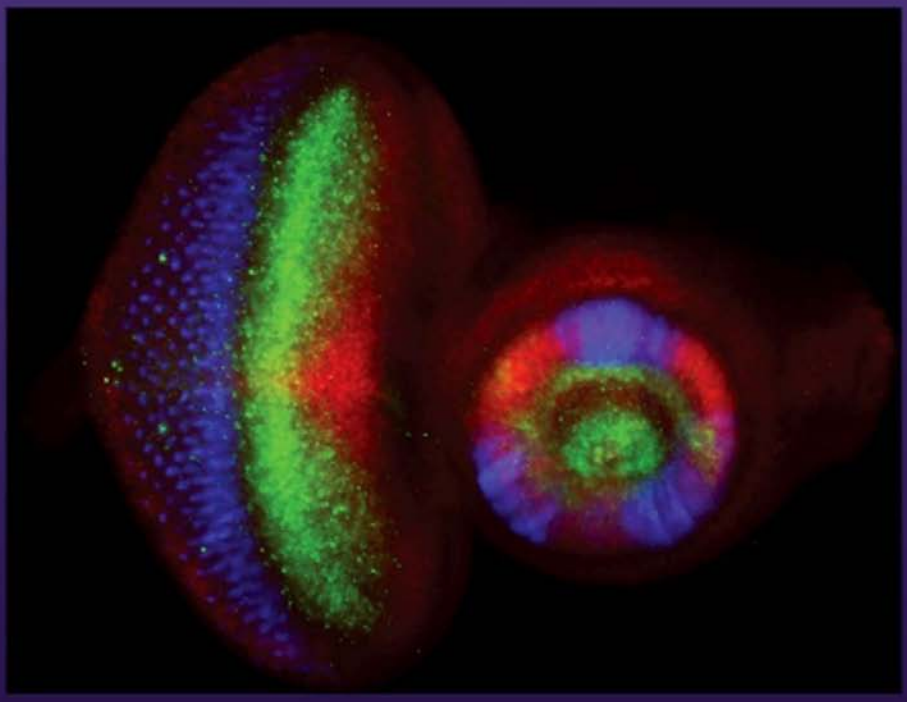
Invertebrate and Vertebrate Eye Development



Edited by

Ross L. Cagan & Thomas A. Reh

VOLUME NINETY THREE

Current Topics in DEVELOPMENTAL BIOLOGY

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Invertebrate and Vertebrate Eye Development

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Preface

Eye development has long been a favorite for exploring mechanisms of cell fate choice, patterning, cell signaling, etc. There are two reasons. First, vision is our primary sensory modality, and so we are naturally curious as to how the visual system assembles. Second, the visual system is in many ways remarkably simple, a repeating assemblage of neurons and support cells that parse the visual field through precision and redundancy. Through this simplicity, the eye has often led the way in our exploration of how an organ is assembled.

In the following set of reviews, several of these topics are on display: really a survey of the exceptional advances over the past twenty years. One theme that has emerged is the marked similarities but also significant differences between the invertebrate and vertebrate eyes. The differences that have emerged were both expected and unexpected. Early expectations were that the various eyes were examples of convergent evolution, making the assumption that a compound eye and a simple camera eye were simply too different structurally to derive easily from a common, recent progenitor. Furthermore, the two retina types are derived from different tissue layers in the head: the compound eye is derived from the overlying ectoderm while the vertebrate camera eye is induced from within deeper neuroectoderm.

These differences are not, however, the whole story. While the issue is not settled, many evolutionary biologists believe that at least most eyes are indeed derived from a common progenitor. The most basic "eye" requires two cells: a neuron for photoreception and a lens or pigmented cell to provide directionality, properties shared by most eyes. Perhaps some of our ancestors had both invertebrate-like rhabdomeric photoreceptor neurons plus vertebrate-like ciliary photoreceptor neurons, leading to an eventual split as divergence of body size necessitated different types of eyes to fit on the available space. But perhaps the strongest evidence for an ancestral link is the similarity in genes and gene networks that are active in both eye types. As described in the chapters in this issue, many of the genes that establish both the eye field and specific cell types are well conserved among diverse species. Although many of the mechanistic details may differ between the way in which the eyes of vertebrates and invertebrates are assembled, they have enough in common to continue to inform us about the fundamental features of the retinal assembly.

In the collection of reviews that follows, we have emphasized younger faculty that represent the emerging future of the field. They have the

advantage of seeing the progress but also have clear views on where their fields are heading. We begin with reviews by Justin Kumar, Michael Zuber, and Sabine Fuhrmann, who explore the mechanisms that establish the eye fields in flies and vertebrates. These reviews emphasize our knowledge of transcription factors and how these generate networks to direct the eye field and associated structures. Particularly in vertebrates we still only poorly understand how these factors connect to the signaling factors between cells and tissues that establish the eye field, but Zuber's and Fuhrmann's reviews emphasize that significant progress has been made.

We next present three reviews that explore cell fate specification. The eye has been a leader in this field due to the relatively small number of cell types coupled with the wave-like nature in which in cell type emerges. Sujin Bao and Tiffany Cook examine the local signals that direct early photoreceptor cell type specification, later photoreceptor type identity, and the emergence of the support cells that are required to establish the ommatidial field. An exciting new topic reviewed by Cook explores the potential of Drosophila "cone cells" to be a model for lens formation, while Bao explores concepts such as cell adhesion that act apparently independent of cell fate specification to move cells within the eye field. Complementing this is Michael Dyer's review of our current state of knowledge regarding the factors that direct vertebrate retinal development. In addition to examining specific factors identified in Drosophila, Dyer is part of a new generation approach that takes a more systems approach to identifying factors and also better defines cell subtypes.

Finally, we have two reviews examining other aspects of eye development. Andreas Jenny reviews the impressive progress made in using the Drosophila eye to examine "planar cell polarity," the mechanisms by which cells and cell groups are orientated with respect to the surface field. Using ommatidial rotation as a readout, the fly eye has played an important role in exploring general aspects of PCP. And no set of eye reviews would be complete without also considering the establishment of retinal connections into the brain. Andrew Huberman examines our current state of knowledge on how processes from the eye find their way to their correct targets, through both pathfinding and selective pruning.

Our objective in these reviews was not to be comprehensive but to explore issues that represent successes and to identify questions that the eye field is beginning to address. We have selected researchers who have been especially active in defining and moving forward these new paradigms.

Ross L. Cagan and Thomas A. Reh

CHAPTER ONE

Retinal Determination: The Beginning of Eye Development

Justin P. Kumar

Contents

Abstract

The road to producing an eye begins with the decision to commit a population of cells to adopting an eye tissue fate, the process of retinal determination. Over the past decade and a half, a network of transcription factors has been found to mediate this process in all seeing animals. This retinal determination network is known to regulate not only tissue fate but also cell proliferation, pattern formation, compartment boundary establishment, and even retinal cell specification. The compound eye of the fruit fly, *Drosophila melanogaster*, has proven to be an excellent experimental system to study the mechanisms by which this

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network regulates organogenesis and tissue patterning. In fact the founding members of most of the gene families that make up this network were first isolated in *Drosophila* based on loss-of-function phenotypes that affect the eye. This chapter will highlight the history of discovery of the retinal determination network and will draw attention to the molecular and biochemical mechanisms that underlie our understanding of how the fate of the retina is determined.

1. Introduction

The retinal determination network in *Drosophila* sets in motion the process by which approximately 20,000 undifferentiated cells are specified and incorporated into the several hundred unit eyes or ommatidia that comprise the adult retina. The genes that are included within the network work to coordinate cell proliferation rates, regulate the initiation and progression of the morphogenetic furrow, specify and maintain individual cell fates, as well as eliminate excess numbers by programmed cell death. The network is a central part of eye development from its beginnings during embryogenesis through its completion within the adult. As retinal precursor cells are initially set aside during embryogenesis (Cohen, 1993; Held, 2002), several members of the network begin the task of canalizing these cells toward adopting an eye fate. And as the morphogenetic furrow later patterns the retina (Lebovitz and Ready, 1986; Ready *et al.*, 1976; Wolff and Ready, 1991), the retinal determination network plays critical roles in its initiation and progression. Later, as individual ommatidia are being assembled behind the furrow (Cagan and Ready, 1989a,b; Tomlinson and Ready, 1986, 1987a,b), a number of these factors play critical roles in the acquisition of photoreceptor neurons, lens-secreting cone, and optically insulating pigment cell fates. And finally, select retinal determination genes function in the adult retina to activate the expression of light-capturing rhodopsin genes (Sheng *et al.*, 1997). This review will introduce the reader to the genes that comprise the retinal determination network in *Drosophila* and will highlight the role that these genes play during eye specification. It will also draw attention to the intricate molecular and biochemical relationships that exist between network members. Particular attention will be placed on emphasizing the spatial and temporal nature of these relationships within the retinal epithelium.

2. Structure and Development of the *Drosophila* Eye

Since its initial structural and developmental description by Ready and coworkers more than 30 years ago, the compound eye has served as an excellent model system for understanding a myriad of developmental

processes including organogenesis, cell proliferation and apoptosis, compartment boundary establishment, pattern formation, cell fate specification, planar cell polarity and cell rotation, as well as axon projection and guidance. Its simple adult structure and stereotyped developmental history have allowed us to also study basic mechanisms of morphogen gradients, gene regulation and signal transduction, and gene regulatory networks. Despite three decades of exploration, the eye continues to provide fertile ground for the discovery of new and exciting cellular mechanisms. Indeed, each passing year brings more than a hundred new papers and this continues to fuel our growing understanding of the mechanisms that underlie the specification and patterning of this near perfect simple nervous system.

The adult retina consists of approximately 800 unit eyes. Each is a six-sided replica of its neighbors; thus the adult retina is a precise hexagonal display that has been described as a neurocrystalline lattice (Fig. 1.1A;

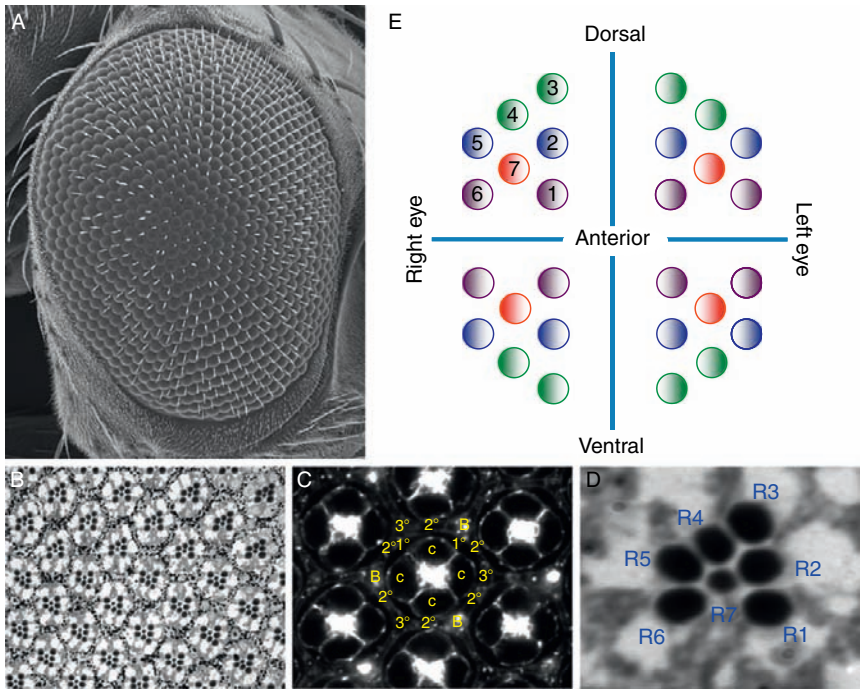

Figure 1.1 Structure of the adult compound eye. (A) Scanning electron micrograph of the adult eye. (B) Section of the adult retina showing the photoreceptor neurons. (C) Section of the pupal retina showing all cone, pigment, and bristle cells. (D) A high magnification of a single ommatidium from the adult retina. Note that each photoreceptor neuron is given a unique identifier number. (E) A schematic describing the orientation of ommatidia in the dorsal and ventral quadrants in the left and right compound eyes. Anterior is to the right in all images.

Ready *et al.*, 1976). Each ommatidium contains approximately 20 cells: 8 photoreceptor neurons and 12 lens-secreting cone cells and optically insulating pigment cells (Fig. 1.1B and C; Ready, 1989). The placement of the photoreceptors within the ommatidium is stereotyped and resembles that of an asymmetric trapezoid (Fig. 1.1D and E; Dietrich, 1909). Each cell within the ommatidium is specified through a combination of cell–cell interactions as well as short- and long-range signals during the final larval instar and pupal stage of the life cycle (Cagan and Ready, 1989a,b; Tomlinson and Ready, 1986, 1987a,b). The template for the pupal and adult retinas is the eye imaginal disk. Prior to the onset of pattern formation, all cells within the disk are unpatterned and undifferentiated. But during the third and final instar, a wave of differentiation initiates at the posterior margin of the disk and proceeds toward the anterior edge of the epithelium. The most anterior edge of this wave is called the morphogenetic furrow and transforms the sea of undifferentiated cells into a tiling of periodically spaced ommatidial rudiments (Fig. 1.2A–C; Ready *et al.*, 1976; Wolff and Ready, 1991). Once the photoreceptors cells have been specified, they will undergo significant morphological changes including the elaboration of their rhabdomeres, which are the light sensitive organelles homologous to the outer segments of vertebrate photoreceptors (Kumar and

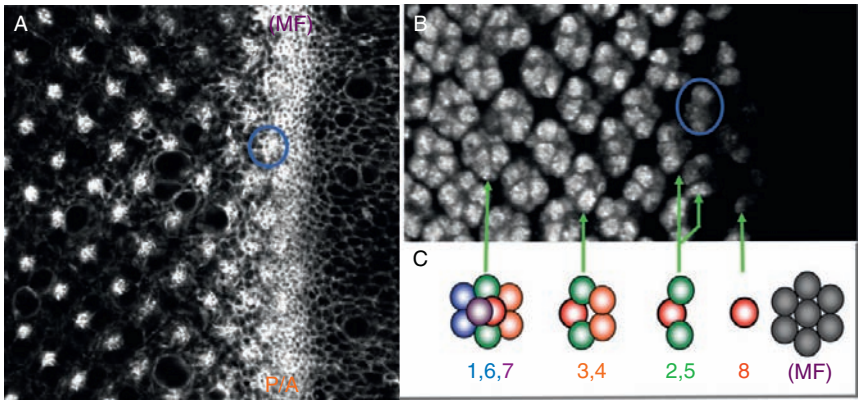

Figure 1.2 The morphogenetic furrow and ommatidial assembly. (A) A confocal section of a third instar larval eye disk that has been stained with phalloidin, which marks F-actin. Note that cells ahead of the furrow are unpatterned while those behind the furrow are organized into ommatidial rudiments. The blue circle marks one individual unit eye. (B) A confocal section of a third instar larval eye disk that has been stained with an antibody that is directed against the ELAV protein. The blue circle marks a unit eye that is in roughly the same position as the one in panel A. (C) A schematic drawing of the order of ommatidial assembly within the eye disk. Anterior is to the right in all images. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this chapter.)

Ready, 1995; Longley and Ready, 1995). These cells will go on to express rhodopsins, which are light-capturing photopigments, while the cone cells will secrete the overlying lens and the pigment cells will provide the bulk of the optically insulating pigment granules (reviewed in Wolff and Ready, 1993).

3. The Retinal Determination Network: Membership Has Its Privileges

Membership within the retinal determination network currently stands at 14 genes, the vast majority of which code for DNA-binding proteins (Fig. 1.3A). These include *eyeless* (*ey*; Quiring *et al.*, 1994), *twin of eyeless* (*toy*; Czerny *et al.*, 1999), *eyegone* (*eyg*; Jun *et al.*, 1998), *twin of eyegone* (*toe*; Aldaz *et al.*, 2003), *sine oculis* (*so*; Cheyette *et al.*, 1994; Serikaku and O'Tousa, 1994), *optix* (Seimiya and Gehring, 2000), *teashirt* (*tsh*: Pan and Rubin, 1998), *tiptop* (*tio*; Laugier *et al.*, 2005), *distal antenna* (*dan*; Curtiss *et al.*, 2007), *distal antenna related* (*danr*; Curtiss *et al.*, 2007), *dachshund* (*dac*; Mardon *et al.*, 1994), and *homothorax* (*hth*; Pai *et al.*, 1997). The remaining two genes encode the *eyes absent* (*eya*) transcriptional coactivator/protein tyrosine phosphatase (Bonini *et al.*, 1993) and the *nemo* (*nmo*) protein kinase (Braid and Verheyen, 2008; Choi and Benzer, 1994). While these factors were initially thought to function as purely selector genes, more recent evidence indicates that these genes play roles in the proliferation of progenitor cells, the differentiation of retinal precursors, and the specification and/or maintenance of photoreceptor neurons (Bessa *et al.*, 2002; Lopes and Casares, 2009; Peng *et al.*, 2009; Pignoni *et al.*, 1997). Nearly every one of these genes is also represented in vertebrates, and many have been implicated in retinal disorders (Fig. 1.3A; Chi and Epstein, 2002; Hanson, 2001; Jean *et al.*, 1998; Kozmik, 2005; Kumar, 2009a,b; Mansouri *et al.*, 1999; Wawersik and Maas, 2000). Additionally, several of these factors also play crucial roles in the development of a broad range of nonretinal tissues and organs (Brodbeck and Englert, 2004; Chi and Epstein, 2002; Christensen *et al.*, 2008; Dressler, 2006; Kawakami *et al.*, 2000), thereby adding to their growing importance in both development and disease.

Historically, the founding members of the retinal determination network were grouped together and considered part of a single regulatory system if they displayed two physical attributes. First, loss-of-function mutations needed to result in a strongly reduced or missing eye phenotype (Fig. 1.3B; Bonini *et al.*, 1993; Cheyette *et al.*, 1994; Dominguez *et al.*, 2004; Jang *et al.*, 2003; Mardon *et al.*, 1994; Quiring *et al.*, 1994; Serikaku and O'Tousa, 1994). Second, forced expression in nonretinal tissues had to

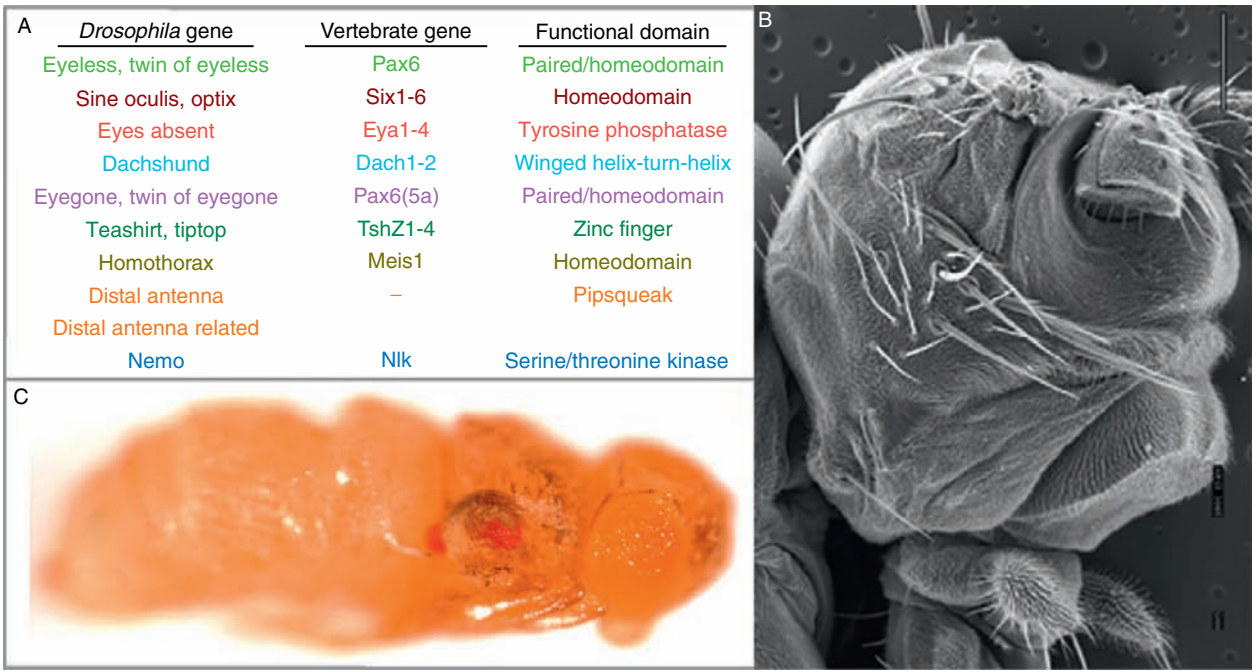

Figure 1.3 The retinal determination network: genes and phenotypes. (A) A list of the known retinal determination genes, the vertebrate homologs, and the known functional domains. (B) A scanning electron micrograph of a *sine oculis* loss-of-function mutant. Note that the compound eyes are missing and have been replaced by head cuticle. (C) A light microscope image of an animal in which the *ey* gene has been expressed in the wing and haltere disks using an ap-GAL4 driver. Anterior is to the right in all images.

be sufficient to force these cells into adopting a retinal fate (Fig. 1.3C; Bonini *et al.*, 1997; Chen *et al.*, 1997; Halder *et al.*, 1995; Pignoni *et al.*, 1997; Weasner *et al.*, 2007). But as the number of genetic screens and genome/proteome-wide studies has grown, new genes have been included in the network based on combinations of criteria that had been expanded to include genetic, molecular, or biochemical interactions with existing pathway members (Bessa *et al.*, 2009; Braid and Verheyen, 2008; Curtiss *et al.*, 2007; Czerny *et al.*, 1999; Datta *et al.*, 2009; Pai *et al.*, 1997; Pan and Rubin, 1998; Seimiya and Gehring, 2000; Yao *et al.*, 2008). As of this writing, the number of genes that are universally accepted as being *bona fide* retinal determination genes stands at 14 (see above). This expanded list of genes has provided us with an opportunity to gain a much more sophisticated understanding of how early decisions in developing organs, particularly that of the eye, are executed. Our appreciation for how an eye is specified will only accelerate exponentially as new genes are identified as playing a role during early retinal differentiation.

