripts. However, despite much lower resolution, proteomic analysis is important for understanding OET because it monitors functional (final) gene products, not mere mRNA intermediates. In addition, it allows for identifying posttranslational modifications, which are undetectable by transcriptome profiling. At the same time, these data illustrate limitations of proteomic studies relying on protein mass, which cannot be amplified like nucleic acids. Thus, further advancement of mammalian OET proteomics requires either isolation of large amounts of individual stages or dramatically increased resolution in terms of protein detection and quantification.

So far, the most comprehensive and informative analysis of mouse OET was produced by Wang et al., who collected 7000 oocytes (fully grown and MII) and zygotes and subjected them to semiquantitative mass spectrometry (Wang et al. 2010). Two to three thousand proteins were identified, providing the first solid insight into proteome changes during OET before zygotic genome activation (Wang et al. 2010). Notably, maternal proteins were quickly degraded after fertilization as suggested by 50 % fewer peptides identified in zygotes relative to MII oocytes despite the same number of zygotes and oocytes being analyzed (Wang et al. 2010). Remarkably, this observation correlates nicely with 30-year-old data showing that protein half-lives become reduced after fertilization from ~18 to ~13 h between the 1-cell and cleavage stages (Merz et al. 1981).

Taken together, research on maternal protein degradation is coming of age. Our current knowledge of protein-degrading mechanisms and genes expressed during OET opens new directions for analyzing, for example, roles of individual F-box proteins in selective protein degradation by ubiquitin-proteasome pathway during OET. In addition, development of more sensitive instruments and new methods for proteomic analysis brings hopes that comprehensive proteome profiling during mammalian OET will become more feasible in the near future.

10.4 Erasure of Parental Epigenetic Marks

Epigenetic information is heritable information not encoded in the DNA sequence. The two key molecular mechanisms discussed further below concern chemical alteration of DNA (DNA methylation) and posttranslational modification of histones (histone marks) (for further reading, see Allis et al. 2007). The zygote is formed upon fusion of two differentiated gametes. Each of them carries its own pattern of DNA methylation and histone modifications, both of which are extensively remodeled during OET (reviewed in detail in Kimmins and Sassone-Corsi 2005; Burton and Torres-Padilla 2010, 2014; Gill et al. 2012; Rivera and

Ross 2013). Significance of unique parent-of-origin epigenetic marks differs across vertebrates. Normal mammalian development requires genomes from both parents (McGrath and Solter 1984; Surani et al. 1984) because only this combination assures functional expression of imprinted genes carrying parentally created epigenetic marks. Such imprinting marks are maintained in the soma and are erased only during germline reprogramming in primordial germ cells in the embryo (reviewed in Hajkova 2011; Leitch et al. 2013). In contrast, zebrafish and *Xenopus* can produce viable uniparental diploid progeny demonstrating epigenetic equality of parental genomes (Cheng and Moore 1997).

As some parental epigenetic marks are retained while others are erased, it has been proposed that reprogramming (i.e., erasure and rebuilding of marks) is balanced with inheritance (i.e., maintenance of parental epigenetic marks) (Gill et al. 2012). To remain within the scope of this chapter, we will focus on erasure of parental epigenetic marks, which we can be seen as a special category of parental products, which are eliminated at different phases of development. Specifically, we will focus on two events during mammalian OET: (1) clearance of parental DNA methylation after fertilization and (2) loss of parental histone modifications during OET. For more details on parental imprinting in vertebrates, the reader is referred to Chap. 12.