4. The Molecular Biology and Biochemistry of Retinal Determination

The genes that govern eye specification are said to constitute a network rather than a cascade or pathway because the experimentally verified genetic, molecular, and biochemical interactions among the various members include several reinforcing positive feedback loops, mutually dampening negative interactions, and self-fortifying autoregulatory circuits (Fig. 1.4; Kumar, 2009a,b). A growing body of evidence is also suggesting that the transcriptional output of each retinal determination gene is controlled by the combined regulatory inputs of other network members (Niimi *et al.*, 1999; Pappu *et al.*, 2005; Pauli *et al.*, 2005). Adding additional layers of regulatory complexity is the fact that numerous signaling pathways are known to integrate into the network: multiple pathways are used to activate/repress transcription of individual genes and each pathway appears to integrate into the network at multiple levels (Chen *et al.*, 1999; Kenyon *et al.*, 2003; Kumar and Moses, 2001a; Kurata *et al.*, 2000). All these interactions (at least the ones that are relevant for eye specification) occur in two places: first, they occur throughout the eye disk prior to the initiation of the morphogenetic furrow and second, once pattern formation has initiated, they occur ahead of the advancing furrow. This section will highlight features of the retinal determination proteins themselves and will draw attention to the molecular and biochemical interactions that are known to exist between the network members.

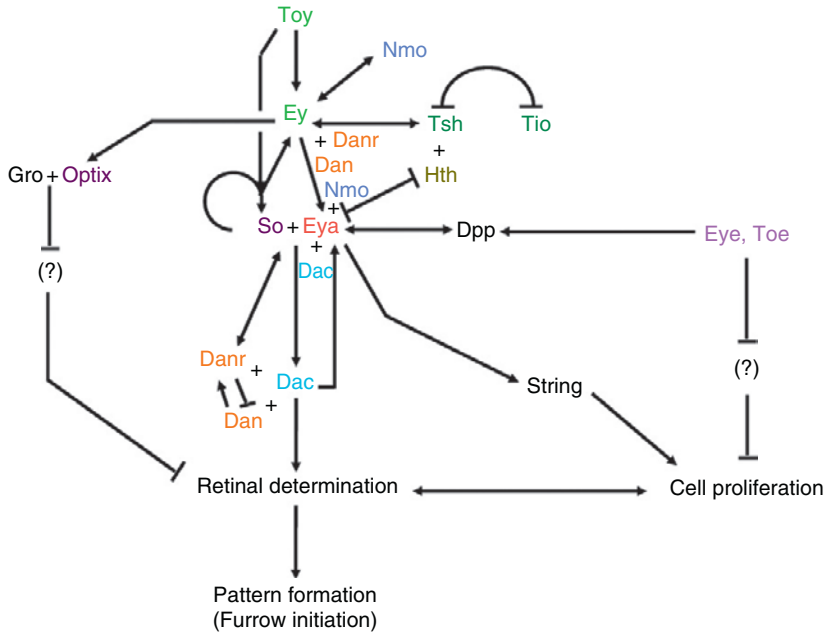

Figure 1.4 The workings of the retinal determination network. A schematic of all known interactions within the retinal determination network. Note that the cascade does not function as a linear pathway but rather contains autoregulatory circuits and feedback loops.

4.1. Eyeless/Pax6: King of kings

The founding member of the retinal determination network was *ey*. Mutations in this gene were discovered nearly 100 years ago based on their no-eye phenotype (Hoge, 1915). Its central role in early eye development was established by its description as a transcription factor with homology to human *Pax6* and murine *Small eye* (Quiring *et al.*, 1994), the demonstration that it could force cell populations in nonretinal tissues to adopt an eye fate (Halder *et al.*, 1995), the revelation that it regulates a large number of target genes in the developing eye (Michaut *et al.*, 2003; Niimi *et al.*, 1999; Ostrin *et al.*, 2006), and the identification of functional orthologs within nearly every phylum of the animal kingdom (Callaerts *et al.*, 1997; Gehring, 1996; Gehring and Ikeo, 1999). Unlike vertebrates, the fly genome contains a second Pax6 gene, *toy*. Its expression precedes that of *ey*, and Toy protein can directly activate *ey* transcription during embryonic development (Czerny *et al.*, 1999; Hauck *et al.*, 1999; Kronhamn *et al.*, 2002). This interaction appears to be unidirectional since Ey can neither feed backward to activate *toy* expression nor bind to its own eye-specific enhancer

(Fig. 1.4; Czerny *et al.*, 1999). These two Pax6 proteins then, either separately or together depending upon the target enhancer, activate the transcription of several other retinal determination genes including *so*, *eya*, *dac*, and *optix* (Fig. 1.4; Halder *et al.*, 1998; Niimi *et al.*, 1999; Ostrin *et al.*, 2006; Pappu *et al.*, 2005). During the first and second larval instars, these interactions take place throughout the entire eye disk. However, by the third larval instar, Toy and Ey are only able to activate their targets within specific regions of the epithelium (see below).

The two proteins cannot fully substitute for each other: expression of *toy* in an *ey* mutant background fails to promote retinal development (Czerny *et al.*, 1999; Kronhamn *et al.*, 2002). While forced expression of either Pax6 gene can direct ectopic eye formation, Ey appears to be able to induce retinal development in a broader topographical range than Toy (Czerny *et al.*, 1999; Halder *et al.*, 1995; Salzer and Kumar, 2010). These differences have been the subject of several molecular dissections (Clements *et al.*, 2009; Punzo et al., 2001, 2004; Weasner *et al.*, 2009), and results from these studies indicate that reported distinctions and disparities can be attributed to different transactivation potentials (Punzo *et al.*, 2004; Weasner *et al.*, 2009) as well as the unique presence of transcriptional repressive activity within Ey (Weasner *et al.*, 2009). Additionally, it appears that the paired DNA-binding domain and the C-terminal segment (which contains the activation domain) are the only segments of either Ey or Toy that are required for the promoting of eye ectopic formation (Punzo *et al.*, 2001; Weasner *et al.*, 2009); this suggests that Pax6 proteins may in fact function in modular fashion with different DNA-binding domains and nonconserved segments being used in different molecular and developmental contexts. Immediately, after *ey* was initially cloned and shown capable of inducing ectopic eye formation, it was crowned as "the master control gene" for eye development. Ey was thought to assume its role as king when transcription of it (and its sister gene *toy*) initiated during embryogenesis (Callaerts *et al.*, 1997; Halder *et al.*, 1995; Quiring *et al.*, 1994). Ey still rules the eye although it must share its throne with the other eye specification genes as they share many of the properties that once were the realm of Ey alone.

4.2. So–Eya: A workhorse complex in the early eye

While Ey has grabbed much of the spotlight over the years, several of the more downstream members of the network such as So, Eya, and Dac may in fact turn out to be just as influential in terms of regulating early eye development. Transcription of each of these three genes is responsive to the expression of *ey* (Halder *et al.*, 1998; Michaut *et al.*, 2003; Ostrin *et al.*, 2006; Pappu *et al.*, 2005; Punzo *et al.*, 2002) with additional evidence supporting direct binding of both Ey and Toy to an eye-specific enhancer within the *so* transcriptional unit (Niimi *et al.*, 1999). So, the founding

member of the Six family is a homeobox containing transcription factor (Cheyette *et al.*, 1994; Serikaku and O'Tousa, 1994) that on its own can bind to DNA but is unable to strongly activate transcription of its target. It forms a biochemical complex with the Eya transcriptional coactivator (Kenyon *et al.*, 2005a,b; Pignoni *et al.*, 1997), which also functions as a protein tyrosine phosphatase (Li *et al.*, 2003; Rayapureddi *et al.*, 2003; Tootle *et al.*, 2003). The So–Eya complex is thought to influence eye specification by regulating the other members of the retinal determination network such as *dac* (Pappu *et al.*, 2005) as well as by feeding back to autoregulate its own transcription and that of *ey* (Pauli *et al.*, 2005). But the influence that So–Eya has on eye development does not stop at retinal determination. In fact, the complex regulates the transcription of genes that play critical roles in the initiation of the furrow (*hedgehog*; Pauli *et al.*, 2005), the cell cycle (*string*; Jemc and Rebay, 2007), and cell fate decisions (*lozenge*; Tanaka-Matakatsu and Du, 2008; Yan *et al.*, 2003; *atonal*, Zhang *et al.*, 2006).

A second Six protein, Optix, is distributed in the eye and can induce ectopic eye formation (Seimiya and Gehring, 2000), but the lack of published loss-of-function phenotypes has made it difficult to define a role for *optix* in retinal development. Despite this, it has been possible to gain some hints into the molecular workings of Optix. Recent reports have indicated that the binding sites for all Six proteins in *Drosophila* are very similar, if not identical, to each other (Berger *et al.*, 2008; Noyes *et al.*, 2008). Thus, it is likely that Optix and So bind and regulate common target genes. However, rescue experiments have indicated that Optix cannot substitute for So in eye development (Weasner *et al.*, 2007). This is due to cryptic functional motifs embedded within the nonconserved C-terminal (Weasner and Kumar, 2009) and differences in binding partner specificity (Kenyon *et al.*, 2005a, b; Weasner *et al.*, 2007). Of particular interest is the ability of So to toggle between activator and repressor by interacting with both Eya and the transcriptional corepressor Groucho (Gro; Kenyon *et al.*, 2005a; Salzer and Kumar, 2009) while Optix appears to be a dedicated repressor as it can only interact with Gro (Kenyon *et al.*, 2005a).

4.3. Dac: A transcription factor in search of a target

While a requirement for *dac* in eye specification and morphogenetic furrow initiation has been well documented (Chen *et al.*, 1997; Mardon *et al.*, 1994; Shen and Mardon, 1997), its molecular and biochemical roles in these processes have remained somewhat elusive, in part, because neither consensus binding sites nor transcriptional targets in the eye have been identified despite a significant body of evidence suggesting that it functions as a *bona fide* transcription factor. For example, sequence analysis suggests that Dac is related to members of the Ski/Sno family of proto-oncogenes

(Hammond *et al.*, 1998), contains a winged helix-turn-helix DNA-binding motif, and can physically interact with double-stranded nucleic acids (Kim *et al.*, 2002). Further evidence indicates that Dac is not only capable of activating transcription of a reporter, on its own, in yeast but can also physically interact with Eya (Chen *et al.*, 1997). Several reports indicate that the vertebrate homolog Dach1 switches between serving as an activator (Ikeda *et al.*, 2002; Li *et al.*, 2003) and functioning as a repressor (Li *et al.*, 2002; Wu *et al.*, 2003). The only potential targets known in the eye are the upstream factors *so* and *eya,* as loss of *dac* at the margin of the eye field leads to the loss of both of these genes (Salzer and Kumar, 2009).

4.4. Hth–Exd and Tsh: Suppressors of eye specification

In *Drosophila*, homeotic (Hox) genes regulate the development of body segments along the anterior–posterior (A/P) axis. They are expressed sequentially along the embryonic A/P axis and encode homeobox containing transcription factors. It was discovered early on that these proteins bind to very similar sequences (reviewed in Mann, 1995). It was thus proposed and later confirmed that Hox genes required cofactors to ensure binding specificity. One such cofactor is the product of the *extradenticle* (*exd*) gene. It also encodes a homeodomain and its binding to Hox proteins can alter their DNA-binding specificity (Chan and Mann, 1996; Chan *et al.*, 1996). Interestingly, Exd is not located in the nucleus of all cells but rather is found both in the cytoplasm and the nucleus (Aspland and White, 1997). The Homothorax (Hth) protein, which shares extensive homology to the murine Meis1/2 proteins, contains a TALE class homeobox, binds to Exd, and translocates it to the nucleus where it can interact with Hox genes (Jaw *et al.*, 2000; Pai *et al.*, 1997; Rieckhof *et al.*, 1997).

The Hth–Exd complex is important for eye development as loss-of-function mutations in either factor lead to the formation of ectopic eyes (Gonzales-Crespo *et al.*, 1998; Pai *et al.*, 1997; Pichaud and Casares, 2000; Rauskolb *et al.*, 1995). This inhibition of normal eye development likely occurs through direct transcriptional repression of retinal determination genes as forced expression of *hth* can repress both *eya* and *dac* (but not *ey*). Presently, the Hth-binding site has not been determined but an effort to identify transcriptional targets has localized Hth to approximately 150 sites on *Drosophila* polytene chromosomes (Cohen and Salzberg, 2008). At the moment, the resolution of this map does not allow for the identification of individual genes but a scan of the listed cytological location suggests that Hth may not be occupying sites within *eya* or *dac.* This does not necessarily rule out a direct regulatory mechanism since the efficiency of repression is synergistically increased with the coexpression of both Ey and the zinc finger transcription factor Teashirt (Tsh), two factors that physically interact with Hth (Bessa *et al.*, 2002). It is likely that some configuration of this

complex is necessary to directly repress both *eya* and *dac* since Hth is a transcriptional activator (Inbal *et al.*, 2001) while both Ey and Tsh can, depending upon the circumstance, function as transcriptional repressors (Bessa *et al.*, 2009; Weasner *et al.*, 2009).

Tiptop (Tio) is structurally related to Tsh (Laugier *et al.*, 2005) and can promote eye development in forced expression assays (Bessa *et al.*, 2009; Datta *et al.*, 2009). In the eye, both genes are expressed in identical patterns and at approximately equal levels. Interestingly, *tio* loss-of-function mutants are completely viable and have no retinal defects. Similarly, *tsh* loss-of-function mutations have little to no effect on the eye (Laugier *et al.*, 2005; Pan and Rubin, 1998). This suggests that these genes are functionally redundant. The loss of each gene leads to an upregulation of the other while elevated expression levels of each gene results in the downregulation of the other (Bessa *et al.*, 2009). Both results provide further evidence that Tsh and also Tio function as transcriptional repressors. Does Tio function to also repress *eya* and *dac* transcription? At present, the answer is not clear as binding sites for neither gene have been identified, but such a mechanism would be consistent with the redundant role that Tio plays in the early eye.

4.5. Nemo: From ommatidial rotation to eye specification

nmo is a founding member of Nemo-like kinase family of proline-directed serine–threonine kinases and was initially studied for its role in ommatidial rotation (Choi and Benzer, 1994). But its expression pattern, which overlapped with several retinal determination genes, hinted of roles in early eye development. A recent report has demonstrated that *nmo* interacts genetically with *ey* and *eya* during normal retinal development as well as during the induction of ectopic eye formation (Fig. 1.4; Braid and Verheyen, 2008). Excitingly, forced expression of *nmo* is sufficient, on its own, to induce ectopic eye formation (Braid and Verheyen, 2008). To date, the only known substrate of Nemo is the Mothers against dpp (Mad) protein, which is a downstream component of the TGFβ signaling pathway (Zheng *et al.*, 1995). A tantalizing model is that one or more retinal determination proteins are a substrate for phosphorylation by Nmo. As *nmo* genetically interacts with both *eya* and *ey*, these two factors would be the likely targets.

4.6. Dan and Danr: An antennal gene regulates the eye

The *dan* and *danr* genes code for Pipsqueak-class DNA-binding proteins and were initially identified as playing critical roles in antennal development (Emerald *et al.*, 2003). Both genes appear to also play important roles in specifying the eye, in part, by participation within the retinal determination network (Curtiss *et al.*, 2007). Both Dan and Danr contribute by regulating the expression of *ey* and *eya*. In turn, the So–Eya–Dac complex activates

expression of both *dan* and *danr* (Fig. 1.4). Furthermore, *dan* and *danr* appear to regulate each other with Dan activating *danr* transcription and Danr repressing *dan* expression (Fig. 1.4; Curtiss *et al.*, 2007). It is not yet known if any of these interactions are direct. In another twist, Dan and Danr form physical complexes with both Ey and Dac (Fig. 1.4; Curtiss *et al.*, 2007). These multiple molecular and biochemical interactions suggest that both Dan and Danr play important roles in specifying the compound eye.

5. One Network yet Several Incarnations

As anyone who has read papers dealing with retinal determination can attest, one will usually find a depiction of the retinal determination network that is not much different than the one in Fig. 1.4, which is replete with activation steps, inhibitory, and autoregulatory loops. But closer inspections of expression patterns and mutant phenotypes indicate that all these interactions cannot and are not happening within the entire eye. In fact, several reports indicate that subsets of interactions are taking place in different geographical locations within the eye (Bessa *et al.*, 2002; Lopes and Casares, 2009; Mardon *et al.*, 1994; Peng *et al.*, 2009; Pignoni *et al.*, 1997; Salzer and Kumar, 2009); therefore, this section will place the aforementioned genetic, molecular, and biochemical interactions in temporal and spatial contexts.

5.1. Embryogenesis

The eye field initiates its development during embryogenesis as a simple cluster of approximately 20 cells (Cohen, 1993; Held, 2002). As these cells delaminate from the surface ectoderm, expression of the four Pax genes (*ey*, *toy*, *eyg*, and *toe*) and the Six gene *optix* is initiated (Aldaz *et al.*, 2003; Czerny *et al.*, 1999; Kronhamn *et al.*, 2002; Kumar and Moses, 2001a; Quiring *et al.*, 1994; Seimiya and Gehring, 2000). Very little is known about the molecular interactions that take place in the embryonic eye disk, save for the initial step in which *toy* expression precedes and presumably activates *ey* through binding at the eye-specific enhancer (Czerny *et al.*, 1999; Hauck *et al.*, 1999; Kronhamn *et al.*, 2002).

5.2. Larval development

Upon hatching of the embryo into a larva, these cells continue to divide rapidly and are self-organized into a monolayer epithelium called the eye-antennal imaginal disk (Fig. 1.2). During the first two larval instars, the disk

is primarily concerned with establishing dorsal–ventral compartment boundaries (Cho and Choi, 1998; Dominguez and de Celis, 1998; Papayannopoulos *et al.*, 1998; Sato and Tomlinson, 2007), while rapidly generating the requisite number of cells that are needed for ommatidial assembly and with molecularly canalizing the epithelium toward an eye fate. This last task is achieved, in part, by the expression of the remaining retinal determination genes (Bonini *et al.*, 1993; Braid and Verheyen, 2008; Curtiss *et al.*, 2007; Kumar and Moses, 2001b; Mardon *et al.*, 1994; Serikaku and O'Tousa, 1994; Singh *et al.*, 2002).

As the larva enters the third and final instar stage, a wave of differentiation initiates at the posterior margin of the disk and proceeds toward the anterior border of the eye field (Ready *et al.*, 1976). As this mobile compartment boundary progresses across the retinal field, the vast expanse of unpatterned and undifferentiated cells are transformed into a highly ordered display of periodically spaced unit eyes or ommatidia (Ready *et al.*, 1976; Tomlinson and Ready, 1987a,b; Wolff and Ready, 1991). This moving current of differentiation can be visualized by an indentation in the tissue and is referred to as the morphogenetic furrow (Ready *et al.*, 1976). As the furrow progresses across the eye field, expression of most retinal determination genes, which was once uniform throughout the entire disk, is now relegated to regions that remain undifferentiated and possibly undetermined (Bessa *et al.*, 2009; Curtiss *et al.*, 2007; Czerny *et al.*, 1999; Datta *et al.*, 2009; Dominguez *et al.*, 2004; Jang *et al.*, 2003; Pai *et al.*, 1997; Pan and Rubin, 1998; Quiring *et al.*, 1994; Seimiya and Gehring, 2000). A few genes such as *so*, *eya*, *dac*, and *nmo* continue to be expressed in cells that now lie behind the furrow and contribute to cell fate choices within developing ommatidia (Bonini *et al.*, 1993; Cheyette *et al.*, 1994; Choi and Benzer, 1994; Mardon *et al.*, 1994; Serikaku and O'Tousa, 1994). The full complexity of retinal determination gene expression can most easily be observed and appreciated when the furrow has advanced roughly halfway across the eye field (Fig. 1.5).