10.4.1 Loss of DNA Methylation

Vertebrate DNA methylation (reviewed in Schubeler 2015; Li and Zhang 2014) employs addition of a methyl group at the carbon 5 of a cytosine followed by guanosine (CpG). This methylation is symmetrical (i.e., occurring also on the cytosine complementary to guanosine in the CpG context) and maintained. DNA methylation is created by de novo DNA methyltransferases (DNMT3 family), which operate on non-methylated substrates. Subsequently, CpG methylation is maintained by the maintenance DNA methyltransferase DNMT1, which recognizes hemi-methylated DNA after replication and renews the symmetrical DNA methylation by placing a 5-methyl cytosine (5mC) on the newly synthesized DNA strand. Thus, DNA methylation is a classical "epigenetic mark"—a molecular modification that does not alter DNA sequence but can carry heritable information. DNA methylation in vertebrate genomes is widespread. As a consequence of conversion of 5mC into thymine, which is a frequent mutation, vertebrate genomes exhibit lower frequency of CpG dinucleotides than expected frequency calculated from GC content of the genome (Jabbari and Bernardi 2004).

While there is an ongoing debate regarding accurate understanding of causes and consequences of DNA methylation and transcriptional silencing (Schubeler 2015), this is beyond the scope of this chapter. In the common view, DNA methylation is associated with transcriptional silencing and contributes to (1) genome maintenance by mediating silencing of parasitic repetitive sequences and (2) control of gene expression by enforcing a silenced state of a methylated promoter.

DNA methylation within the mammalian germline cycle undergoes two reprogramming episodes, during which the existing DNA methylation is largely eliminated (reviewed in Surani et al. 2007). After demethylation during primordial germ cell reprogramming, maternal and paternal genomes accumulate DNA methylation, including parent-of-origin-specific methylation in imprinting control regions. However, a large portion of 5mC methylation is lost during early mammalian development. Genome-wide demethylation manifests as a loss of signal in cleaving mouse zygotes stained with an antibody recognizing 5mC (Mayer et al. 2000). There were two distinct demethylation patterns observed in paternal and maternal genomes. The paternal genome rapidly lost 5mC signal (6–8 h after fertilization) suggesting an active demethylation process. In contrast, staining of the maternal genome showed gradual reduction of the signal through cleavages, implying passive DNA demethylation due to absent DNA methylation maintenance (Mayer et al. 2000; Oswald et al. 2000). Notably, the zygotic genome is not being completely demethylated, presumably due to continuous de novo DNA methylation of specific loci. Apart from imprinted loci, which retain their parent-of-origin methylation pattern in imprinting control regions, it was shown that intracisternal A particle (IAP), an active and aggressive murine endogenous retrovirus, remains methylated despite the global demethylation (Lane et al. 2003).

Passive DNA demethylation of the maternal genome is supported by staining of metaphase chromosomes from cleaving embryos, which revealed asymmetrical labeling of sister chromatids (Rougier et al. 1998). Passive DNA demethylation is also consistent with behavior of DNMT1, which remains retained in the cytoplasm of the zygote until the 8-cell stage (Cardoso and Leonhardt 1999).

Active paternal DNA demethylation in the zygote is found in many mammals, but it is perhaps not entirely conserved (Dean et al. 2001; Beaujean et al. 2004; Lepikhov et al. 2008). The mechanism of active DNA demethylation in 1-cell mammalian embryos involves genome-wide oxidation of 5mC to 5-hydroxymethylcytosine (5hmC) by Tet3 dioxygenase (Gu et al. 2011; Iqbal et al. 2011; Wossidlo et al. 2011). The reason why active methylation targets only paternal DNA is that maternal DNA is apparently protected by PGC7/Dppa3/Stella complex (Wossidlo et al. 2011). Loss of maternal Tet3 results in reduced fertility (in terms of both successful matings and litter size) suggesting that oxidation of the paternal 5mC is important but not essential (Gu et al. 2011). At this point, it is not clear whether the phenotype reflects a nonessential function of maternal Tet-3 or whether there is some redundancy in 5mC oxidation.