Based on the expression patterns of genes that are known to regulate eye specification, furrow progression, and cell fate specification, the mid-third instar eye field can be divided into several broad zones. However, there is some disagreement as to the physical extent of each expression pattern and the role that each gene may play in the proliferation of progenitor cells versus their role in the specification of precursor cells. This has in turn resulted in differences in the labeling/nomenclature of individual zones (Bessa *et al.*, 2002; Braid and Verheyen, 2008; Lopes and Casares, 2009; Pappu and Mardon, 2004; Silver and Rebay, 2005). Based on the published expression domains of each retinal determination gene and on loss-of-function phenotypes, we subdivide the eye into six zones (A–F; Fig. 1.5). We discuss each zone in turn starting with the region of the disk that borders the antennal segment.

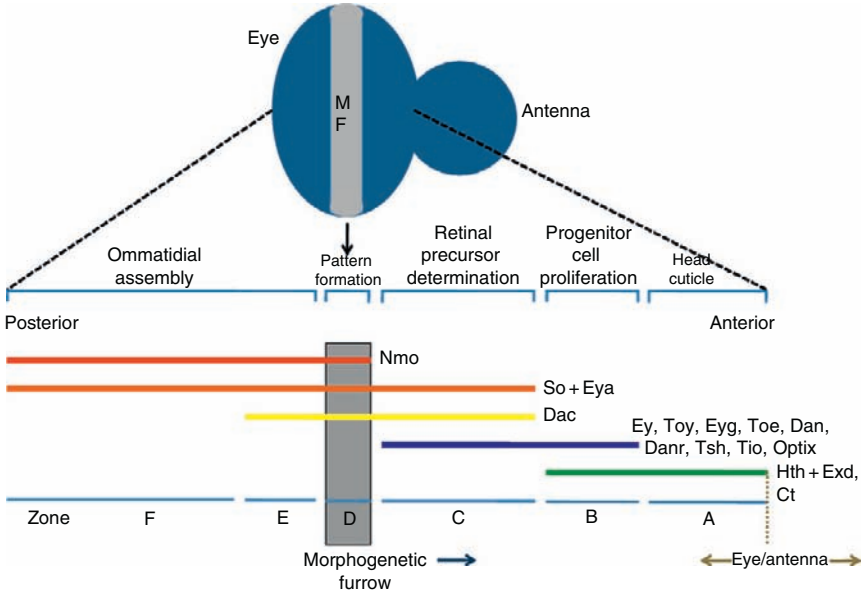

Figure 1.5 Expression patterns of retinal determination genes within the developing eye field. The eye disk is divided into six zones based on the expression patterns and functions of the known retinal determination genes. The zones are listed at the bottom of the figure. The expression patterns of each gene are represented by the colored horizontal lines. The ongoing developmental processes within each zone are listed at the top of the figure. The morphogenetic furrow is shown in gray. Anterior is to the right in this schematic diagram. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this chapter.)

The most anterior edge of the eye primordium (Zone A) actually will not yield retinal tissue at all but rather will give rise to head cuticle (Fig. 1.5; Haynie and Bryant, 1986). Cells within this zone appear not to express any retinal determination genes, save for one exception, *hth*, and its cofactor *exd* (Bessa *et al.*, 2002; Pai *et al.*, 1997; Pichaud and Casares, 2000). The Hth–Exd complex appears to normally block retinal development as loss-of-function clones of either gene result in ectopic eyes where head cuticle is normally produced (Gonzales-Crespo *et al.*, 1998; Pai *et al.*, 1997; Rauskolb *et al.*, 1995). Similarly, simultaneously coexpressing both factors leads to an inhibition of normal eye development (Pai *et al.*, 1997). In addition to the Hth–Exd complex, the antennal specifying factor *cut* (*ct*) is also expressed within Zone A thereby serving as an additional block on retinal development (Blochlinger *et al.*, 1993; Dong *et al.*, 2002). Thus, the combined efforts of these factors result in the proliferation of precursor cells that will be simultaneously blocked from adopting a retinal fate while being directed toward giving rise to head cuticle.

Directly adjacent to the head cuticle producing cells lie a swath of cells (Zone B) that are eventually slated to adopt an eye fate (Fig. 1.5). These cells, in addition to *hth* and *exd*, now begin to express most retinal determination genes including *ey/toy* (Czerny *et al.*, 1999; Quiring *et al.*, 1994), *eyg/toe* (Aldez *et al.*, 2003), *tsh/tio* (Bessa *et al.*, 2009; Datta *et al.*, 2009; Pan and Rubin, 1998), *dan/danr* (Curtiss *et al.*, 2007), and *optix* (Seimiya and Gehring, 2000). However, since the transcription of several key genes such as *so*, *eya*, and *dac* is not yet activated, the cells within this region of the disk will be held in a highly proliferative state while being temporarily blocked from being specified (Fig. 1.5; Lopes and Casares, 2009; Peng *et al.*, 2009). There appears to be several simultaneously acting mechanisms for promoting cell proliferation. First, Notch signaling promotes growth through at least one Pax6(5a) variant Eyg (Chao *et al.*, 2004; Dominguez *et al.*, 2004). Second, a complex containing Hth and Yorki (Yki), the most downstream member of the Hippo tumor suppressor pathway, stimulates growth by targeting and activating transcription of the *bantam* microRNA which in turn results in the downregulation of the cell death gene *head involution defective* (*hid*: Peng *et al.*, 2009). And finally, a transcription complex containing Hth, Tsh, and potentially Ey also contributes to cell proliferation rates in this region of the disk. Support for this last input comes from experiments showing that mutant *hth* clones rarely survive anterior to the furrow (Bessa *et al.*, 2002) and that disruptions with Ey function via developmental pathway interference can be partially rescued by the expression of several cell cycle genes such as *cyclin E* (*cycE*) and *myc* (Jiao *et al.*, 2001). It should be noted that this last complex is also thought to play a role in repressing *so*, *eya*, and *dac*, each of which is critical for the proper specification of the eye. The rare *hth* loss-of-function clones that do survive anterior to the furrow ectopically express these three genes in Zone B and this in turn promotes precocious eye development (Bessa *et al.*, 2002; Lopes and Casares, 2009; Pai *et al.*, 1997; Pichaud and Casares, 2000). Additionally, the coexpression of different combinations of these three genes (Tsh–Hth, Tsh–Ey, and Hth–Ey) is sufficient to inhibit expression of *so*, *eya*, and *dac* (Bessa *et al.*, 2002).

Cells that lie closest to the morphogenetic furrow (Zone C—also referred to as the preproneural [PPN] zone), while still proliferating, are on the cusp of being incorporated into the growing neurocrystalline lattice (Lebovitz and Ready, 1986). This transition, however, requires the presence of So, Eya, and Dac, as mutations in these genes affect the initiation and progression of the morphogenetic furrow (Mardon *et al.*, 1994; Pignoni *et al.*, 1997; Salzer and Kumar, 2009). These genes are activated by the downregulation of *hth* (Bessa *et al.*, 2002) although the exact mechanism of *hth* transcriptional inhibition remains unknown. At this point in the disk, the activation of all retinal determination genes is sufficient to transition cells from being undifferentiated and undetermined to adopting cellular fates that

are appropriate for the retina (Kumar and Moses, 2001b). As these cells prepare to be assembled into ommatidial units, several mechanisms for regulating the movement of the furrow and for selecting the first photoreceptor are initiated and are discussed in other chapters. Briefly, within Zone C, the bHLH protein Hairy (H) and the HLH protein Extramacrochaete (Emc) function together to prevent the pace of the patterning from outstripping the rate of cell proliferation ahead of the furrow (Brown *et al.*, 1995). There is evidence now to indicate that the loss of *emc*, on its own, is sufficient to accelerate the pace of the furrow (C.M. Spratford and J.P. Kumar, unpublished data). Emc and its vertebrate homologs (inhibitors of differentiation) function by sequestering DNA-binding proteins away from target promoters (Benezra *et al.*, 1990; Ellis *et al.*, 1990; Garrell and Modolell, 1990). At present, the targets of sequestration by Emc ahead of the furrow are yet to be found. Their identification will be a huge advance in understanding how the rate of pattern formation in the eye is regulated. In addition to Emc holding the furrow to a sustainable pace across the disk, the Wingless (Wg) signaling pathway prevents pattern formation from initiating inappropriately from the lateral margins of the epithelium. Wg is expressed at the margins and its loss leads to the initiation of ectopic morphogenetic furrows (Ma and Moses, 1995; Treisman and Rubin, 1995).

Within the morphogenetic furrow itself (Zone D), cells are arrested in the G1 phase of the cell cycle. As they transition to positions behind the furrow, a subset of cells will exit the cell cycle and begin to initiate their development as the first five cells of the ommatidium (R8, R2, R5, R3, and R4) while the remainder will undergo one last round of synchronous cell division which will in turn lead to the production of the remaining photoreceptor neurons (R1, R6, and R7), the nonneuronal cone and pigment cells, as well as the cells of the bristle complex (Wolff and Ready, 1991). The G1 arrest within the furrow is established and maintained by high levels of the TGFβ ligand Decapentaplegic (Dpp: Fig. 1.5; Horsfield *et al.*, 1998). In addition to affecting the cell cycle, Dpp signaling is also required for the initiation and progression of the furrow across the eye field (Chanut and Heberlein, 1997a,b; Heberlein *et al.*, 1995). One attractive model for linking Dpp signaling (originating within the furrow) to cells just ahead of the furrow invokes a situation in which Dpp would regulate retinal determination gene expression, particularly that of *so*, *eya*, and *dac*. In fact, an eye-specific enhancer within the eighth intron of *dac* is responsive to Dpp signaling (Pappu *et al.*, 2005), and loss of Dpp signaling leads to reductions in dac, *eya*, and *so* transcription (Curtiss and Mlodzik, 2000). Unfortunately, the regulation of these three retinal determination genes by Dpp appears to occur only at the margins of the disk during furrow initiation. Not only is expression of *so*, *eya*, and *dac* normal within internally located *Mad* clones but also the furrow progresses normally through this tissue (Curtiss and

Mlodzik, 2000; Greenwood and Struhl, 1999). Thus, the molecular link between signaling pathway cells in the furrow (Zone D) and the retinal determination network in cells that lie just anterior (Zone C) remains unresolved. As a consequence, the morphological mechanism by which cells are canalized into the furrow also remains an open issue. Also, the exact roles of the retinal determination network in the furrow itself are not known with any degree of certainty.

The area behind the furrow can be divided into two sections (Zones E and F). The first encompasses cells that lie just posterior to the furrow (Zone E) and can be molecularly marked by the expression of four retinal determination genes, *so*, *eya*, *dac*, and *nmo* with the *dac* expression profile defining the most posterior edge of this zone (Fig. 1.5). The second (Zone F) begins where *dac* expression terminates and extends all the way to the posterior edge of the eye field. These cells continue to express only *so*, *eya*, and *nmo* (Fig. 1.5). The role played by the retinal determination genes in cells behind the furrow has not been extensively studied and is not well understood. However, an analysis of mutant phenotypes suggests that these factors contribute to at least two processes. The first involves a potential role for the *so*, *eya*, and *dac* genes in the acquisition and maintenance of individual cell fates within developing ommatidia. The loss of either *so* or *eya* within cells that are born in the second mitotic wave results in the loss of R1, R6, and R7 photoreceptors (Pignoni *et al.*, 1997). It is not clear, however, if the So–Eya complex functions to specify these cell fates or to maintain cell identity. Also, a role for So–Eya in cells of the precluster (R8, R2, R5, R3, and R4) has not been described. Downstream of the So–Eya complex lies *dac*, so it is thought that it might play a role in photoreceptor cell specification and/or maintenance. However, the loss of *dac* in the middle of the eye field does not appear to inhibit the movement of the furrow or have a significant effect on the initial steps in photoreceptor specification (Salzer and Kumar, 2009).

In addition, each unit eye is executing dorsal–ventral patterning signals by assuming a chiral form that is appropriate for its location within either the dorsal or ventral half of the retina. Also, ommatidia in the two compartments must rotate in opposite directions. The combined effects of chirality and rotation result in ommatidia in the dorsal half being positioned in a mirror-image orientation to those in the ventral section (Fig. 1.5; reviewed in Fanto and McNeill, 2004; Mlodzik, 1999; Strutt and Strutt, 2003; Wolff and Ready, 1993). The Nmo kinase, while functioning to promote eye formation (Braid and Verheyen, 2008), also plays a major role in ommatidial rotation as loss-of-function mutations prevent ommatidia from rotating past the first 45° (Choi and Benzer, 1994; Fiehler and Wolff, 2008). It should be noted that the expression of all other retinal determination genes is extinguished at the furrow and thus do not appear to play roles in cell fate specification or ommatidial rotation.

6. Taking Instructions from Higher Authorities

The nuclear retinal determination network, while functioning as a unit, does not do so in isolation. Rather, it is a nexus point for integrating instructions that are being transmitted across the disk by diffusible morphogens and signal transduction pathways. These cascades are used reiteratively during eye development and intersect with the retinal determination network at multiple levels (reviewed in Kumar, 2001; Voas and Rebay, 2004). This final section will briefly bring to light the known interactions between signaling pathways and the eye specification network.

6.1. Notch and the EGF receptor: A role in sensory organ identity

As mentioned above, there is a unidirectional flow of information between *toy* and *ey* with the former residing molecularly upstream of the latter. Within this section of the network, there does not appear to exist any feedback loops or autoregulatory circuits. It raises the issue of how these genes are initially transcribed in the eye anlagen and how their expression patterns restricted are to just the eye field after being initiated throughout the entire eye-antennal epithelium. It appears that Notch signaling activates and maintains *ey* expression while in the second instar, the EGF receptor (EGFR) pathway functions to restrict *ey* transcription to the developing eye (Kumar and Moses, 2001b; Kurata *et al.*, 2000). Alterations of either signaling cascade during this stage results in the transformation of the eye into an antenna (Kumar and Moses, 2001b). The latter half of the second instar appears to be a key developmental window for organogenesis, as manipulations of EGFR signaling within the wing disk during this same interval can convert the developing notum into wing tissue (Baonza *et al.*, 2000).

A role for the EGFR in retinal determination does not stop with the regulation of *ey* expression. In fact, the pathway also goes on to regulate *eya* at multiple levels. This signaling cascade appears to regulate eya expression, possibly directly, as mutations in *yan* and *pointed* (*pnt*), which encode Ets type transcription factors, lead to up- and downregulation of *eya* transcription, respectively, in both the embryo and the developing eye (Salzer *et al.*, 2010). The EGFR pathway also modulates the activity of Eya protein through phosphorylation by MAPK (Hsiao *et al.*, 2001). This modification is critical for the ability of Eya to support normal and ectopic eye development.

6.2. Hedgehog and Dpp: Required for ectopic eye formation

Since the very first reports of ectopic eye formation, it has been noted that widespread expression of any retinal determination gene only transforms portions of some imaginal disks. A recent systematic effort to document this

phenomenon identified nine cell populations that can support eye formation in the eye-antenna, leg, wing, and haltere disks (Salzer and Kumar, 2010). Several of these transformation "hot spots" appear to coincide geographically with several previously identified transdetermination weak points (Maves and Schubiger, 1998, 2003). These points seem to be under the control of several signaling pathways including Wg and Dpp. The coincidental location of the transdetermination weak points and the transformation hot spots suggests that the ability of the retinal determination network to support eye development within the hot spots and even in the normal eye may require that these cell populations be primed by the expression and activity of short- and long-range diffusible signals. Two efforts have attempted to confirm this hypothesis and uncover the identity of the morphogens. First, clones expressing retinal determination genes were induced in random locations within the wing disk. The only clones that contained ectopic retinal tissue were ones that were contained within the posterior compartment, which expresses the Hh morphogen (Kango-Singh *et al.*, 2003). Second, coexpression of *ey* with *dpp* in cells that surround the developing wing pouch expanded the range of cells that can be transformed into retinal tissue (Chen *et al.*, 1999). Together, these two reports suggest that the Hh and Dpp pathways are required in cells prior to the onset of the retinal determination network. While two pathways do play important roles in promoting eye development, the signaling requirements are likely to be more complicated, as several of the recently identified cell populations that can support eye development lie well outside the *hh* and *dpp* expression zones (Salzer and Kumar, 2010).

6.3. Wingless: A repressive signal

The eye imaginal disk gives rise to more than just the compound eye; the surrounding head cuticle is also derived from this epithelium (Haynie and Bryant, 1986). The bulk of retinal determination genes, save for *hth*, are not expressed within the tissue that will give rise to head cuticle (see above). So how does the epithelium subdivide itself in this way? The Wg signaling pathway is known to inhibit eye development by blocking ectopic morphogenetic furrow initiation (Ma and Moses, 1995; Treisman and Rubin, 1995). Further analysis of its working in the eye indicated that Wg prevents the expression of *so*, *eya*, and *dac* as clones of *axin* or *armadillo* (*arm*) lead to their upregulation and ectopic *ug* expression downregulates their transcription in the eye (Baonza and Freeman, 2002). This effect is not direct as the Wg pathway activates *hth* expression, which in turn blocks initiation of *so*, *eya*, and *dac* in conjunction with *tsh* (Bessa *et al.*, 2002; Singh *et al.*, 2002). So while the Notch, EGFR, Hh, and Dpp signaling cascades promote eye development, the Wg pathway acts to balance these effects and subdivide the eye imaginal disk into the eye proper and head cuticle.

7. Concluding Remarks

The past 30 years have seen remarkable advances in our understanding of how a simple nervous system, the insect compound eye, is specified and patterned. The retina has been an enduring model for studying a myriad of developmental processes including organogenesis. This review has attempted to summarize the known molecular and biochemical events that lead to the specification of the eye. In addition, special emphasis has been put on placing these interactions within temporal and spatial contexts of the developing eye field. Despite the enormous progress that has been made on understanding how the retina is determined, we still have a long way to go before we will have a complete understanding of how the eye is constructed. Based on past experience, it will not be long before new genes are identified as retinal determination factors and their roles in eye development are elucidated. The advent of new technologies, application of high throughput assays, and the implementation of creative genetic, molecular, and biochemical screens will only accelerate this process. In time, we will surely unravel the mystery of how gene regulatory networks function to coordinate the fates of large groups of undifferentiated cells and produce a unique organ or tissue.

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CHAPTER TWO

Eye Field Specification in *Xenopus laevis*

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Abstract

Vertebrate eyes begin as a small patch of cells at the most anterior end of the early brain called the eye field. If these cells are removed from an amphibian embryo, the eyes do not form. If the eye field is transplanted to another location on the embryo or cultured in a dish, it forms eyes. These simple cut and paste experiments were performed at the beginning of the last century and helped to define the embryonic origin of the vertebrate eye. The genes necessary for eye field specification and eventual eye formation, by contrast, have only recently been identified. These genes and the molecular mechanisms regulating the initial formation of the *Xenopus laevis* eye field are the subjects of this review.

1. Introduction

The vertebrate eye consists of a number of tissues with distinct origins. The cells that eventually form retina, the light sensing tissue of the eye, progress through a series of three states, which include retinal competence, specification, and determination. In *Xenopus laevis*, nine animal blastomeres of the 32-cell stage embryo are competent, but not yet committed, to form the differentiated cells of each retina (Huang and Moody, 1993; Moody, 1987a). For example, one animal blastomere normally generates the majority (>50%) of retinal cells. However, if it is transplanted to the vegetal side of the embryo, it no longer generates any retinal cells (Gallagher *et al.*, 1991). Now, fast-forward approximately 11 h to stage 12.5, when gastrulation is nearly complete and the neural plate is forming. If cells from an anterior region of the neural plate called the eye field are cultured in a neutral environment or even transplanted to a ventral region of the embryo, differentiated retinal tissue and eyes form (Fig. 2.1; reviewed in Spemann, 1938; Li *et al.*, 1997; Lopashov and Stroeva, 1964). If the same region is explanted or transplanted even a few hours earlier, it does not form retina or eyes. Molecular changes take place in these few hours that transform the frog neuroectoderm into cells committed to form retina—a process called eye field specification.

This chapter begins with an introduction to the tools for working with, and eye formation in *Xenopus*. Next is a description of the key transcription factors that regulate early eye formation, and how together these genes are sufficient to generate functional eyes in the frog. The final section will provide a few examples of the mechanisms used by these transcription factors to regulate eye field and eye formation. To include all that is known of early eye formation in all vertebrate species is beyond the scope of this review. Consequently, I have focused on experiments performed in *X. laevis* and select experiments using other species, with apologies to those whose work could not be included.

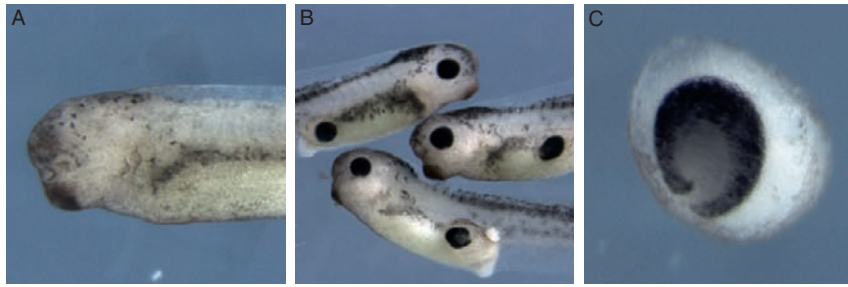


Figure 2.1 Eye field removal, transplantation, and culturing are easily accomplished using *Xenopus laevis*. One-half of an eye field can be removed from a stage 15 embryo. (A) The tadpole develops normally, but lacks an eye on the operated side. (B) If transplanted to the flank of host embryos, eye fields form eyes (shown here approximately 2 days after transplantation). (C) This eye formed from an eye field explant cultured for 3 days. These simple experiments show the remarkable, self-organizing nature of the eye field.

2. Tools for Studying *Xenopus* (and Eye) Development

Amphibian species in general and *Xenopus* more recently have served as powerful model systems for the study of early embryonic events. Early investigators were drawn to amphibia due to their remarkable ability to regenerate complex body parts. Experiments as early as the 1780s, for example, reported regeneration of the eye after partial removal from the salamander *Triton* (noted by King, 1905). In addition to cell and tissue ablation experiments, amphibia also recover remarkably well from other surgical manipulations including tissue isolation (explants grown in culture), recombination (swapping tissues on the same embryo), and transplantation experiments (grafting tissues from one embryo to another). These manipulations are made possible due to the fact that eggs are externally fertilized and can be cultured on the lab bench at room temperature in simple salt solutions. Early embryologists were dependent on eggs and embryos collected seasonally. In the 1960s, the African Clawed Frog *X. laevis* became the favored model, providing researchers with the ability to perform experiments year round since hormone-injected females could be induced to lay eggs when it was convenient for the investigator. *Xenopus laevis* eggs are relatively large (~1½ mm) and can be collected and synchronously fertilized by the thousands, if necessary. Embryos develop rapidly. From fertilization to eye field specification takes ~14 h, and a functioning eye forms in less than three more days (Witkovsky *et al.*, 1976). Embryo development can be

easily accelerated or slowed by raising or lowering the culture temperature within reason (as low as 14 °C to as high as 27 °C).

2.1. *Xenopus* developmental biology in the molecular age

Using amphibia, embryologists established some of the most important tenets of developmental biology. Included among these are the concepts of determination, specification, body axis formation, regulative development, and embryonic induction to name only a few. The move to *Xenopus* and the development of molecular tools have allowed the experimenter to investigate in fine detail the cellular and molecular mechanisms driving the above and other developmental processes. *Xenopus* eggs can be injected with DNA, RNA, and proteins individually or in any combination. Researchers have been highly inventive, modifying constructs to express proteins with constitutively active, dominant negative, hypermorphic, or antimorphic activities to investigate the gain- and loss-of-function phenotypes of specific genes and signaling systems (Sive *et al.*, 2000). Antisense RNA, RNAi, and antisense morpholino oligonucleotides can be used to reduce the level of one, or multiple, target proteins in individual animals (Heasman *et al.*, 2000; Nakano *et al.*, 2000; Steinbeisser *et al.*, 1995; Zhou *et al.*, 2002). Lineage tracing experiments have fate mapped the progeny of individual blastomeres to specific embryonic tissues, and regions of the neural plate to the tadpole brain (Eagleson and Harris, 1990; Eagleson *et al.*, 1995; Moody, 1987a,b). Therefore, by varying the injection location one can more specifically target the cells, tissues, and developing organs of interest. Injected RNAs are most often translated straightaway, resulting in immediate protein activity. Constructs fusing hormone-binding domains to the protein of interest can alleviate this potential problem. The properly timed addition of hormone to the culture media activates the fusion protein, thereby avoiding what might otherwise result in toxicity at an earlier developmental stage (Sive *et al.*, 2000). Introduction of DNA, RNA, and morpholinos via lipofection and electroporation into the neural plate and eye field has also been used to test gene function at these developmental stages and later (Ohnuma *et al.*, 2002; Sasagawa *et al.*, 2002b). In addition to observing developmental alterations, qualitative, and quantitative changes in gene expression can be easily monitored using *in situ* hybridization and/or RT-PCR in gain- and loss-of-function experiments.

2.2. Genetic and genomic tools for studying development in *Xenopus*

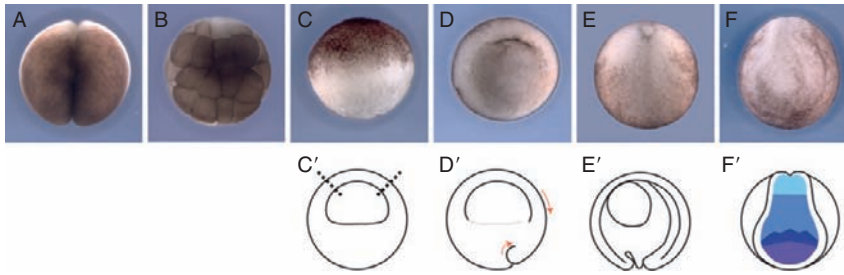
A relatively recent advance in the study of *Xenopus* is the ability to generate transgenic animals using a variety of techniques (Chesneau *et al.*, 2008). Hundreds of transgenic animals can be generated by a single investigator in a day. This level of efficiency allows for rapid promoter analysis and the ability

to generate lines of animals expressing any manner of transgene (fluorescent reporters, substrate dependent toxins, modified, or fusion proteins with constitutively active or dominant negative functions) under the control of cell type specific promoters. In recent years, technologies previously reserved for mice and fish have been developed in transgenic *Xenopus*, including gene silencing, FLP-FRT, Cre-lox, steroid-, tet-, and heat-shock regulatable systems (Chae *et al.*, 2002; Li and Rohrer, 2006; Ridgway *et al.*, 2000; Roose *et al.*, 2009; Waldner *et al.*, 2006; Werdien *et al.*, 2001). *Xenopus* lines constitutively expressing green, yellow, or red fluorescent proteins have made problems distinguishing donor and host cells in transplantation experiments a thing of the past, since cells continue to fluoresce (unlike injected lineage tracers) for the lifetime of the donor cells and all their progeny (Marsh-Armstrong *et al.*, 1999; Sakamaki *et al.*, 2005; Waldner *et al.*, 2009).

The relatively slow generation time (1–2 years) and pseudotetraploid genome of *X. laevis* has lead to the more recent use of the smaller and more rapidly developing (as short as 4 months) diploid *Xenopus tropicalis*, allowing for the rapid generation of breeding lines (Amaya *et al.*, 1998; Bisbee *et al.*, 1977; Graf and Kobel, 1991). The addition of *X. tropicalis* has resulted in the development of a number of new approaches and genomics tools for developmental biologists working with either species. The sequencing of the *X. tropicalis* genome has been completed and there is an initiative to sequence the *X. laevis* genome. High-density microarrays have been generated for both species and gene sequences are similar enough to allow cross-hybridization of probes for *in situ* hybridization and microarray analysis (Chalmers *et al.*, 2005; Khokha *et al.*, 2002). Genomics approaches typically reserved for traditional genetic model systems are now being used in frog, including gene trapping, genetic screens and genetic mapping of the *X. tropicalis* genome, global gene expression profiling, whole-genome, and promoter tile path microarrays to investigate transcription factor binding during development (Akkers *et al.*, 2010; Baldessari *et al.*, 2005; Bronchain *et al.*, 1999; Goda *et al.*, 2006; Khokha *et al.*, 2009; Noramly *et al.*, 2005). These new technologies, the ever expanding computer based and online *Xenopus* resources (http://www.xenbase.org/), coupled with the powerful "old school" embryological manipulation, make *Xenopus* an ideal model system for identifying the mechanism driving eye field specification (Bowes *et al.*, 2008; Gerth *et al.*, 2007; Gilchrist *et al.*, 2009; Pollet *et al.*, 2000, 2003, 2005; Segerdell *et al.*, 2008; Vize, 2001).

3. *Xenopus* Eye Formation

Once fertilized *Xenopus* embryos progress through a series of rapid cell divisions (the cleavage stages) resulting in the formation of smaller cells called blastomeres (Fig. 2.2). Generally speaking, the first cleavage plane



Stage 2	Stage 6	Stage 9	Stage 10	Stage 12.5	Stage 15
2-Cell (animal)	32-Cell (animal)	Blastula (lateral)	Early gastrula (vegetal)	Late gastrula (dorsal)	Neurula (anterior)
1.5 hpf	3 hpf	7 hpf	9 hpf	14.5 hpf	17.5 hpf

Figure 2.2 Early development of *Xenopus laevis*. (A–F) Bright field images of embryos from stages 2 to 15. (C′) Schematic cross section at stage 9 showing the tissue collected for animal cap assays. (D′) Stage 10 embryo at the start of gastrulation. Arrows indicate the direction of cell movements. (E′) Stage 12.5 embryo illustrating the layers of internal tissues that form. (F′) By stage 15 the anteroposterior axis of the neural plate is specified into the forebrain (purple), midbrain (dark blue), hindbrain (blue) and spinal cord (light blue). The table shows staging per Nieuwkoop and Faber (1994), and hours postfertilization (hpf).

demarcates the right and left half of the embryo. Injection into one of the two blastomeres provides both an experimentally treated side as well as a convenient internal (uninjected) control side. Subsequent cleavages generate progressively smaller blastomeres with more restricted fates. The fifth cleavage results in the 32-cell stage embryo 3 h after fertilization (Fig. 2.2B). Although the eye field does not become well established until neurula stages (~1/2 day later), lineage tracing, transplantation, and ablation experiments have demonstrated that a subset of nine blastomeres are already competent to contribute progeny to the retina (Gallagher *et al.*, 1991; Huang and Moody, 1993; Moody, 1987a). Cell–cell interactions are involved in determining the location and size of the retinogenic zone and recent experiments have also identified animal and vegetal determinants that restrict the location of the retinogenic zone to the animal side of the embryo (Lee *et al.*, 2006; Moore and Moody, 1999; Moore *et al.*, 2004; Yan and Moody, 2007).

Through the cleavage stages (~7 h), the embryo does not increase in volume, it simply generates more cells via continued divisions. Cell division is faster in the animal hemisphere where the fluid filled cavity of the blastocoel forms (Fig. 2.2C′). The animal pole (cap) cells above the blastocoel are pluripotent and responsive to a variety of inducing signals. Untreated, these cells form atypical epidermis in culture. However,

if treated with the appropriate inducer, they can form endoderm, mesoderm, or ectodermal cell types (Green, 1999). These cells provide a convenient tool for testing the effects of candidate molecules on cell fate *in vitro*. Treated cap cells can also be grafted to an embryo to determine their fate *in vivo*.

During gastrulation, cells that involute and migrate along the inside of the blastocoel toward the animal pole induce the overlying cells to form neural tissue (Fig. 2.2D′ and E′). Neural induction is regulated by bone morphogenetic protein (BMP) inhibitors, fibroblast growth factors (FGFs) and Wingless-Int proteins (Wnts). At midgastrula (stage 11.5), all regions of the presumptive neural plate can form eyes, indicating an anterior neural bias throughout the developing plate at these early stages. It is only at later stages that eye formation is restricted to the most anterior regions (Li *et al.*, 1997; Saha and Grainger, 1992). This specification of the eye field, and the patterning of other presumptive brain regions, in the neural plate is regulated by a number of signaling systems including BMPs, FGFs, Wnts, Nodals, hedgehogs, and retinoic acid (RA). These molecules and their inhibitors modulate signaling gradients that pattern the neural plate, along both anteroposterior and dorsoventral axes (Fig. 2.3). The loss or overactivity of any of these signaling systems can result in abnormal patterning of the neural plate and forebrain and ultimately affect eye field specification and eye development.

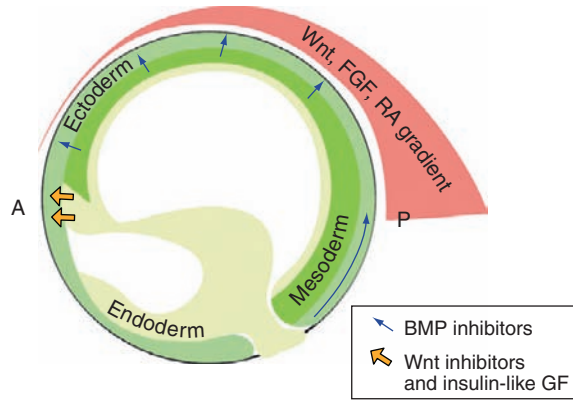


Figure 2.3 Signaling systems regulating neural patterning. Schematic transection of a *Xenopus* gastrula shows in red gradients of caudalizing signaling systems in the embryo (Wnt, FGF, and retinoic acid, RA). Wnt inhibitors (e.g., Cerebrus, Frzb, Dickkopf) and insulin-like growth factors (GF; yellow arrows) are expressed in the anterior endomesoderm, causing head formation. Purple arrows indicate BMP inhibitors (e.g., Follistatin, Noggin, Chordin) that neuralize the ectoderm. A, anterior; P, posterior.

It is during neurulation that the first morphological signs of the eye can be detected. As the initial flat sheet that is the neural plate begins to fold to form the neural tube, a bilateral evagination of the forebrain-derived diencephalon (called the optic vesicle) can be seen. The optic vesicle continues to grow, makes contact with the surface ectoderm and invaginates to form the optic cup. Having folded back into itself, the optic cup now has two layers of tissue—the outer part of the cup forms the retinal pigment epithelium (RPE) and the inner forms the retina. The surface ectoderm having made contact with the optic vesicle forms the lens.

The retinas of higher vertebrates (human included) and frog share striking similarities in structure, function, and development in spite of the dramatic differences in developmental time scale. For example, all seven of the retinal cell classes in humans are also found in the frog eye. The retinas of both species are organized into three distinct cellular layers (Dowling, 1987). Photoreceptors (rods and cones) of the outer nuclear layer (ONL) convert light into an electrical signal. Inner nuclear layer (INL) cells (horizontal cells, bipolar cells, amacrine cells, and Müller glia) pass the electrical signal to retinal ganglion cells (RGCs). Ganglion cells, which lie closest to the lens, then relay the electrical signal to the brain. In addition to these structural similarities, homologous, eye field, and retina-specific genes are required for normal eye development in multiple vertebrate species.

4. Transcription Factors Regulating Eye Field and Early Eye Formation

Chapter 3 of Hans Spemann's (1938) book *Embryonic Development and Induction* is titled "The Development of the Vertebrate Eye as an Example of a Composite Organ." In it, Spemann details what was known at the time about the origin of the amphibian eye. His own work, and the isolation and transplantation experiments of H. Bautzmann, J. Holtfreter, O. Mangold, H. B. Adelmann, and others established the timing of eye field specification (Spemann, 1938). Spemann summarizes Mangold's findings from 1931: "From his experiments we may conclude 'that, before the commencement of neurulation, the determination of the eye-rudiment is labile, that it gradually becomes stable during gastrulation, and that it becomes finally established in the neurula stage.'" Given the tools available at the time, only guesses could be made as to the mechanisms driving eye field formation and the above passage concisely summarizes what was known of vertebrate eye field specification for decades to come.

With respect to molecular mechanisms, the 1990s proved to be the golden age for identifying transcription factors that are expressed in the eye field, required for vertebrate eye formation, and in some cases sufficient

for generating eye-like structures. Originally cloned from numerous species, these genes were found to be highly conserved through evolution. Homologs of the vertebrate eye field transcription factors (EFTFs) are among the retinal determination genes of *Drosophila melanogaster*. Although the members of these gene sets are not identical, similarities in their actions and the genetic networks they form are striking. Although the focus here is on vertebrate eye formation, it is important to acknowledge the work in *Drosophila* (reviewed elsewhere in this volume), which has in many cases served as an excellent template for identifying the molecules and genetic mechanisms of vertebrate eye field specification (reviewed in Erclik *et al.*, 2009; Finkelstein and Boncinelli, 1994; Gehring, 2004; Halder *et al.*, 1995b; Kumar, 2001; Kumar and Moses, 2001; Wawersik *et al.*, 2000). Here the focus is on a set of vertebrate transcription factors and their roles in *Xenopus* early eye formation. For consistency and clarity, symbols approved by the HUGO Gene Nomenclature Committee (HGNC) have been used (prefaced with X to indicate *Xenopus*).

4.1. Orthodenticle homeobox 2 (Otx2)

Xotx2 (a *Xenopus* homolog of the *Drosophila orthodenticle* gene) expression is detected prior to gastrulation in the dorsal marginal zone (Gammill and Sive, 1997; Pannese *et al.*, 1995). During gastrulation, *Xotx2* is expressed first in the involuting mesoderm and later, in the overlying ectoderm that will eventually form the rostral brain and eye field. Immediately before eye field specification (stage 12), *Xotx2* expression extends from the presumptive cement gland into the midbrain (Fig. 2.4A). During early eye field specification, the expression pattern changes rapidly. Although its expression continues to be detected anteriorly (presumptive cement gland) and posteriorly (presumptive midbrain), a distinct gap develops in the forebrain. Between stages 12 and 13 (<90 min) expression of *Xotx2* is lost in the forming eye field (Kablar *et al.*, 1996; Pannese *et al.*, 1995; Zuber *et al.*, 2003). The result is a "hole" in the otherwise uniform expression domain (Fig. 2.4B). The location and timing of its formation are synchronous with the expression of several early EFTFs (see below).

In *Xenopus*, knockdown of XOtx2 expression using morpholino oligonucleotides results in abnormal development of anterior structures including the eyes (Carron *et al.*, 2005). Eye formation is also blocked if XOtx2 expression is not repressed in the presumptive eye field. Misexpression of *Xotx2* by RNA microinjection induces ectopic cement glands—the most anterior structure in which it is normally expressed, while more posterior structures are lost or develop abnormally (Gammill and Sive, 1997; Pannese *et al.*, 1995). At eye field stages, *Xotx2* (and the fly homolog *otd*) represses eye field expression of EFTFs and eye formation *in vivo* (Casarosa *et al.*, 1997; Lunardi and Vignali, 2006; Pannese *et al.*, 1995).

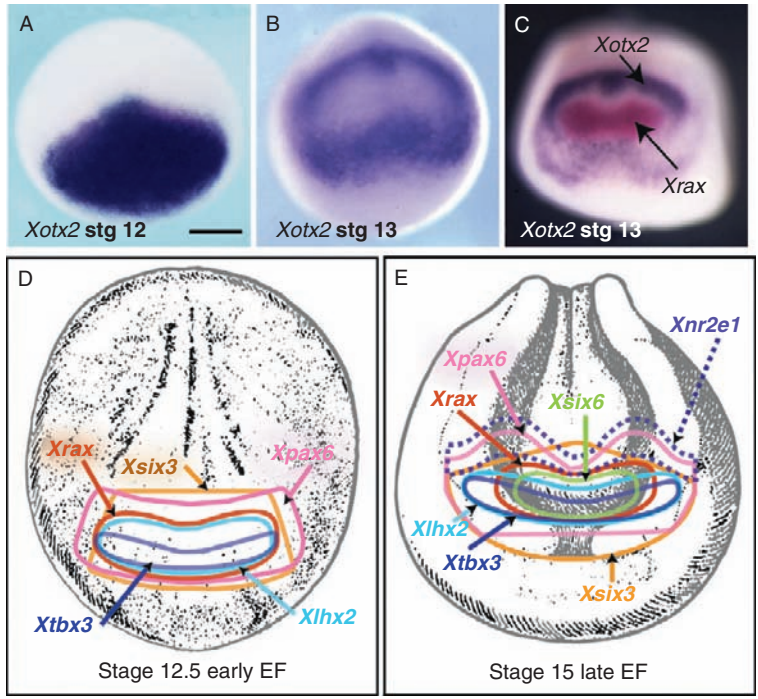


Figure 2.4 *Xotx2* and EFTF expression during eye field specification. (A) *In situ* hybridization of *Xotx2* shows it is expressed in the anterior neural plate prior to eye field specification. (B) Central *Xotx2* expression is rapidly repressed as EFTF expression is first detected in the 90 min between stages 12 and 13. (C) Double *in situ* hybridization for *Xotx2* (purple) and *Xrax* (red) illustrates their mutually exclusive expression domains. (D–E) Illustrations adapted from Nieuwkoop and Faber (1994), showing the dynamic, nonidentical overlapping expression patterns of the EFTFs at early and late stages of eye field specification. The expression domains of these genes outside the eye field at these stages have not been included for clarity.