10.4.1.1 DNA Methylation During Early Development of Nonmammalian Vertebrates

In contrast to mice, *Xenopus* maintains high global levels of DNA methylation during early development (Veenstra and Wolffe 2001). Staining with an antibody recognizing 5mC confirmed that the paternal genome of *Xenopus* embryos is not actively demethylated (Stancheva et al. 2002). It was also found that high levels of

parental DNA methylation of several promoters progressively decline during cleavage, resulting in the lowest global methylation levels at MBT and during gastrulation (Stancheva et al. 2002). The loss of parental methylation of ribosomal genes and specific protein-coding genes correlates with their activation during MBT (Stancheva et al. 2002; Bird et al. 1981). However, finer mapping of DNA methylation at blastula and gastrula using affinity capture of methylated DNA suggested that parentally methylated promoters can transiently support transcription (Bogdanovic et al. 2011).

Zebrafish genome is extensively methylated, but initial analysis of DNA methylation during early development did not show the genome-wide changes observed in mammals (Macleod et al. 1999). However, a more detailed study suggested that reduced DNA methylation occurs postfertilization followed by a rapid increase in DNA methylation (Mhanni and McGowan 2004). This observation is consistent with later genome-wide analysis by bisulfite sequencing (Potok et al. 2013). Analysis of promoter methylation during ZGA by methylated DNA immunoprecipitation (MeDIP) showed that promoters methylated in the sperm become demethylated in the embryo, while nearly all genes methylated in the embryo are also methylated in the sperm (Andersen et al. 2012).

A comprehensive insight into the genome-wide DNA methylation reprogramming in zebrafish was provided by Potok et al. who performed whole genome shotgun bisulfite sequencing in germ cells and early embryo stages (2–16-cell, 64-cell, 256-cell (all before ZGA), and sphere stage (after ZGA)) (Potok et al. 2013). They found demethylation reaching the lowest level at the 64-cell stage followed by remethylation occurring at the 256-cell stage and restoration of the global genome methylation by the sphere stage, which was consistent with earlier data (Mhanni and McGowan 2004). Remarkably, the maternal genome methylation became apparently remodeled by ZGA to the paternal state (Potok et al. 2013). Furthermore, parental DNA methylation prevents precocious expression of specific genes during ZGA—providing a distinct regulatory role for removal of parentally deposited DNA methylation (Potok et al. 2013).

Taken together, parental methylation is removed to a different extent in vertebrate early development. Active paternal DNA demethylation in 1-cell embryos is present in many mammals, while in other vertebrates the loss of parental methylation is much less dramatic and appears much more restricted. A common theme for vertebrate early embryos seems to be the loss of parental methylation at promoters that will be utilized during early development.

10.4.2 Loss of Histone Modifications

Histone modifications serving as active and inactive chromatin marks offer a unique system for recording additional information concerning the histone-associated DNA. The so-called histone code may be used in many roles, such as regulation of transcription or DNA repair (reviewed, e.g., in Jenuwein and Allis 2001;

Tessarz and Kouzarides 2014). Importantly, functionality of key histone modifications is more conserved across eukaryotic kingdoms than DNA methylation (for further reading, see Allis et al. 2007). Importantly, not every histone modification is a true epigenetic mark, i.e., exists as a stable mark maintained through S-phase and cell division. Some of the marks are simply deposited as a consequence of some event (e.g., transcriptional activation, DNA damage) but are not specifically maintained. Next, we will provide a brief overview of chromatin remodeling during mammalian OET (reviewed in detail in Kimmins and Sassone-Corsi 2005; Burton and Torres-Padilla 2010, 2014; Gill et al. 2012; Rivera and Ross 2013) with a specific focus on clearance of parental histone marks, which can be seen as a path toward erasure of ancestral cellular identity and establishment of pluripotency.

During germ cell development in the mouse, histone modifications undergo a major "reset" upon entry of primordial germ cells into the genital ridge. Next, differentiating male and female germ cells deposit various histone modifications across their genomes. At the end of germ cell development and at the onset of OET, parentally deposited histone modifications take dramatically different paths in the two differentiating germ cells.