XOtx2 can act as both a transcriptional activator and repressor. For example, XOtx2 is a direct activator of XCG (a cement gland marker), but also represses the posterior genes *Xcad3* and *Xbra in vivo* and *in vitro* (Gammill and Sive, 1997, 2001; Isaacs *et al.*, 1999). The activator function of XOtx2 appears to be required for eye formation. Misexpression of a repressor-only form of XOtx2 (XOtx2–EnR) results in eyeless tadpoles. Eye formation can be rescued by coinjection of wild-type *Xotx2* (Isaacs *et al.*, 1999).

Together, these results illustrate that XOtx2 is first required to specify anterior structures, but must then be repressed to allow for eye field specification.

4.2. Six homeobox 3 (Six3)

Xenopus laevis Six3 is expressed broadly (relative to the other EFTFs) in the developing neural plate at all stages (Ghanbari *et al.*, 2001; Zhou *et al.*, 2000; Zuber *et al.*, 2003). *Xsix3* is first detected by *in situ* hybridization at stage 12 along with the other early expressed EFTFs (this group also includes *Xpax6*, *Xrax*, *Xlhx2*, and *Xtbx3*) (Zuber *et al.*, 2003). *Xrax*, *Xlhx2*, and *Xtbx3* are contained centrally within the *Xsix3* expression domain (Fig. 2.4D). X*pax6* has a broader lateral expression domain, while *Xsix3* extends to more posterior and anterior regions. By midneurula stages, the *Xsix3* expression domain continues to encompass most other EFTFs' domains—the exceptions being *Xnr2e1* and X*pax6*, which are expressed more posteriorly on either side of the midline (Fig. 2.4E).

XSix3 misexpression has dose-dependent effects. An increase in eye field and eye size is observed at low doses, while structures both anterior and posterior to the eye field have altered fates at high doses. XSix3 expands the expression domains of *Xpax2* in the presumptive optic stalk, while simultaneously repressing its expression at the midbrain/hindbrain boundary (Bernier *et al.*, 2000). *Engrailed 2* (*Xen2*) is normally expressed in the caudal midbrain and midbrain/hindbrain border. XSix3 represses *Xen2* expression while expanding X*pax6* and *Xrax* expression into the midbrain. Thus, excessive XSix3 appears to impart retinal character to part of the midbrain (Bernier *et al.*, 2000).

In *Xenopus*, misexpression of a transcriptional activator form of XSix3 by fusion with the VP16 activation domain (XSix3–VP16) blocks *Xrax* and X*pax6* expression and dramatically reduces or even eliminates eye formation, suggesting that XSix3 normally acts as a repressor with respect to eye field and eye formation (Gestri *et al.*, 2005).

The relatively broad expression domain and range of phenotypes observed following its inactivation indicate that XSix3 is required not only for eye field specification, but also for much of rostral brain formation.

4.3. Retina and anterior neural fold homeobox (Rax)

Xenopus laevis Rax was originally identified by two groups and named *Xrx1* and *Xrx* (*Xenopus retinal homeobox 1*; Casarosa *et al.*, 1997; Mathers *et al.*, 1997). Two highly homologous *Rax* genes named *Xrx1* and *Xrx2* were originally identified (Mathers *et al.*, 1997). More recently, the names *Xenopus Rx1a* and *Rx2a* have been used (other synonyms include *rx1* and *Xrax;* Wu *et al.*, 2009). *Xrx1/2* proteins are greater than 95% identical and the mRNAs appear to have identical expression patterns. The presence of these two genes is likely to have resulted from the partial duplication of the *Xenopus* genome (Bisbee *et al.*, 1977). For simplicity, I will use the nomenclature of *Xrax* for these two genes. A third *Xenopus* gene originally named

Rx-L (*Rx-like*) shares 62% amino acid identity with *Xrax* (Pan *et al.*, 2006). Rx-L (current nomenclature XRax2) is smaller in size than XRax, as it lacks an octapeptide motif thought to facilitate protein × protein interactions (Pan *et al.*, 2006). XRax2 is required for photoreceptor formation, but is not expressed until optic vesicle stages and thus not involved in eye field specification (Pan *et al.*, 2006; Wu *et al.*, 2009).

Detected as early as stage 12 by *in situ* hybridization, *Xrax* expression is limited to the presumptive forebrain and the structures it will eventually form including the telecephalon, hypothalamus, eyes, and diencephalon, but not in the more anterior cement gland (Casarosa *et al.*, 1997; Mathers *et al.*, 1997). The early *Xrax* expression domain lies within that of *Xpax6* and *Xsix3*, but encompasses both *Xlhx2* and *Xtbx3* (Mathers *et al.*, 1997; Zuber *et al.*, 2003). By stage 15, only the late expressed *Xsix6* domain lies within that of *Xrax* (Fig. 2.4E; Zuber *et al.*, 2003).

Overexpression of *Xrax* by RNA microinjection results in retinal hyperproliferation, which is sometimes so severe that the retina folds onto itself to form a duplicated retina (Mathers *et al.*, 1997). Ectopic RPE is also observed in regions between the retina and the brain suggesting the formation of ectopic retinal tissue (Andreazzoli *et al.*, 1999; Mathers *et al.*, 1997). *Xrax* misexpression does not dramatically alter *Xpax6* or *Xsix3* expression at early eye field stages (stage 13), but by tailbud stages (stage 23) the *Xpax6* and *Xsix3* domains are both expanded (Andreazzoli *et al.*, 1999).

In contrast to *Xpax6* and *Xsix3*, the expression of *Xotx2* is dramatically modified by *Xrax* from an early stage. The timing and region of *Xrax* expression matches closely the location and timing at which *Xotx2* expression is lost—their expression patterns being complementary during these stages (Fig. 2.4C). Consistent with a role in shaping the *Xotx2* expression domain, XRax can repress *Xotx2* expression at these early eye field stages (Andreazzoli *et al.*, 1999; Zuber *et al.*, 2003).

Interfering with XRax function results in a reduction in eye size and in extreme cases, tadpoles lacking eyes (Andreazzoli *et al.*, 1999, 2003). XRax contains a carboxy-terminal region named the paired tail or OAR domain (*Otp*, *Aristaless*, and *Rax*), which functions as a transactivation/inhibition domain (Amendt *et al.*, 1999; Furukawa *et al.*, 1997; Norris and Kern, 2001; Simeone *et al.*, 1994). Misexpression of XRax lacking the OAR domain (ΔOAR) or a fusion of XRax lacking the OAR domain to the *Drosophila* engrailed repressor domain (to generate a putative antimorph XRaxΔOAR–EnR), as well as injection of *Xrax* antisense RNA or morpholinos all result in abnormal eye formation. XRax–EnR reduces the expression domains of *Xpax6*, *Xsix3,* and *Xotx2* in stage 13 embryos and of *Xpax6* and *Xotx2* in neuralized animal caps (Andreazzoli *et al.*, 1999). However, XRax does not appear to act exclusively via *Xpax6* or *Xotx2*, since neither of these genes can rescue phenotypes induced by XRax–EnR misexpression (Andreazzoli *et al.*, 1999). Developmental defects are not

restricted to the eyes when XRax function is disrupted since telencephalon and diencephalon formation is also abnormal (Andreazzoli *et al.*, 1999).

In *Xenopus*, Rax controls proliferation as well as neurogenesis in the anterior neural plate, explaining why it is required for normal development of the eyes and other forebrain derivatives (Andreazzoli *et al.*, 2003).

4.4. Paired box 6 (Pax6)

Xenopus laevis Pax6 is first detected in the embryo by *in situ* hybridization at stage 12 before gastrulation is complete. In the anterior portion of the presumptive neural plate, *Xpax6* is expressed in a continuous and symmetrical band across the embryonic midline. Expression is not restricted to this region, but is also detected in two broad stripes in the presumptive neural tube (Hirsch and Harris, 1997; Li *et al.*, 1997). *Xpax6* is the most laterally expressed of the early EFTFs and at this stage encompasses the expression of *Xrax*, *Xlhx2*, and *Xtbx3*. Only *Xsix3* expression extends more anteriorly and posteriorly (Fig. 2.4D). By stage 14 (neural plate stage), the anterior crescent of expression includes not only the eye field, but also regions that will eventually form the telencephalon, diencephalon, and olfactory bulbs. Expression is also detected in the presumptive lens ectoderm and presumptive hindbrain (Grainger *et al.*, 1997; Hirsch and Harris, 1997; Zygar *et al.*, 1998).

Consistent with its predicted role in vertebrate lens formation and its early expression in the *Xenopus* presumptive lens ectoderm, an early reported phenotype of *Xpax6* overexpression in the frog was lens induction (Altmann *et al.*, 1997). Misexpression of XPax6 in both *Xenopus* embryos and animal caps induced expression of the lens specific marker β-crystallin. XPax6 did not induce the expression of either neural or mesodermal markers, indicating a direct role in lens induction (Cvekl *et al.*, 2004; Zygar *et al.*, 1998).

Given its strong expression in the eye field, its requirement for normal eye formation in mouse, and the ability of a vertebrate Pax6 to induce ectopic eyes in the fly, it was to some extent surprising that early misexpression studies found no evidence of ectopic frog eyes or retinal tissue formation (Altmann *et al.*, 1997; Halder *et al.*, 1995a; Hirsch and Harris, 1997; Mathers *et al.*, 1997; Zuber *et al.*, 1999). Later, detailed experiments however, showed that XPax6 could indeed induce ectopic eye-like structures in *Xenopus*. By injecting one blastomere at the 16-cell stage or two blastomeres at the 32-cell stage ectopic eye-like structures did form (Chow *et al.*, 1999). Concentration and location appeared to be critically important in the ability of XPax6 to induce ectopic structures. Previous investigators typically injected at the four-cell stage (Altmann *et al.*, 1997; Hirsch and Harris, 1997; Mathers *et al.*, 1997; Zuber *et al.*, 1999). In addition to inducing *Xotx2*, *Xsix3*, and *Xrax* expression, XPax6 also induced its own

expression in these experiments indicating that it can trigger the expression of multiple genes required for eye formation (Chow *et al.*, 1999).

In *Xenopus*, targeted misexpression of a dominant negative form of XPax6 called XPax6ΔCT blocks the proximal eye defects and ectopic eye formation induced by wild-type XPax6. When injected alone, *Xpax6ΔCT* also reduced or blocked endogenous eye formation (Chow *et al.*, 1999). Other neural structures were not reported to be affected, possibly due to the targeted expression of XPax6ΔCT in the eye forming region (Chow *et al.*, 1999).

Its expression domain, requirement for eye formation, ability to induce EFTFs and ectopic eye-like structures, all indicate a key role for XPax6 during eye field specification.

4.5. LIM homeobox 2 (Lhx2)

Xenopus laevis Lhx2 is also one of the early expressed EFTFs (Fig. 2.4). *Xlhx2* mRNA is first detected in *Xenopus* embryos between stages 12 and 12.5 (Viczian *et al.*, 2006). Initial expression is detected as a single uniform strip in the anterior of the embryo over the early eye field. *Xlhx2* expression at this stage encompasses that of *Xtbx3* only, while *Xrax*, *Xpax6*, and *Xsix3* have progressively larger expression domains indicating that *Xlhx2* expression is restricted to the presumptive forebrain (Zuber *et al.*, 2003). At neural plate stages expression is more intense but reduced medially when compared to stage 12.5. *Xlhx2* continues to encircle *Xtbx3* expression, but extends more anteriorly and laterally than *Xrax* and the later expressed *Xsix6*.

Neither knockdown nor misexpression phenotypes have been reported for *Xenopus* Lhx2. *Xlhx2* expression is induced by five of the six EFTFs in *Xenopus* animal caps—only the late expressed XSix6 does not induce *Xlhx2* (Zuber *et al.*, 2003). Consistent with this result, 100% of frog embryos injected with the EFTFs (and the neural patterning gene *Xotx2*) ectopically express *Xlhx2*. However, it is unclear if XLhx2 expression is required for the retinal tissue that forms on approximately 90% of these EFTF-injected tadpoles (Zuber *et al.*, 2003). In contrast to the frog, a requirement for *Lhx2* in mouse eye formation is clear. $Lhx2^{-/-}$ mice lack eyes (Porter *et al.*, 1997). In addition, *Lhx2* is required for normal development of a variety of brain regions and the liver, though not all these tissues express *Lhx2* in *Xenopus* (Bulchand *et al.*, 2001; Chou *et al.*, 2009; Hirota and Mombaerts, 2004; Mangale *et al.*, 2008; Monuki *et al.*, 2001; Porter *et al.*, 1997; Saha *et al.*, 2007; Viczian *et al.*, 2006; Wandzioch *et al.*, 2004; Zhao *et al.*, 2010).

In summary, the importance of XLhx2 in *Xenopus* eye formation has not been extensively investigated. Although it is expressed in a variety of other tissues, its regulation by nearly every *Xenopus* EFTF and the dramatic eye phenotype observed in mice lacking Lhx2, strongly suggests a role in early (but not necessarily eye field) formation.

4.6. T-box 3 (Tbx3)

Xenopus Tbx3 was first identified as *ET* (eye T-box) in a degenerate PCR screen to isolate new T-box genes (Li *et al.*, 1997). *Xtbx3* is first detected at stage 12 in two distinct expression domains, the early eye field and more anteriorly in the region that will form the cement gland (Li *et al.*, 1997; Zuber *et al.*, 2003). When compared to the other early expressed EFTFs, *Xtbx3* has the most restricted domain in the anterior neural plate, forming a relatively thin strip of expressing cells at stage 12.5 (Fig. 2.4). Between stages 12.5 and 15, the expression domain remains thin, but extends laterally. At stage 15, *Xtbx3* expression is overlapped by all other EFTFs but *Xnr2e1* (Zuber *et al.*, 2003).

Misexpression of *Xtbx3* in frog embryos by RNA microinjection at the two-cell stage results in abnormal eye morphogenesis, loss of ventral retinal markers, and when expressed medially, fused retinas (Takabatake *et al.*, 2002; Wong *et al.*, 2002). *Xtbx2*, the most closely related member of the T-box family, generates similar overexpression phenotypes (Takabatake *et al.*, 2002). *In vivo*, XTbx3 (and XTbx2) regulates components of the sonic hedgehog (XShh) signaling pathway and the expression of ventral retina markers (Takabatake *et al.*, 2002; Wong *et al.*, 2002). Reciprocally, XShh signaling modulates *Xtbx3* (and *Xtbx2*) expression (Lupo *et al.*, 2005; Sasagawa *et al.*, 2002a; Takabatake *et al.*, 2002). The XTbx3 misexpression phenotype is consistent with these results since XShh signaling is required for separation of the eye field into the two distinct eye primordia. Other extrinsic factors (BMPs, FGFs, and RA) also modulate dorsoventral patterning of the *Xenopus* eye (Lupo *et al.*, 2005; Sasagawa *et al.*, 2002a; Takabatake *et al.*, 2002). These signaling systems can all alter the expression domain of *Xtbx3* at eye field (neurula) and later stages. XTbx3 can act as a transcriptional repressor (He *et al.*, 1999). However, relatively little is known of its function in early eye field specification. In isolated *Xenopus* animal caps, XTbx3 is able to induce the expression of EFTFs *Xrax*, *Xlhx2*, and *Xnr2e1* (Zuber *et al.*, 2003). Misexpression of XTbx3 in frog embryos represses the expression of *Xotx2*. Since XRax can also repress *Xotx2*, an early model proposed that *Xotx2* regulation by XTbx3 might be via XRax at early eye field stages (Zuber *et al.*, 2003). Alternatively, XTbx3 (a known repressor) may repress *Xotx2* independent of, with, or in addition to XRax (Takabatake *et al.*, 2002; Zuber *et al.*, 2003). Notably, XTbx3 misexpression has not been reported to induce ectopic retina, or even expand the retinal domain in *Xenopus* embryos. Although knockdown experiments result in headless tadpoles, no eye phenotype has been reported in $Tbx3^{-/-}$ mice (Davenport *et al.*, 2003; Rana *et al.*, 2006; Ribeiro *et al.*, 2007).

In summary, the lack of an early eye specific phenotype suggests XTbx3 may not be required for vertebrate eye field specification. In spite of this evidence, *Xtbx3* is expressed in the early eye field, regulates the expression of

other EFTFs *in vivo* and *in vitro* and, as we will see below, is a crucial component of a cocktail of EFTFs sufficient to induce ectopic eye formation.

4.7. Six homeobox 6 (Six6)

Xenopus laevis Six6 was originally called *Xoptx2* (optic six gene 2) after a previously identified chicken ortholog (Toy *et al.*, 1998; Zuber *et al.*, 1999). By PCR and whole mount *in situ* hybridization, *Xsix6* is the last EFTF expressed in the eye field (Zuber *et al.*, 2003). Although *Xnr2e1* and *Xsix6* expressions begin at approximately the same developmental stage (stage 14/15), their expression patterns do not overlap, suggesting independent regulation. *Xnr2e1* expression lies more posterior, completely outside of the presumptive eye field, while *Xsix6* has the smallest and most eye field-centric expression domain (Fig. 2.4E). The expression domains of all other EFTFs overlap and are larger than that of *Xsix6* (Zuber *et al.*, 2003).

In *Xenopus*, overexpression of XSix6 dramatically increases eye field and eye size (Zuber *et al.*, 1999). XSix6 overexpression expands the expression domains of *Xpax6*, *Xrax*, and *Xtbx3*. However, due to their earlier expression, it is unlikely *Xsix6* is required for the initial expression of these genes. Eye field enlargement can be blocked by the mitotic inhibitor hydroxyurea suggesting the increase is dependent on cell proliferation. Moreover, eye field retinoblasts transfected with *Xsix6* generate twice as many cells as controls. At the molecular level, XSix6 acts as a repressor in the eye. Fusion of the XSix6 homeodomain (XSix6HD) to engrailed mimics XSix6, while XSix6HD fused to VP16 reduces eye field and eye size. XSix6 has also been proposed to transform midbrain to retina in *Xenopus*. Like *Xsix3*, high doses of mouse *Six6* RNA repress expression of the midbrain marker *Xen2* in frog embryos. In its place, both *Xpax6* and *Xrax* expression is induced, and eventually, eye-like structures form (Bernier *et al.*, 2000).

In summary, XSix6, like the earlier expressed XSix3, regulates retinal progenitor proliferation—possibly via similar mechanisms (see below). However, its relatively late expression suggests that XSix6 (like XNr2e1 of the next section) is required for normal development of the eyes only after eye field specification.

4.8. Nuclear receptor subfamily 2, group E, member 1 (Nr2e1)

Xenopus Nr2e1 was originally identified as *Xtll* (*Xenopus tailless*), a homolog of the *Drosophila* gene *tailless* (Hollemann *et al.*, 1998; Pignoni *et al.*, 1990). *Xnr2e1* is detected late relative to the early expressed EFTFs. No expression is detected in the early eye field (stages 12–12.5). By neurula stages (stages 14–16), *Xnr2e1* is detected by *in situ* hybridization in the prechordal region of the neural plate (Fig. 2.4E). The pattern is distinct from the other EFTFs in that it is the most posteriorly expressed of the EFTFs. *Xnr2e1* expression

does not overlap that of *Xrax*, *Xlhx2*, *Xtbx3*, or *Xsix6*. Only *Xsix3* (medially) and *Xpax6* (laterally) are coexpressed with *Xnr2e1*.

Knockdown phenotypes have not been reported for *Xenopus Nr2e1*. However, fusion of the XNr2e1 zinc-finger (XNr2e1-ZF) (DNA binding) domain to engrailed (XNr2e1-ZF–EnR) reduces eye size in a dose-dependent manner. In extreme cases, eye formation is completely blocked (Hollemann *et al.*, 1998). Neither fusion of the zinc-finger domain to the E1A activator nor full-length XNr2e1 alter eye formation. In *Xnr2e1-ZF–EnR* injected embryos, *Xpax6* expression is unaffected at eye field stages (14 and 16) but is reduced after neurulation (stages 19 and 23). Residual *Xpax6* expressing cells fail to evaginate and form an optic vesicle, suggesting that eye formation requires normal XNr2e1 function after eye field specification (Hollemann *et al.*, 1998).

Xsix3 and *Xpax6* are broadly expressed in the anterior neural plate prior to *Xnr2e1* and both can induce *Xnr2e1* expression in *Xenopus* animal caps (Hollemann *et al.*, 1998; Zuber *et al.*, 2003). Conversely, XNr2e1 can induce the expression of both *Xsix3* and *Xpax6*, as well as other early EFTFs with which it is not coexpressed in the stage 15 eye field (*Xrax* and *Xtbx3*) (Zuber *et al.*, 2003). Why XNr2e1 induces *Xrax* and *Xtbx3* is unclear. These *in vitro* experiments may be evidence of inductive events that normally take place at later developmental stages, when all these genes are coexpressed in regions of the optic vesicle (Hollemann *et al.*, 1998).