10.4.3 Protamine/Histone Exchange During Spermatogenesis

Parental histone modifications are essentially completely erased together with the nucleosomal chromatin structure at the end of spermatogenesis during the so-called protamine/histone exchange (reviewed in Kimmins and Sassone-Corsi 2005; Braun 2001). Protamines are proteins used for tight packaging of paternal genomes in sperm heads. During the final stages of spermatogenesis, paternal chromatin undergoes complex remodeling. Its final stage involves sperm-specific transition proteins, which are subsequently replaced by protamines (Kimmins and Sassone-Corsi 2005). While the reason for protamine/histone exchange is unknown, it has clear consequences for the histone code, which is almost completely erased.

However, detailed analysis of sperm chromatin revealed that 1–10 % of histones are retained in the mouse and human spermatozoa, respectively (Brykczynska et al. 2010). Furthermore, retained histones carried specific modifications, and histone retention was not random but rather associated with a distinct promoter set in human sperm (Brykczynska et al. 2010). In particular, H3K4me2 (active) mark was found in promoters of genes involved in spermatogenesis and cellular homeostasis, while H3K27me3 (repressive) mark was associated with developmental promoters. It was proposed that residual histones contribute to the paternal heterochromatin formation in human zygotes (van de Werken et al. 2014). However, subsequent studies suggested that nucleosome retention is predominantly associated with gene deserts and not developmental promoters (summarized in Saitou and Kurimoto 2014). Therefore, the exact contribution of nucleosomal retention to the early embryo still needs to be elucidated.

10.4.4 Erasure of Maternal Chromatin Modifications During OET

Oocyte retains normal nucleosomal chromatin structure and has a global histone modification pattern similar to that of somatic cells (reviewed in Burton and Torres-Padilla 2014). A detailed catalogue of histone modification dynamics during OET has been provided elsewhere (Burton and Torres-Padilla 2010, 2014); here, we will only focus on the loss of maternal histone modification marks during mouse OET.

There is not much evidence for robust global change in histone modifications during NSN/SN transition. Despite establishment of transcriptional quiescence during NSN/SN transition, histone H3 and H4 acetylation and H3K4me3 (marks observed at active promoters) remain high in fully grown oocytes with SN chromatin configuration (Kageyama et al. 2007; Zuccotti et al. 2011). This phenomenon was observed in different species (reviewed in Gu et al. 2010; Bonnet-Garnier et al. 2012).

The first main global elimination histone modifications formed during oocyte growth appear upon resumption of meiosis. Histone acetylation (observed at active genes) is removed during resumption of meiosis (Kim et al. 2003). However, this change is not a part of epigenetic reprogramming of gene expression. Instead, histone deacetylation at the onset of meiosis is important for proper meiotic chromosome segregation (Kim et al. 2003; De La Fuente et al. 2004; Akiyama et al. 2006). Although most of histone acetylation is removed from the oocyte chromatin upon its condensation and entry into meiosis, certain histone residues retain the acetylation mark (e.g., H4K8 Gu et al. 2010). Whether this specific residue becomes deacetylated after fertilization in mammals remains to be determined.

Another global removal of histone modifications occurs after fertilization when heterochromatin histone marks are removed from the maternal chromatin. This includes H4K20me3 and H3K64me3, which are rapidly lost by the 2-cell stage (Kourmouli et al. 2004; Daujat et al. 2009), and H3K9me3, which is reduced in pericentric heterochromatin until the 8-cell stage, when it becomes highly enriched again (Puschendorf et al. 2008). Removal of these histone marks suggests that erasure of maternal constitutive heterochromatin marks is a part of embryonic chromatin reprogramming into a totipotent state. This notion is consistent with open chromatin observed during early development (Cho et al. 2002; Ahmed et al. 2010), which is reminiscent of chromatin structure as it is observed in pluripotent embryonic stem cells (Ahmed et al. 2010; Martens et al. 2005).