Given its relatively late expression, *Xnr2e1* is not required for the initial specification of the eye field. However, its ability to regulate the expression of other EFTFs and eye development at late developmental stages suggest that *Xnr2e1*, like *Xsix6*, is required for later steps in eye formation and maintenance.

In summary, most of the transcription factors described above are required for normal eye formation in *Xenopus*. The expression patterns of these genes are distinct and dynamic during and after eye field formation. Not one of these genes, however, is exclusively expressed in, and required only for, retina formation. In fact, their expression patterns, and the phenotypes resulting from their misexpression and absence suggest that in addition to their importance in eye formation, they also have distinct roles in the formation of other neural structures.

4.9. Coordinated expression of EFTFs is sufficient for eye formation

Xenopus Otx2, Six3/6, Rax, Pax6, Lhx2, Tbx3, and Nr2e1 are most homologous to the fly *orthodenticle* (*otd*), *sine oculis/optix* (*so/optix*), *Drosophila Rx* (*drx*), *eyeless/twin of eyeless* (*ey/toy*), *apterous* (*ap*), *optomotor-blind* (*omb*), and *tailless* (*tll*), respectively. As mentioned earlier, some *Xenopus* EFTFs were originally identified as homologs of *Drosophila* genes required for fly

eye formation. Only a subset (*so*, *optix*, *ey*, *toy*) of the fly homologs, however, are considered retinal determination genes. Nevertheless, *otd*, *drx*, *omb*, and *tll* are required for normal visual system and/or brain formation in the fly and intriguing similarities exist between the fly and vertebrate homologs, which, in some cases can functionally substitute for each other (Acampora *et al.*, 2001; Daniel *et al.*, 1999; Davis *et al.*, 2003; Eggert *et al.*, 1998; Leuzinger *et al.*, 1998; Lunardi and Vignali, 2006; Mathers *et al.*, 1997; Nagao *et al.*, 1998; Pflugfelder *et al.*, 1992).

In *Drosophila*, work from a large number of labs has demonstrated the fly retinal determination genes act coordinately to regulate eye development, and form a network with hierarchical components as well as regulatory feedback loops (reviewed in another chapter in this volume). The expression patterns of many fly retinal determination genes overlap in the *Drosophila* eye field during its specification. As described in the previous section, the timing and expression patterns of the *X. laevis* EFTFs correlate remarkably well with the classical fate mapping and transplantation experiments that determined the timing and location of eye field specification in a variety of amphibian species (reviewed in Holtfreter and Hamburger, 1955; Spemann, 1938). Subsequent fate mapping and expression studies in *X. laevis* confirm the correlation between eye field specification and the *Xenopus* EFTF expression domains (previous section; Eagleson and Harris, 1990; Eagleson *et al.*, 1995). These observations prompted us to ask, "Since the EFTFs are coordinately expressed in the vertebrate eye field at the time of its specification, do they, like the fly retinal determination genes, form a regulatory network required for vertebrate eye field specification?"

To our surprise, we found that coordinated misexpression of the *Xenopus* EFTFs Six3, Rax, Pax6, Tbx3, Six6, and Nr2e1 with the anterior neural patterning gene *Xotx2* was sufficient to induce ectopic eye fields and eye-like structures (Zuber *et al.*, 2003). *Xlhx2* was intentionally left out of the cocktail since we needed an early marker for eye field specification. In preliminary experiments, we noticed that *Xlhx2* (despite its requirement for mouse eye formation) was not necessary for the phenotypes we observed. The EFTF cocktail induced *Xlhx2* expression in 100% of injected embryos. When embryos were cultured to later developmental stages, ectopic RPE and eye-like structures formed on the injected side of approximately 90% of the tadpoles. Approximately 20% of injected embryos developed quite large ectopic eyes—as large as or even larger than the endogenous eyes (Fig. 2.5A). The ectopic tissues had the cup-like structure of the normal eye, including the expected trilayered structure of a retina, including ganglion, inner nuclear, and ONLs. These tissues expressed markers for differentiated retinal cell classes including retinal ganglion, rod, and cone photoreceptor cells as well as RPE and lens markers. Intriguingly, *Xlhx2* expression and ectopic eyes were detected both in and outside the nervous system. From these results, it was concluded that coordinated

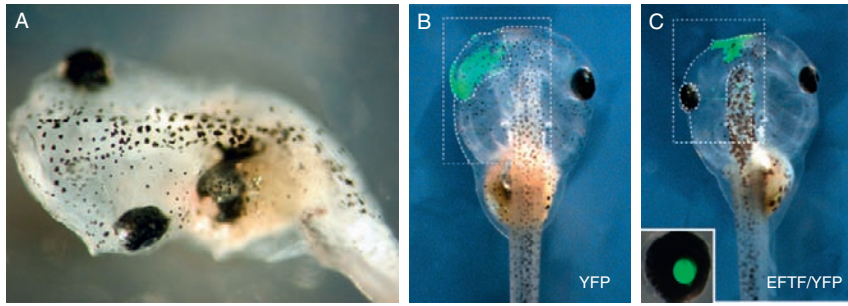


Figure 2.5 XOtx2 and the EFTFs are sufficient to induce eye formation from pluripotent cells. (A) RNAs coding for XOtx2 and the EFTFs is sufficient to induce eye formation when injected directly into one cell of a two-cell embryo. (B) Replacement of an eye field with YFP-only expressing animal cap cells generates only epidermis. (C) In contrast, cells expressing the EFTFs with XOtx2 form a functional eye.

expression of the vertebrate EFTFs with *Xotx2* is sufficient for induction of ectopic eye fields and ultimately eye-like structures (Zuber *et al.*, 2003).

These early studies could not, however, address several questions, "Are these really eyes? Are they functional? If not, can you really call them eyes?" In addition, it was not possible to definitively identify all retinal cell types in the ectopic structures that formed due to the lack of appropriate molecular markers. Lastly, the embryonic origin of the cells that generated the eye-like structures could not be identified. Did "ectopic eyes" first form in the neural plate and, due to abnormal morphogenesis during development, end up outside the nervous system? To address these questions we took a different approach.

As described in a previous section, animal cap cells isolated from blastula stage embryos are pluripotent. We transplanted EFTF-expressing donor caps to the flank of host embryos. To distinguish between the donor and host cells, we generated the donor cells from transgenic embryos constitutively expressing yellow fluorescent protein (YFP; Sakamaki *et al.*, 2005). While control cells only formed epidermis (the normal fate of isolated animal cap cells), EFTF-expressing cells formed eye-like structures similar to those generated by direct injection of EFTFs into embryos at the two-cell stage. To determine if all retinal cells were generated, EFTF-expressing cap cells were transplanted directly into the eye field of stage 15 embryos to generate mosaic retinas containing a mixture of endogenous and EFTF/YFP-expressing donor cells (Viczian *et al.*, 2009). This allowed a direct comparison of endogenous retinal cells with those generated using the EFTFs. Control, YFP-only expressing cells never formed retina, even when transplanted directly into the eye field. By contrast, EFTF-expressing cells generated all seven classes of retinal cells normally found in the

Xenopus retina. Together, these results demonstrated that EFTF-expressing pluripotent cells were determined (like the endogenous eye field) to form all the retinal cell types, and even eye-like structures when transplanted directly to a region outside the nervous system (Viczian *et al.*, 2009).

Despite the ability of XOtx2 and the EFTFs to induce eye-like structures on the tadpole flank, it was not possible to test the function of these retinas. Due to their abnormal location, RGC axons from EFTF-induced eyes could not reach their normal tectal targets. In addition, it was not possible to record electroretinograms (ERGs) due to their small size. Therefore, a different approach was needed. In the hope of generating larger eyes that would project ganglion cell axons to the brain, we replaced endogenous eye fields with EFTF-expressing cells. As demonstrated previously by the classical cut-and-paste experiments, *Xenopus* embryos from which one-half of an eye field has been removed survive, develop normally, but lack an eye on the operated side (Fig. 2.1). When eye fields were replaced with EFTF-expressing cap cells, however, morphologically normal eyes formed (compare Fig. 2.5B vs. C). More importantly, using both ERGs and a vision-based behavioral assay, we were able to show these eyes were functional. These results demonstrate that the EFTFs are sufficient to direct pluripotent cells to an eye field-like lineage. The induced cells then differentiate into a retina with all the cell classes and circuitry necessary for vision.

5. Mechanisms Regulating Eye Field Specification and Early Eye Formation

It can be concluded from the previous section that *XOtx2* and the *Xenopus* EFTFs are required and, in the proper context, sufficient for retinal and even functional eye formation in *Xenopus*. But why are they required and what are the mechanisms by which they regulate eye field formation and eye development? Evidence suggests that these genes regulate the early signaling system that pattern the nervous system, the mobility of cells within the neural plate, the proliferative state of eye field cells, and lastly, each others' expression. The following examples illustrate these mechanisms and how the collective expression of the EFTFs drive and maintain eye field and early eye formation.

5.1. Controlling the signaling systems that pattern the nervous system

BMPs, FGFs, Wnts, Nodals, and RA all regulate neural patterning events. Consequently, these genes and their signaling systems can regulate the expression domains of the EFTFs, and in some cases, the EFTFs regulate the neural patterning systems.

Inhibition of BMP signaling is required during neural induction and during normal patterning of the forebrain, but must also be controlled even after neural plate formation. If beads coated with BMP4 are transplanted to the anterior neural plate at eye field stages, the expression of neural markers including *Xotx2*, *Xrax*, and *Xpax6* are repressed (Hartley *et al.*, 2001). Similarly, BMP4 expression driven under the control of the *Xpax6* promoter represses *Xotx2*, *Xrax* expression and eye formation in greater than 90% of transgenic tadpoles (Hartley *et al.*, 2001).

As described previously, XSix3 stimulates proliferation, and misexpression of *Xsix3* RNA expands the size of the neural plate (Bernier *et al.*, 2000; Gestri *et al.*, 2005). However, expansion of the neural plate is only partially blocked by mitotic inhibitors, indicating cell proliferation is not the lone mechanism (Gestri *et al.*, 2005). XSix3, in defense of the eye field, also represses *Xbmp4* expression. XSix3 binds directly to the *Xbmp4* promoter and can rescue eye formation in a mutant fish with anterior neural plate defects resulting from excessive BMP signaling. Consistent with these results, when embryos are injected with *Xsix3* morpholinos or *Xsix3–VP16* (*XSix3* normally acts as a transcriptional repressor), *Xbmp4* expression invades the presumptive neural plate and the eye field is reduced in size (Gestri *et al.*, 2005). Therefore, the extrinsic factors that pattern the vertebrate neural plate continue to influence the eye field (sometimes negatively) at later developmental stages. The evidence suggests that one role of the EFTFs is to protect the early eye field from these disrupting signaling systems.

5.2. Regulation of cell migration in the anterior neural plate

Cell movements are dramatic during gastrulation and neurulation. Therefore, mechanisms must be in place, to not only form, but also maintain the eye field in its proper location. The EFTFs appear to play a role in the mobility of cells that will eventually form the retina.

Dorsal animal blastomeres of the 32-cell *Xenopus* embryo generate the vast majority (greater than 99%) of the retina in each eye (Huang and Moody, 1993; Moody, 1987a). In contrast, more ventral animal blastomeres normally contribute to epidermal and placodal fates. If, however, XOtx2, XRax, or XPax6 are misexpressed in ventral animal blastomeres, their progeny generate retina (Kenyon *et al.*, 2001). This is due to a change in cell movements during gastrulation. XOtx2, XRax, and XPax6-expressing cells migrate anteriorly into the neural plate and eye field. The BMP-inhibitor Noggin, which can strongly induce EFTF expression, has a similar effect (Kenyon *et al.*, 2001). The FGF, ephrin, and Wnt signaling pathways are also required for proper positioning of retinal progenitors, emphasizing the importance of controlling cell movements during early eye field formation (Lee *et al.*, 2006, 2009; Moore *et al.*, 2004). Several EFTFs are either regulated by, or can regulate these signaling systems. Although it

is interesting to speculate, there has been no direct evidence linking these signaling systems via EFTFs (or vice versa) to retinal progenitor movements.

When an endogenous eye field is replaced with a control (uninjected) animal cap, the donor cells form only epidermis—the surface skin of the host tadpole (Viczian *et al.*, 2009). Therefore, control cells migrate out of the neural plate, most likely prior to neurulation (when the neural plate folds in upon itself to form the neural tube). In contrast, EFTF-expressing animal cap cells generate retina and other neural tissue (in addition to some epidermis). These results indicate the EFTFs may not only be able to direct prospective retinal progenitors into the presumptive eye field, but also keep them there.

5.3. Maintaining the proliferative state of eye field cells

In *Xenopus*, neural differentiation starts in the posterior neuroectoderm soon after gastrulation. In contrast, eye field cells and retinal progenitors continue to proliferate for an extended period of time in order to generate a normal sized eye. Proneural genes, which can drive neuronal differentiation are excluded from the eye field. The expression of proneural genes such as *Xngnr-1* and *Xdelta-1* border, but are not expressed within the eye field. Expression of XRax in regions outside the presumptive forebrain represses the expression of *Xngnr-1* and *Xdelta-1*, which inhibits neural differentiation. Conversely, *Xrax* loss-of-function (XRax–EnR) results in the expansion of *Xngnr-1* expression into the anterior neural plate and reduces proliferation in, and the size of, the eye field. In addition to repressing the expression of proneural genes, XRax also induces the expression of *Xzic2* and *Xhairy2* (antineurogenic transcription factors) and represses the cell cycle inhibitor *p27Xic1* (Andreazzoli *et al.*, 2003).

XSix3 and XSix6 also regulate the proliferation of retinal progenitors (Bernier *et al.*, 2000; Gestri *et al.*, 2005; Zuber *et al.*, 1999). In addition to its role in repressing *Xbmp4*, XSix3 promotes neural plate proliferation by modulating the expression the antineurogenic genes *Xzic2* and *Xhairy2*, and the cell cycle regulators *XcyclinD1* and *p27Xic1* (Gestri *et al.*, 2005)

A screen for cofactors of Six3 and Six6 identified Groucho family members Tle1 and Aes as interacting partners of Six3 and Six6 (Lopez-Rios *et al.*, 2003; Tessmar *et al.*, 2002). In medaka fish (*Oryzias latipes*), *Otle1* and *Oaes* expression patterns overlap those of *Osix3* and *Osix6* in the developing fish eye. OTle1, which can expand eye field size on its own, acts synergistically with both OSix3 and OSix6. In contrast, OAes (which is thought to act as a dominant negative form of Tle) reduces eye size and inhibits the ability of OSix3 and OSix6 to expand the eye field. Another direct Six3 interactant identified from the screen was geminin (Gem)—an inhibitor of DNA replication (Del Bene *et al.*, 2004). Interestingly, blocking

OGem expression increases cell proliferation resulting in eye enlargement, whereas misexpression of OGem results in a dose-dependent loss of forebrain and eye structures. OSix3 can rescue the eye loss caused by misexpression of OGem. Six3 forms protein × protein complexes with, and antagonizes the ability of Gem to inhibit DNA replication, thereby controlling the balance between proliferation and differentiation in the early eye (Del Bene *et al.*, 2004).

Together the above examples illustrate how EFTFs use multiple mechanisms (repression of neurogenesis, stimulation of proliferation, etc.) to maintain cells of the eye field in a proliferative state.

5.4. Cross-regulation of the EFTFs

EFTFs also regulate each others' expression *in vitro* and *in vivo*. XSix6 can act coordinately with XPax6 to regulate eye size. XPax6, alone, does not increase eye size. However, XPax6 potentiates the eye enlarging activity of XSix6, suggesting they may act together to regulate common downstream targets, XPax6 might induce *Xsix6* expression, or both (Zuber *et al.*, 1999). In *Xenopus* animal caps, XSix6 is unable to induce the expression of any other EFTF, while XPax6 and XRax both induce *Xsix6* expression. Although *Xrax* is expressed in the anterior neural plate as early as stage 12, *Xsix6* is not detected until stage 14/15 (Zuber *et al.*, 1999, 2003). These results prompted speculation that XRax and/or XPax6 might be responsible for the initial expression of *Xsix6* (Zuber *et al.*, 2003). This hypothesis was certainly consistent with the ability of XRax to induce proliferation in the *Xenopus* eye field and retina (Andreazzoli *et al.*, 2003; Casarosa *et al.*, 2003; Mathers *et al.*, 1997). These ideas have since been confirmed as subsequent experiments have demonstrated that XRax is required for the initial expression of *Xsix6* in *Xenopus* (Terada *et al.*, 2006). In addition, Pax6 and Lhx2 directly coregulate the transcription of *six6* in mouse (Tetreault *et al.*, 2009).

Recent evidence suggesting *Xrax* is transcriptionally regulated by *Xotx2* may explain phenotypic similarities in patients with mutations in the human orthologs of these genes. Approximately 2 kb 5′ of the *XRax* coding region lies an evolutionarily conserved sequence (CNS1) that contains consensus binding sites for both XOtx2 and XSox2 (Danno *et al.*, 2008). A GFP reporter under the control of the *Xrax* 5′ flanking region mimics the endogenous *Xrax* expression pattern. Removal of CNS1 from the transgene markedly reduced GFP expression. Reporter and DNA binding assays performed in *Xenopus* animal cap cells and HEK293T cells demonstrate XOtx2 and XSox2 physically interact and together bind to CNS1. Binding is not observed when Sox2 containing a missense mutation known to cause ocular malformations in human patients. The authors suggest the interdependence of XOtx2 and XSox2 in regulating *Xrax* expression during eye

development in *Xenopus* may explain the similarities in human ocular malformations resulting from mutations in any one of these three genes (Danno *et al.*, 2008).

All the EFTFs have been highly conserved through evolution, and functional inactivation of Otx2, Six3, Rax, Pax6, Lhx2, Six6, Tbx3, or Nr2e1 result in frogs, fish, rodents, and/or humans with abnormal or no eyes. As in *Xenopus*, the expression patterns of the EFTFs in other model systems also overlap in the presumptive eye field during, and immediately following, its specification. Although some species-specific differences have been observed, clear evidence for the mechanisms described above in *Xenopus* have also been observed in other model systems. Consequently, *Xenopus* has served, and will no doubt continue to serve, as a valuable model system for identifying and understanding the cellular and molecular mechanisms driving early eye formation.

6. Conclusions

More than 100 years have passed since embryologists first identified the location and timing of vertebrate eye field specification. The past 20 years, in particular, have seen great strides in our understanding of the molecules and molecular mechanisms driving eye field and early eye formation. The genes described here are all expressed at a time and in a place that would not surprise researchers from even a century ago. In contrast, little more of what we now know could have been predicted. Individually these genes are required for eye formation. They control aspects of neural patterning, cell migration, proliferation as well as each other expression. Nevertheless they are also collectively needed, and at least in *Xenopus*, collectively sufficient to specify a frog eye field and ultimately a functional eye.

Despite these advances, much has yet to be discovered. What are the upstream controllers regulating EFTF expression? What are the downstream targets controlled by the EFTFs, and how and why are they collectively required for eye formation? Some tantalizing (sometimes unexpected) clues have emerged. Neural and head inducers including Noggin, Wnts, and IGFs can induce the expression of EFTFs and ectopic eyes in *Xenopus* (Gessert *et al.*, 2007; Lan *et al.*, 2009; Maurus *et al.*, 2005; Onuma *et al.*, 2002; Pera *et al.*, 2001; Rasmussen *et al.*, 2001; Richard-Parpaillon *et al.*, 2002; Viczian *et al.*, 2009). More surprising is the discovery of a new purine-mediated signaling pathway required and sufficient for early EFTF expression and eye formation (Masse *et al.*, 2007). Given its already long history, it is not difficult to image that the next 20 years could easily be spent unraveling the genetic and molecular mechanisms regulating the 90 minutes during which a small patch of cells are specified to begin eye formation.

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CHAPTER THREE

Eye Morphogenesis and Patterning of the Optic Vesicle

Sabine Fuhrmann

Contents

Abstract

Organogenesis of the eye is a multistep process that starts with the formation of optic vesicles followed by invagination of the distal domain of the vesicles and the overlying lens placode resulting in morphogenesis of the optic cup. The late optic vesicle becomes patterned into distinct ocular tissues: the neural retina, retinal pigment epithelium (RPE), and optic stalk. Multiple congenital eye disorders, including anophthalmia or microphthalmia, aniridia, coloboma, and retinal dysplasia, stem from disruptions in embryonic eye development. Thus, it is critical to understand the mechanisms that lead to initial specification and differentiation of ocular tissues. An accumulating number of studies demonstrate that a complex interplay between inductive signals provided by tissue–tissue interactions and cell-intrinsic factors is critical to ensuring proper specification of ocular tissues as well as maintenance of RPE cell fate. While several of the

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extrinsic and intrinsic determinants have been identified, we are just at the beginning in understanding how these signals are integrated. In addition, we know very little about the actual output of these interactions. In this chapter, we provide an update of the mechanisms controlling the early steps of eye development in vertebrates, with emphasis on optic vesicle evagination, specification of neural retina and RPE at the optic vesicle stage, the process of invagination during morphogenesis of the optic cup, and maintenance of the RPE cell fate.

1. Introduction

The vertebrate eye is formed through coordinated interactions between neuroepithelium, surface ectoderm, and extraocular mesenchyme, which originates from two sources: neural crest and mesoderm. Following eye field formation, the neuroepithelium of the ventral forebrain evaginates, resulting in the formation of bilateral optic vesicles (Fig. 3.1A). The distal

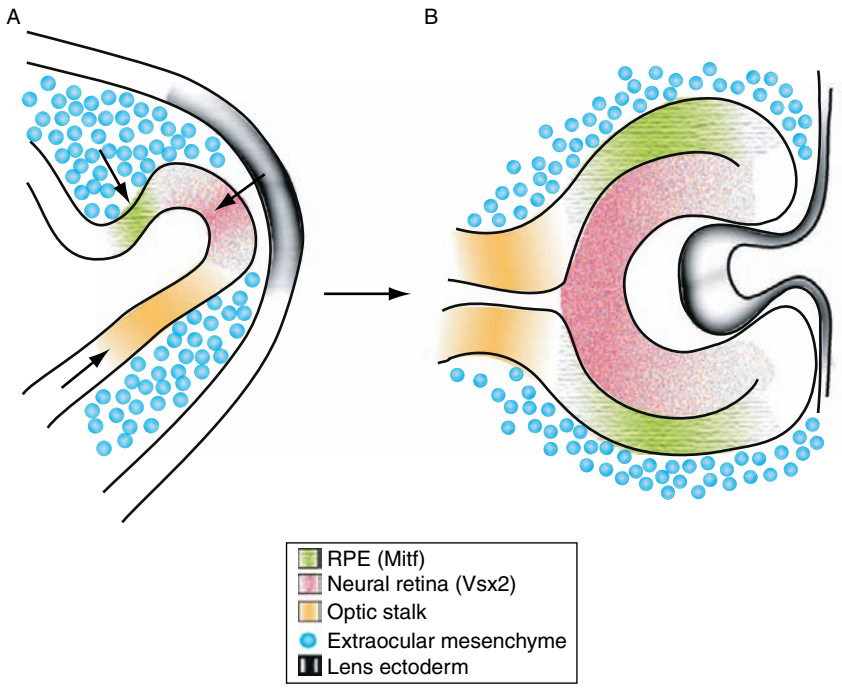

Figure 3.1 Summary of early eye development in vertebrates. (A) Factors from surrounding tissues (extraocular mesenchyme, optic stalk, and surface ectoderm) regulate patterning of the neural retina and RPE in the vertebrate optic vesicle, which then expresses the specific transcription factors Vsx2 and Mitf, respectively. (B) Invagination of the distal optic vesicle (presumptive retina) and the overlying lens placode results in formation of the optic cup and lens vesicle (for details, see text). Modified from Fuhrmann, 2008.

portion of the vesicle makes contact with the overlying surface ectoderm (lens ectoderm), which is then induced to form the lens placode. This interaction results in invagination of the lens placode and distal optic vesicle leading to formation of a bilayered optic cup (Fig. 3.1B). The neural retina develops from the inner layer of the optic cup, and the retinal pigment epithelium (RPE) is derived from the outer layer. The margin between the two layers gives rise to peripheral structures, the iris epithelium and ciliary body. The most proximal part of the optic vesicle, the optic stalk, "narrows" to become the optic fissure. The lens vesicle eventually separates from the surface ectoderm and differentiates into the mature lens. Tissue–tissue interactions, mediated by extracellular factors and intrinsic signals such as transcription factors, control differentiation of ocular tissues starting at the optic vesicle stage.

Much progress has been made in recent years elucidating the mechanisms involved in evagination and proximodistal patterning of the optic vesicle, and morphogenesis of the optic cup; therefore, these topics will be the focus of this chapter. The reader is referred to many excellent reviews for discussions of other aspects of early eye development, such as lens and optic stalk formation, dorsoventral and nasotemporal patterning of the optic vesicle, as well as differentiation of ciliary body and iris epithelium (Adler and Canto-Soler, 2007; Davis-Silberman and Ashery-Padan, 2008; Donner *et al.*, 2006; Hyer, 2004; Lang, 2004; Morcillo *et al.*, 2006; Takahashi *et al.*, 2009; Yang, 2004; Zhao *et al.*, 2010).

2. Evagination of the Optic Vesicles

The first morphological sign of eye morphogenesis is evagination of the optic vesicles, which occurs in the ventral forebrain during the final stages of neural tube formation (Fig. 3.1A). Detailed analyses have revealed changes in cell behavior that take place during the evagination process. In mouse, the cellular shape of optic vesicle cells changes dramatically, accompanied by transient alterations in basal lamina composition (Svoboda and OShea, 1987). In fish and frogs, ocular cells undergo extensive movements that are essential for organogenesis of the eye. Fish retinal progenitor cells first display a directed movement toward the midline, followed by an outward turn into the evaginating optic vesicles (Rembold *et al.*, 2006). It still needs to be determined whether these directed migrations occur in other vertebrates such as chick and mouse. Taken together, these findings are evidence that coordinated changes in cell shape and cellular behavior are required for evagination of the optic vesicles.

At the molecular level, it has been shown that the retinal homeodomain transcription factor Rx/RAX mediates some of these cell behaviors.

Null or hypomorphic *Rx* alleles display anophthalmia in humans (*RAX*), mouse (*Rx*), frog (*Rx1*), zebrafish (*Rx3/chokh*), and medaka (*eyeless*) (Andreazzoli *et al.*, 1999; Furukawa *et al.*, 1997; Kennedy *et al.*, 2004; Loosli *et al.*, 2003; Mathers *et al.*, 1997; Tucker *et al.*, 2001; Voronina *et al.*, 2004), indicating that *Rx* genes are essential for early eye development. Work in zebrafish and medaka indicates why early eye development fails. In Rx3 null mutants, optic vesicle evagination is disrupted in a cell autonomous manner; specifically, the outward directed movement of cells appears to be disturbed (Loosli *et al.*, 2003; Rembold *et al.*, 2006; Stigloher *et al.*, 2006; Winkler *et al.*, 2000). These data suggest that Rx/RAX is involved in the extensive cell movements that are integral to evagination.

How does Rx/RAX work? Information about its role can be gleaned through identification of target genes. Three lines of evidence demonstrate that the Ig-domain cell adhesion molecule, Nlcam, is a potential direct target of Rx3 in zebrafish. First, predicted Rx3 binding sites are found in the Nlcam locus (Brown *et al.*, 2010). Second, in *Rx3/chk* mutants, Nlcam is ectopically upregulated in the eye field, suggesting that Rx3 represses NIcam expression. Third, similar to *Rx3* null mutants, overexpression of Nlcam results in smaller eyes, and cell tracking experiments revealed that mutant cells display increased convergence at the midline (Brown *et al.*, 2010). Thus, downregulation of Nlcam expression by Rx3 may be necessary to enable retinal progenitor cells in the eye field to move away from the midline and outward to contribute to the evaginating optic vesicles. It was also recently shown that Rx-deficient cells are excluded from optic vesicle domains in embryonic mouse chimeras consisting of wild-type and mutant Rx cells (Medina-Martinez *et al.*, 2009). Together with the zebrafish data, these findings are consistent with the idea that Rx controls segregative behavior of retinal progenitor cells.

There are other possible mechanisms by which Rx/RAX may act during early eye development. Work has shown that Rx participates in suppressing the canonical Wnt pathway to prevent the induction of posterior fates of the anterior neural plate, and it promotes noncanonical Wnt signaling that control morphogenetic movements of ocular cells (Martinez-Morales and Wittbrodt, 2009). Another potential function of Rx is the regulation of proliferation (Stigloher *et al.*, 2006). Optx2/Six6, which controls proliferation in the eye field, is dependent on Rx function (Zuber *et al.*, 1999, 2003. Finally, Rx is essential for the expression of other key regulators of early eye formation such as Lhx2, Pax6, Mab21l2, and Six3 to control, directly or indirectly, specification of retinal progenitor cells in the optic vesicle. In summary, while Rx is clearly essential during optic vesicle formation, additional potential mechanisms of Rx function need further examination.

3. Patterning of the Optic Vesicle into RPE and Neural Retina

During evagination of the optic vesicle, the neural retina and RPE domains are specified (Fig. 3.2). The neural retina develops from the distal/ventral portion of the optic vesicle, while the RPE emerges from the dorsal region (Hirashima *et al.*, 2008; Kagiyama *et al.*, 2005). At the optic vesicle stage, the neuroepithelium is bipotential; the presumptive retina is competent to develop into RPE (Araki and Okada, 1977; Clayton *et al.*, 1977; Horsford *et al.*, 2005; Itoh *et al.*, 1975; Opas *et al.*, 2001; Rowan *et al.*, 2004; Westenskow *et al.*, 2010) and, conversely, the presumptive RPE can differentiate into retina (Coulombre and Coulombre, 1965; Reh and Pittack, 1995; Stroeva, 1960; Stroeva and Mitashov, 1983). Interestingly, studies in chick reveal that the dorsal and ventral portions of the optic vesicle have distinct developmental potency; after removal of the dorsal optic vesicle, the anterior ventral domain can regenerate both retina and RPE, while the dorsal portion can only develop into a pigmented, RPE-like vesicle that does not invaginate (Hirashima *et al.*, 2008). Thus, the anterior ventral domain of the optic vesicle may be the driving force for morphogenesis of the eye and proper specification of ocular tissues.

The earliest genes that show domain-specific expression for the retina and RPE are the homeobox gene Vsx2 (formerly Chx10) and the bHLH transcription factor Mitf, respectively (Fig. 3.2; Burmeister *et al.*, 1996; Green *et al.*, 2003; Hodgkinson *et al.*, 1993; Nguyen and Arnheiter, 2000). In mouse, Mitf is initially expressed throughout the optic vesicle and is subsequently downregulated in the distal domain when Vsx2 expression is initiated (Nguyen and Arnheiter, 2000). Both transcription factors are essential for early patterning and maintenance of cell fate in the optic vesicle (see below), and Vsx2 also controls other aspects of retinal development such as proliferation (Green *et al.*, 2003; Sigulinsky *et al.*, 2008).

The earliest known patterning gene is the LIM homeobox transcription factor Lhx2, which is first expressed in the eye field and is required for expression of Mitf and for retinal determinants in the optic vesicle (Yun *et al.*, 2009; Zuber *et al.*, 2003). In Lhx2 mouse mutants, expression of other eye field transcription factors initiates normally, but eye development arrests at the optic vesicle stage, and the lens fails to form (Porter *et al.*, 1997; Tetreault *et al.*, 2009; Yun *et al.*, 2009). Recently, a more detailed analysis revealed that expression of optic vesicle regional patterning markers is severely disturbed (Yun *et al.*, 2009). For example, the expression of Mitf, Chx10/Vsx2, and Tbx5 is never initiated, while expression of Pax2, Vax2, and Rx is initiated but not maintained. Interestingly, mosaic analysis of conditionally inactivated Lhx revealed that gene functions cell autonomously to promote Chx10 and Mitf expression

(Yun *et al.*, 2009). Thus, Lhx2 is uniquely required in the early optic vesicle for specification into both neural retina and RPE and to regulate optic cup formation (see below).

3.1. RPE specification

The RPE is required for growth of the eye, it controls proper lamination of the retina, and it regulates differentiation of the photoreceptors (Bharti *et al.*, 2006; Martinez-Morales *et al.*, 2004; Strauss, 2005). Genetic ablation of the RPE or disruption of RPE specification genes result in microphthalmia, RPE-to-retina transdifferentiation, and coloboma during murine eye development (Bumsted and Barnstable, 2000; Martinez-Morales *et al.*, 2001; Nguyen and Arnheiter, 2000; Raymond and Jackson, 1995; Scholtz and Chan, 1987).

The RPE is specified at the early optic vesicle stage, long before pigmentation becomes obvious (Fig. 3.2A). Two key players in RPE specification are the transcription factors Mitf and orthodenticle homeobox 2 (Otx2). Mitf is the first gene that is specifically expressed in the presumptive RPE in the optic vesicle (for reviews, see Bharti *et al.*, 2006; Martinez-Morales *et al.*, 2004). Mitf is a key regulator of pigment cell development in the RPE and neural crest; it transactivates crucial genes for terminal pigment differentiation (e.g., Dct, Tyrp1, and tyrosinase). Otx2 is expressed in the eye field and expression appears to persist until the late optic vesicle stage when it is downregulated in the presumptive retina, similar to Mitf. Otx2 is required for Mitf expression and transactivates expression of pigment genes in cooperation with Mitf (Martinez-Morales et al., 2001, 2003, 2004). Recent studies demonstrate that RPE specification requires interaction with extraocular tissues, however, the exact mechanism is not resolved (Buse and de Groot, 1991; Fuhrmann *et al.*, 2000; Lopashov, 1963; Muller *et al.*, 2007; Stroeva, 1960).

In chick, some progress has been made in clarifying the role of extraocular tissues and signaling pathways regulating RPE development; however, some of the results are controversial. Robust Mitf expression in chick is detectable at the optic vesicle stage; however, in contrast to mouse, expression is restricted to the presumptive RPE domain (Fuhrmann *et al.*, 2000; Ishii *et al.*, 2009; Mochii *et al.*, 1998; Muller *et al.*, 2007). Previous studies, including our own, indicate that the adjacent extraocular mesenchyme is required for expression of RPE-specific genes such as Mitf, the Mitf target melanosomal matrix protein MMP115 and Wnt13, in explant cultures of chick optic vesicles (Fuhrmann *et al.*, 2000; Kagiyama *et al.*, 2005). In the absence of extraocular mesenchyme, the TGFβ family member activin can restore RPE marker expression (Fuhrmann *et al.*, 2000). Since the explants were prepared before Mitf is robustly expressed in the presumptive RPE domain, we conclude that the extraocular mesenchyme is essential for

induction of Mitf expression/RPE fate in the chick optic vesicle (Fuhrmann *et al.*, 2000; Mochii *et al.*, 1998).

Some of our findings are at odds with other published work. Previously, it was shown that Mitf can be expressed earlier at low levels in the entire evaginating chick optic vesicle when the neuroepithelium is in close contact with the overlying ectoderm and mesenchyme is still absent (Muller *et al.*, 2007). The surface ectoderm expresses BMPs and BMP-coated beads can induce ectopic Mitf expression, when implanted adjacent to the optic vesicle (Hyer *et al.*, 2003; Muller *et al.*, 2007). Therefore, it was proposed that BMP secreted by the surface ectoderm acts as an inducer of RPE fate in the optic vesicle (Muller *et al.*, 2007). However, optic cup morphogenesis is disturbed in BMP-treated eyes and BMP can induce apoptosis in the optic vesicle, which may confound these results (Hyer *et al.*, 2003; Muller *et al.*, 2007; Trousse *et al.*, 2001). In addition, loss-of-function studies show that BMP signaling is required for RPE differentiation in the ventral optic cup, but these experiments do not address a role during earlier stages, when the RPE is specified (Adler and Belecky-Adams, 2002b; Muller *et al.*, 2007). Thus, further experiments in chick are required to elucidate the true nature of interacting tissue and which actual RPE-inducing signal mediates this interaction.

In contrast to chick, the mouse extraocular mesenchyme surrounds the budding optic vesicle at very early stages when Mitf starts to become expressed (Bassett *et al.*, 2010; our own unpublished observations). Consistent with our previous work suggesting a role for extraocular mesenchyme in RPE specification in chick, we observed in mouse optic vesicle explant cultures that removal of extraocular mesenchyme interferes with Mitf expression (unpublished observations). Further, other studies demonstrate that mutations in genes critical for extraocular mesenchyme development are accompanied by ocular malformations and abnormal development of the mouse RPE (Bassett *et al.*, 2007; Evans and Gage, 2005; Gage *et al.*, 1999; Grondona *et al.*, 1996; Kastner *et al.*, 1994; Kitamura *et al.*, 1999; Kume *et al.*, 1998; Matt *et al.*, 2008; Mori *et al.*, 2004; Moser *et al.*, 1997; West-Mays *et al.*, 1999). However, in mouse, a direct role for extraocular mesenchyme in induction of the RPE has not been shown so far. Interestingly, interference with BMP signaling does not interfere with RPE development in mouse suggesting that, while the utilization of a common signaling pathway (TGFβ) may be conserved, the actual signal can be different depending on the species (Furuta and Hogan, 1998; Morcillo *et al.*, 2006; Wawersik *et al.*, 1999).

In addition to extracellular signaling, a few intrinsic determinants are known to regulate early aspects of RPE development. In zebrafish, certain Rx3 alleles can interfere specifically with RPE development and it was proposed that Rx confers competence on the presumptive RPE to respond to inducing signals from the mesenchyme (Rojas-Munoz *et al.*, 2005). In Pax2/Pax6 compound mutant mice, Mitf is not expressed in the optic

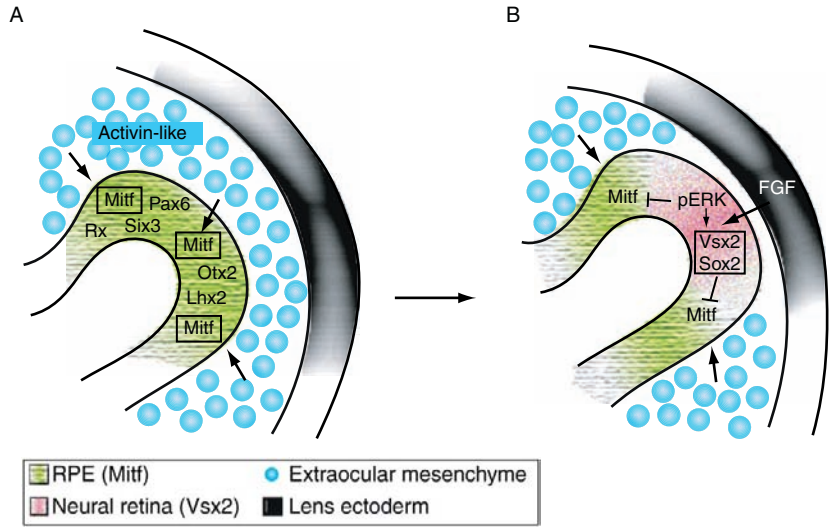

Figure 3.2 Specification of RPE and retina in the optic vesicle. The eye field transcription factors Pax6, Rx, Otx2, Six3, and Lhx2 are required in the optic vesicle to respond to inducing signals. (A) RPE specification in mouse (early optic vesicle): the extraocular mesenchyme, possibly by producing an activin-like factor, induces Mitf expression in the entire optic vesicle. (B) Retina specification in mouse and chick (late optic vesicle): subsequentially, activation of ERK, potentially through FGF secreted from the lens ectoderm, induces/maintains Vsx2 and Sox2 expression in the distal optic vesicle to promote retina development, which requires Vsx2-mediated suppression of Mitf.

vesicle, the RPE transdifferentiates into retina and the optic vesicle does not invaginate to form an optic cup (Baumer *et al.*, 2003). Interestingly, Otx2 expression persists but is not sufficient to promote RPE formation. Furthermore, both Pax2 and Pax6 bind to and activate the Mitf-A enhancer that controls expression of Mitf in the RPE (Baumer *et al.*, 2003). These results suggest that Pax2 and Pax6 are redundantly required for RPE specification in mouse however, it is not clear whether they act upstream or downstream of a potential signal from the mesenchyme. In summary, these studies indicate that several upstream regulators, intrinsic and extrinsic, ensure that Mitf is sufficiently expressed to promote RPE cell fate in the optic vesicle (Fig. 3.2A).

3.2. Neural retina specification

The MAP kinase FGF signaling pathway is important for different steps of neural retina development (Fig. 3.2B). First, it is essential for patterning of the retina in the distal optic vesicle, and second, for initiation of retinal neurogenesis. FGF ligands and receptors are abundantly expressed in ocular

and extraocular tissues, and specifically, FGF1 and FGF2 show strong expression in the lens ectoderm (de Longh and McAvoy, 1993; Pittack *et al.*, 1997; Vogel-Hopker *et al.*, 2000). Removal of the surface ectoderm in chick embryos interferes with neuronal marker expression in the distal domain (Hyer *et al.*, 1998; Pittack *et al.*, 1997). In addition, eyes develop microphthalmic as pigmented vesicles with a few neuronal cells intermingled suggesting that proximodistal patterning of the optic vesicle into retina and RPE is disturbed. However, retinal differentiation capacity appears to be preserved since expression of neuronal markers is rescued when the preplacodal lens ectoderm is replaced by a source of FGF (Hyer *et al.*, 1998).