10.4.4.1 Elimination of Specific Histone Variants

Histone modifications are not the only way to mark chromatin. A similar effect can be achieved by deposition of specific histone variants, which are essentially adapted homologues of histone proteins, which can be deposited at specific loci in replication-dependent and replication-independent manner (reviewed in Henikoff

and Smith 2015). An example of removal of maternal deposition of a specific histone variant is histone H3.3, a replication-independent histone variant associated with active genes, which transiently disappears from the maternal genome after fertilization (Akiyama et al. 2011). Similarly, a maternal store of histone variant macroH2A is eliminated from zygotes shortly after fertilization (Chang et al. 2005). Remarkably, macroH2A is associated with transcriptional repression (Costanzi and Pehrson 1998; Buschbeck et al. 2009) and acts as a barrier to induced pluripotency (Gaspar-Maia et al. 2013).

The last example of maternal chromatin mark is an oocyte-specific linker histone variant H1foo, which has homologues among vertebrates (Tanaka et al. 2001). The mammalian H1foo shows highest expression levels in germinal vesicle oocytes, but it is gradually lost after fertilization (Tanaka et al. 2001; McGraw et al. 2006), when it is replaced with the somatic linker histone H1 (Gao et al. 2004). The oocyte-specific linker histone B4 in *Xenopus*, a homologue of H1foo, also accumulates during oogenesis and becomes eliminated around the MBT when it is replaced by somatic-type linker histones (Dimitrov et al. 1993). Interestingly, the elimination of oocyte-specific linker histones correlates with the time of embryonic genome activation in the mouse, bovine, and *Xenopus*. This implies that the elimination of H1foo might be prerequisite for successful embryonic genome activation. This notion is supported by impaired pluripotency upon ectopic expression of H1foo in embryonic stem cells (Hayakawa et al. 2012).

10.5 Elimination of Other Parental Products

The last section will review controlled elimination of yolk and paternal mitochondria, as unique examples of clearance of parental structures associated with nutrition/energy support.

10.5.1 Yolk Consumption

Yolk (reviewed in Anton 2013) is the common nutrient supply for externally developing embryos. Yolk is a supramolecular assembly of lipids and proteins, which form dense, membrane-bound yolk platelets. The major yolk proteins are derived from the conserved lipoprotein vitellogenin (Vg), which is not synthesized in the oocyte but is internalized by the oocyte and then stored in specialized organelles, the yolk platelets (Anton 2013). Although yolk platelets appear to be modified lysosomes, the content of yolk platelets is not degraded until specific developmental stages, sometimes weeks after their formation. Degradation of yolk proteins mainly serves as a source of amino acids during development (Anton 2013). Two factors appear to be responsible for inducing yolk degradation in the developing embryo: pH and enzymatic latency (Fagotto 1995).

The yolk amount determines the cleavage type, organization of the cleaving embryo, and distribution of the yolk in the developing embryo. *Xenopus* oocytes are mesolecithal with yolk concentrated mainly in the vegetal half and undergo radial holoblastic (complete) cleavage, which results in sequestering yolk platelets into blastomeres of the cleaving embryo. Yolk consumption in *Xenopus* embryos does not appear to be triggered by embryonic cells declining to a critically small size, and it is among the earliest aspects of differentiation (Jorgensen et al. 2009). The yolk contribution to development in the zebrafish goes even farther. Zebrafish oocytes are telolecithal and undergo partial discoidal cleavage (cleavage furrows do not reach into the yolk), and the early embryo essentially develops around the yolk cell, which not only has a nutritional role but also, together with the associated extraembryonic syncytial layer, participates in germ layer patterning (Chen and Kimelman 2000; Ober and Schulte-Merker 1999).

Taken together, there is a great variability in yolk deposition and consumption in vertebrate embryos, ranging from negligible amounts of yolk in mammalian oocytes through different amounts and distributions of yolk deposits in fish, amphibian, reptile, and bird eggs. Importantly, in addition to its nutritional role, yolk may contribute to developmental control, as exemplified by embryonic patterning in the zebrafish model. Finally, with respect to the clearance of parental products, yolk proteins deposited in the oocyte are probably among the longest-lasting maternal factors, which are being used up by the developing embryo.

10.5.2 Elimination of Paternal Mitochondria

Mitochondria are semiautonomous energy-generating organelles, which are in most animals inherited maternally, although some species exhibit strong paternal inheritance (Zouros et al. 1992). The uniparental inheritance may be an adaptation to avoid an intragenomic conflict between parental populations of mitochondria (Hurst 1992) and/or maintenance of mitochondrial genome integrity through segregation of wild-type and mutant mitochondrial DNA during oocyte development (Cree et al. 2008). Mechanisms controlling uniparental mitochondrial inheritance are diverse (reviewed in Luo et al. 2013a; Song et al. 2014; Sato and Sato 2013); here we will mainly focus on mammals.

In mammals, mitochondria are almost exclusively transmitted through the female, although exceptions were observed in interspecific crosses of mice (Gyllensten et al. 1991) and livestock (Zhao et al. 2001b), and there is one well-documented human case report (Schwartz and Vissing 2002). Although many reports implied and repeated that sperm mitochondria do not enter the embryo, the evidence shows the opposite (reviewed in Ankel-Simons and Cummins 1996). Although the midpiece of the giant Chinese hamster sperm is excluded from the zygote (Yanagimachi et al. 1983), in other mammals the midpiece is detectable in the cleaving zygote through several cleavages (Szollosi 1965; Fleming et al. 1986;

Shalgi et al. 1994; Sutovsky et al. 1996). Thus, mitochondria from the sperm enter the embryo, but their contribution to the mitochondrial pool in the zygote is minimal, if any. One contributing factor is simply the minuscule contribution of sperm-derived mitochondria to the mitochondrial pool in the zygote. The mouse oocyte contains ~10^5 mitochondria (Piko and Matsumoto 1976), which provides a huge excess over less than hundred mitochondria in the midpiece (Ankel-Simons and Cummins 1996). Furthermore, paternal mitochondria are actively degraded in the zygote (reviewed in Cummins 2000).

Both ubiquitination and autophagy were implicated in paternal mitochondria destruction in animals. In mammals, Sutovsky et al. noted ubiquitination of the bovine sperm mitochondria inside oocyte cytoplasm and proteolysis during pre-implantation development (Sutovsky et al. 1999). According to their model, mitochondrial membrane proteins are already tagged with ubiquitin in haploid spermatocytes, hence allowing their recognition in the zygote, and subsequent targeting for polyubiquitination and destruction might involve proteasome activity or autophagy (mitophagy) or both. It was also found that degradation of porcine sperm mitochondria is sensitive to proteasome inhibitor MG132 (Sutovsky et al. 2003), while tracking of bovine sperm mitochondria suggested association with lysosomes (Sutovsky et al. 2000). However, tracking autophagosomes and sperm mitochondria during early development challenged the role of autophagy in clearance of paternal mitochondria (Luo et al. 2013b). The current view is that mammals might employ a two-way mechanism of sperm mitochondria degradation involving both proteasomal and lysosomal proteolysis (Song et al. 2014). Of note is that studies in *Drosophila* and *C. elegans* demonstrated involvement of autophagy in degradation of paternal mitochondria (Politi et al. 2014; Sato and Sato 2011; Al Rawi et al. 2011).

Finally, another layer of regulation preventing the transmission of sperm-derived mtDNA to the offspring can be active digestion of the paternal mtDNA in the mitochondria. A study from *Oryzias latipes* (Japanese rice fish, medaka fish) showed that digestion of paternal mtDNA takes place before the destruction of the sperm mitochondrial body (Nishimura et al. 2006). The nuclease responsible for this digestion has not yet been identified. Whether this mechanism exists across vertebrates is unclear; there is no indication that paternal mitochondrial DNA would be degraded before the mitochondrial body in mice (Hecht et al. 1984).

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Index

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