This finding was interpreted to mean that FGF derived from the lens ectoderm is necessary to maintain the retina domain in the distal optic vesicle (Hyer *et al.*, 1998). However, it is also possible that an unknown signal from the lens ectoderm activates FGF signaling in the presumptive retina. Moreover, studies in zebrafish and chick suggest that the ocular neuroepithelium itself may be an FGF signaling center that controls the onset and progression of retinal neurogenesis in the optic cup (Martinez-Morales *et al.*, 2005; McCabe *et al.*, 1999; Picker and Brand, 2005; Vinothkumar *et al.*, 2008). In contrast to chick, FGF/tyrosine receptor activation is not required for the progression of neurogenesis in the mouse central retina (Cai *et al.*, 2010). Thus, the signals driving neurogenesis may be distinct in different species.

MAPK FGF signaling also regulates retina specification, a function conserved in all vertebrates (Fig. 3.2B). Previously, it was shown that in FGF9 mouse mutants, the RPE expands into the presumptive retina suggesting that FGF9 helps to define the boundary between retina and RPE (Zhao *et al.*, 2001). While this result is informative, it is possible that redundant FGFs may compensate for the loss of FGF9. Furthermore, the precise ligands and receptors that regulate these processes are not known. To address this concern, Cai *et al.* (2010) analyzed the role of general FGF pathway activation during optic vesicle and optic cup morphogenesis by manipulating tyrosine phosphatase Src homology 2 (Shp2), which associates with FGF receptor tyrosine kinases and is required for complete activation of FGF signaling. Conditional inactivation of Shp2 at the early optic vesicle stage in mouse (before retinal specification) results in loss of Vsx2 expression in the distal portion. Instead, Mitf expression persists, and the affected part of the optic cup acquires RPE-like morphology and becomes pigmented (Cai *et al.*, 2010). Further analysis shows that Shp2 acts downstream of FGF; ectopic Ras activation can genetically rescue retinal development in Shp2 mutant eyes. This is the first evidence for a direct requirement of FGF signaling in retina specification in the optic vesicle.

This role for FGF was also shown using a different approach. Strikingly, gain-of-functions studies in frog, chick, and rodents demonstrate that

activation of the MAPK FGF pathway can cause the presumptive RPE to transdifferentiate into retina with fully differentiated cell types, such as ganglion cells and photoreceptors (Galy *et al.*, 2002; Guillemot and Cepko, 1992; Hyer *et al.*, 1998; Mochii *et al.*, 1998; Nguyen and Arnheiter, 2000; Park and Hollenberg, 1989; Pittack *et al.*, 1991, 1997; Reh *et al.*, 1987; Sakaguchi *et al.*, 1997; Spence *et al.*, 2007; Vergara and Del Rio-Tsonis, 2009; Vogel-Hopker *et al.*, 2000; Yoshii *et al.*, 2007; Zhao *et al.*, 1995, 2001). The finding that FGF cannot induce transdifferentiation of RPE into retina in optic vesicle cultures of Vsx2 null mutant mice shows that Vsx2 is required to mediate the effect of FGF either directly or indirectly (Horsford *et al.*, 2005). Consistent with this, loss of Vsx2 gene activity mimics FGF loss-of-function, resulting in a retina-to-RPE transdifferentiation (Horsford *et al.*, 2005; Rowan *et al.*, 2004). Vsx2 may act by directly suppressing transactivation of the *Mitf* gene in the distal optic vesicle (Bharti *et al.*, 2008). This would put FGF in a single pathway upstream of Vsx2, which then acts to repress Mitf expression, allowing the distal optic vesicle to develop into retina (Fig. 3.2B).

BMP signaling may also participate in early steps of retina development. This hypothesis is supported by the finding that BMP7 null mice display varying incidents of microphthalmia or anophthalmia, depending on the genetic background. In anophthalmic BMP null mice, the expression of retina-specific genes are downregulated in the optic cup, with concomitant ectopic expression of RPE genes such as Mitf (Morcillo *et al.*, 2006). While this phenotype could be due to a failure of lens induction (see below), the possibility that BMP signaling cell autonomously induces or maintains expression of retina genes in the distal optic vesicle cannot be excluded. Bolstering this idea, Murali *et al.* (2005) showed that in compound mutant mice with homozygous inactivation of BMP receptor types Ia and Ib, Vsx2 expression is downregulated in the optic cup and retinal neurogenesis fails to initiate. While Vsx2 expression was not analyzed at earlier stages, retinal specification may be already disturbed in the distal optic vesicle since, for example, the retina-specific marker FGF15 is not expressed in the mutant eye. Therefore, BMP signaling may be involved in retina specification or maintenance of the retina domain in the mouse optic vesicle.

4. Optic Cup and Lens Morphogenesis

The distal portion of the optic vesicle makes contact with the overlying surface ectoderm, resulting in the specification of the lens ectoderm (preplacodal stage). This interaction leads to invagination of the lens placode and distal optic vesicle resulting in formation of a bilayered optic cup

(Figs. 3.1B and 3.3). The neural retina and RPE develop from the inner and outer layer of the optic cup, respectively. The lens vesicle eventually separates from the surface ectoderm and differentiates into the mature lens. In this section, we will discuss recent progress that has been made with respect to the process of optic vesicle invagination and the role of tissue–tissue interactions mediated by extracellular factors.

4.1. Coordination of cell shape changes during morphogenesis

Following optic vesicle evagination, the distal optic vesicle and lens ectoderm (lens pit) invaginate, forming the optic cup. The mechanics behind the morphogenesis of invagination are just beginning to be understood. Work in different organisms suggests that there are various ways to generate force and tension that are integral to invagination. A study conducted in chick indicates that invagination is a Ca^{2+}-dependent process. The authors of this work suggest that "apical bands of microfilament" exist in the retinal cells that contract, thus generating the force to enable optic cup formation (Brady and Hilfer, 1982). In Medaka fish, a mutant, *ojoplano*, was recently identified that exhibits several morphogenesis defects, including improper invagination or folding of the optic cup (Martinez-Morales *et al.*, 2009). *Ojoplano* encodes a novel transmembrane protein with partial homology to a candidate gene for orofacial clefting syndrome, which is also associated with some eye abnormalities (Mertes *et al.*, 2009). In *ojoplano* mutants, the expression of focal adhesion proteins such as the integrin beta1 receptor in the basal surface of the retina appeared reduced, which may cause reduced tension and a change in cellular shape in retinal progenitors (Martinez-Morales *et al.*, 2009). Since a partial optic cup is formed in these mutants, additional mechanisms may exist that regulate the process of invagination. Future studies will need to address what kind of signal initiates invagination and how conserved the particular mechanism directed by ojoplano protein is across vertebrates.

Filopodia also provide mechanical force necessary for invagination. During the coordinated invagination of the optic vesicle and lens pit, both tissues are in tight apposition. Recent studies revealed that basal filopodia that mostly originate from the lens ectoderm transiently tether the presumptive lens and retina to coordinate invagination of the lens pit (Chauhan *et al.*, 2009). Production of these filopodia is dependent on the Rho family member GTPase cdc42 and the cdc42 effector IRSp53, and a failure of filopdia formation leads to defects in lens pit invagination (Chauhan *et al.*, 2009). Further studies will likely discover additional mechanisms that aid in this dramatic morphogenetic process.

4.2. Tissue–tissue interactions important for optic cup morphogenesis

The process of invagination is dependent upon tissue–tissue interactions. When preplacodal ectoderm is ablated from the optic vesicle, distal optic vesicle invagination is perturbed, but the retina is specified (Hyer *et al.*, 2003). What is more, Vsx2 expression remains normal, indicating that patterning of the optic vesicle and invagination are independent processes (Hyer *et al.*, 2003). Conversely, if surface ectoderm ablation occurs later, at the lens placode stage, an optic cup forms without a properly formed lens (Hyer *et al.*, 2003; Smith *et al.*, 2009). These experiments show that invagination requires specific, precisely timed interactions between retina and surface/lens ectoderm.

4.3. Genes and signaling pathways

The discovery that preplacodal lens specification is critical to invagination of the distal optic vesicle is confirmed by genetic studies in mouse. Similar to the preplacodal lens ablation experiments, disruption of expression of the homeobox transcription factors Six3 or Pax6 or the HMG transcription factor Sox2 during the preplacodal stage results in failure of thickening of the lens placode and lens formation, and disrupted invagination that leads to arrest at the optic vesicle stage (Ashery-Padan *et al.*, 2000; Grindley *et al.*, 1995; Kamachi *et al.*, 1998; Smith *et al.*, 2009). Recent studies revealed that Six3 is expressed in the surface ectoderm before Pax6, and without Six3, Pax6 is downregulated and Sox2 never expressed (Liu *et al.*, 2006). Furthermore, using Chromatin immunoprecipitation, EMSA, and luceriferase reporter assays, it was demonstrated that Six3 directly activates Pax6 and Sox2 expression (Liu *et al.*, 2006). These and other findings indicate that Six3, Pax6, and Sox2 act in a complex regulatory network to regulate each other during lens induction and specification (Fig. 3.3; for review, see Donner *et al.*, 2006; Lang, 2004; Liu *et al.*, 2006).

Induction of Sox2 in lens ectoderm is indirectly controlled by the TGFβ family member BMP7, which signals either upstream or downstream of Pax6 (Fig. 3.3; Faber *et al.*, 2001; Furuta and Hogan, 1998; Gotoh *et al.*, 2004; Wawersik *et al.*, 1999). Different lines of mice with a null mutation in the *BMP7* gene can exhibit severe eye defects such as arrest at the optic vesicle stage and failure of lens formation (Dudley and Robertson, 1997; Morcillo *et al.*, 2006; Wawersik *et al.*, 1999). While some regional patterning of the optic vesicle appears normal (e.g., expression of Lhx2 and the RPE marker Dct), expressions of Sox2 and Pax6 are not maintained in the preplacodal lens ectoderm (Morcillo *et al.*, 2006; Wawersik *et al.*, 1999).

A role for BMPs in lens induction is also supported by the phenotype of Lhx2 mouse mutants. In these animals, eye development arrests at the optic

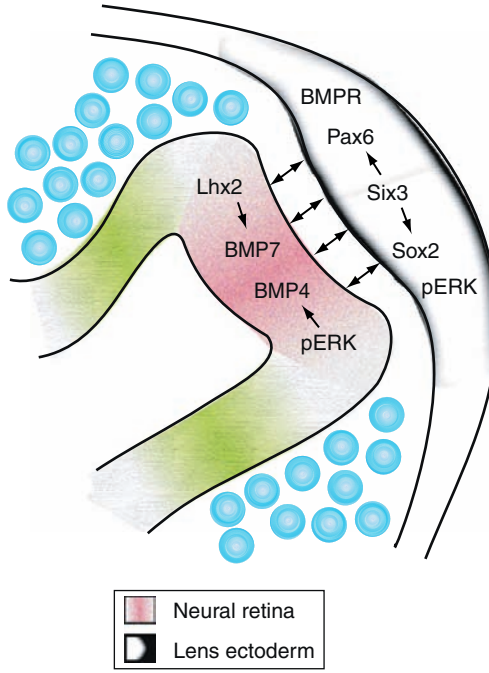

Figure 3.3 Optic cup morphogenesis. Invagination of the lens placode requires correct specification of the lens ectoderm that is dependent on Six3-mediated maintenance and activation of Pax6 and Sox2, respectively. FGF and BMP signaling may also be required for lens induction. In the distal optic vesicle, BMP4 and BMP7 expression is crucial for specification of the lens ectoderm and for optic vesicle invagination. BMP expression requires activation by Lhx2 and FGF signaling. Not all of the mechanisms involved in the invagination process are shown here (for further explanation, see text).

vesicle stage and the lens never forms (Porter *et al.*, 1997; Yun *et al.*, 2009). However, expression of Lhx2 in the surface ectoderm is not required, since lens-specific disruption of Lhx2 has no obvious effect (Yun *et al.*, 2009). What, then, is the source of the defect? Analysis of several pathways implicated in lens induction revealed that signaling downstream of BMP is specifically disrupted in the optic vesicle and in lens ectoderm. Further, in Lhx2 mutants, some but not all aspects of the eye phenotype can be rescued by treatment of Lhx2 mutant explants with exogenously added BMPs (Yun *et al.*, 2009). These results imply not only that BMP signaling regulates lens induction, but also indicates that other unknown factors are involved.

A potential downstream target of BMPs during lens induction is MAB21l2. MAB21l2 is similar to the *Caenorhabditis elegans* MAB-21 cell fate-determining gene, a downstream target of TGFβ signaling. In the developing eye, it is first expressed in the dorsal optic vesicle and expression

extends later, in the optic cup, to the RPE, retina, and optic stalk (Yamada *et al.*, 2004). In Mab21l2 null mice, the area of contact between the surface ectoderm and optic vesicle is reduced, and an optic cup and lens never forms, resulting in an eye rudiment. This phenotype is due in part to the fact that the optic cup displays no Vsx2 expression resulting in a severe proliferation defect (Yamada *et al.*, 2004). Other direct or indirect downstream targets of BMP signaling are not known and have yet to be determined.

FGF signaling is also required for Pax6 expression in the lens placode (Faber *et al.*, 2001; Gotoh *et al.*, 2004). This is demonstrated by the finding that disruption of the major docking protein FGF receptor substrate 2 alpha (FRS2alpha), that links FGFR2 with several downstream targets, can result in defective invagination of the optic vesicle. The concomitant loss of BMP expression in the optic vesicle may enhance the eye phenotype (Gotoh *et al.*, 2004). Defective invagination may be due to reduced FGF signaling in the lens ectoderm, since a significant reduction of Shp2 activation in the distal optic vesicle does not interfere with lens induction (Cai *et al.*, 2010). Interestingly, it has been suggested that retina-derived N-cadherin could act as an alternative ligand for FGF receptor signaling in the lens (Smith *et al.*, 2010). However, the source of the actual FGF receptor ligand(s) is not clear and it is possible that a high degree of redundancy exists.

In addition, retinoic acid (RA) signaling is required for optic cup morphogenesis (for review, see Duester, 2009). Retinaldehyde dehydrogenases (Raldh1, 2, 3) mediate the final step of retinoic acid synthesis. Raldh2 is present in the mesenchyme and Raldh3 is expressed in the RPE (Molotkov *et al.*, 2006). Both enzymes synthesize retinoic acid, providing an essential signal to the neural retina required for morphogenetic movements that lead to ventral invagination of the optic cup (Molotkov *et al.*, 2006). Retinoic acid is further required to induce apoptosis in the extraocular mesenchyme, and one target gene in the extraocular mesenchyme is the transcription factor Pitx2, which is also required for RPE differentiation (Gage and Zacharias, 2009; Gage *et al.*, 1999; Matt *et al.*, 2005). These and other findings indicate that retinoic acid synthesis and signaling is complex during development of the eye (Duester, 2009).

5. RPE Maintenance in the Optic Cup

Subsequent to initial establishment of the RPE in the optic vesicle, proliferation in the presumptive RPE ceases, leading to the formation of a single layer of cuboidal cells that become pigmented. As development proceeds, a period of differentiation and further maturation follows that results in dramatic morphological, structural, and functional changes of the RPE tissue such as formation of tight junctions, expansion of apical microvilli and invagination of the basal membrane, establishment of polarity and

retinoid recycling machinery (Burke and Hjelmeland, 2005; Finnemann, 2003; Marmorstein, 2001; Marmorstein *et al.*, 1998; Rizzolo and Kwang, 2007; Strauss, 2005). The RPE fate is reversible for several days following the initial activation of differentiation as evidenced by a propensity to hyperproliferate and to differentiate into retina, for example, by treatment with FGF (Stroeva, 1960; Zhao *et al.*, 1997). These results suggest that maintenance of the RPE fate is controlled by the concerted effort of multiple factors during this prolonged period (Fig. 3.4).

The sonic hedgehog (shh) signaling pathway is required for maintenance of RPE fate in the ventral optic cup. In chick and mouse, reduced shh signaling may not affect RPE specification but results subsequently in loss of RPE marker expression, increased proliferation of the RPE, and transdifferentiation into retina (Huh *et al.*, 1999; Zhang and Yang, 2001). RPE differentiation defects are also observed in frog, when shh signaling is downregulated (Perron *et al.*, 2003). Growth arrest specific 1 (Gas1), a GPI-anchored cell surface protein that binds shh, may be a positive coregulator of shh signaling (Allen *et al.*, 2007; Lee *et al.*, 2001b; Martinelli and Fan, 2007). Disruption of Gas1 results in RPE defects that are very similar to effects caused by defective shh signaling as described above, for example, ectopic proliferation and transdifferentiation into retina (Lee *et al.*, 2001a). In Gas1 mutants, RPE specification occurs normally in the optic vesicle, but subsequently in the optic cup, the ventral RPE fails to slow down proliferation. This proliferation defect precedes transdifferentiation into retina, suggesting that Gas1 is required for downregulation of proliferation in the ventral RPE (Lee *et al.*, 2001a). The dorsal RPE in Gas1 mutants is not affected; therefore, differentiation in the dorsal and ventral RPE may be controlled by distinct mechanisms.

Other pathways involved in the maintenance of the RPE in the ventral optic cup are retinoic acid and BMP signaling (Fig. 3.4). Retinoic acid signal transduction occurs via the retinoic acid receptors (RARα, β, γ) that bind to RXR receptors and form heterodimers when bound to the RA response element in target genes. RAR mutants display a range of severe eye defects, including microphthalmia and transdifferentiation of RPE into retina (Lohnes *et al.*, 1994; Matt *et al.*, 2008). Furthermore, disruption of BMP signaling by overexpression of the BMP antagonist noggin caused transdifferentiation of the ventral RPE (Adler and Belecky-Adams, 2002a). These findings further support the notion that dorsal and ventral RPE development is regulated by distinct mechanism.

Recent work demonstrates that the Wnt/β-catenin pathway also controls differentiation of the RPE in the optic cup. In brief, activation of the Wnt/β-catenin pathway results in cytoplasmic stabilization of β-catenin, ultimately converting TCF/LEF transcription factors from repressors into activators (Nusse, 2009). In the differentiating zebrafish, chick, and mouse RPE, TCF/LEF-responsive reporters are activated, and several pathway

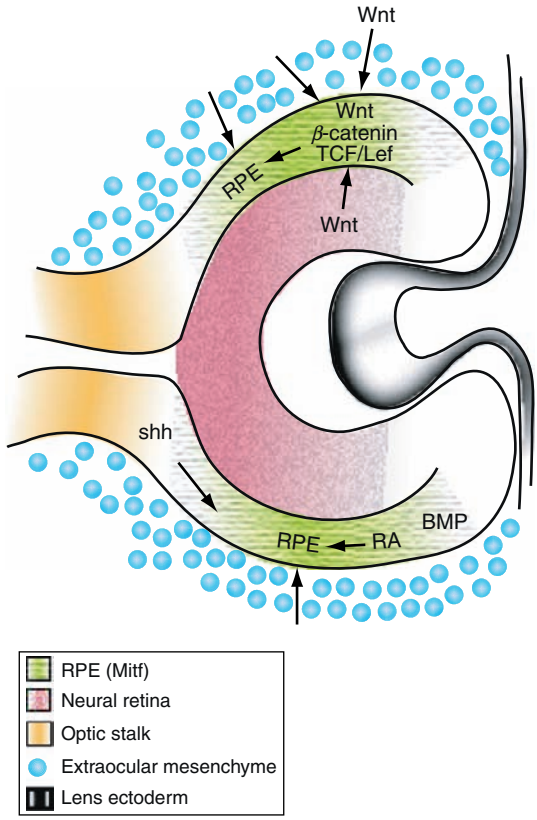

Figure 3.4 Maintenance of the RPE in the optic cup. Several signaling pathways can regulate maintenance of cell fate in the presumptive RPE. For explanation, see text.

components are expressed during RPE development (Chang *et al.*, 1999; Cho and Cepko, 2006; Dorsky *et al.*, 2002; Lee *et al.*, 2006; Liu *et al.*, 2003; Wang *et al.*, 2001; Yasumoto *et al.*, 2002). We and others observed that interference with Wnt/β-catenin signaling in chick and mouse RPE of the optic cup causes loss of TCF/LEF reporter activation and severe eye defects such as microphthalmia and transdifferentiation of the RPE into retina (Fujimura *et al.*, 2009; Westenskow *et al.*, 2009, 2010).

Further analysis using chromatin immunoprecipitation and transactivation assays revealed that a complex of β-catenin/TCF/LEF binds to and activates those Mitf and Otx2 enhancers that regulate expression in the RPE (Fujimura *et al.*, 2009; Westenskow *et al.*, 2009, 2010). It was in this context that it was recently shown that the orphan receptors COUPTFI (Nr2f1) and perhaps COUPTFII (Nr2f2) of the steroid/thyroid hormone receptor

superfamily are also directly regulated by β-catenin/TCF/LEF (Fujimura *et al.*, 2009). In agreement, double mutant COUPTFI/II receptor mice show severe developmental abnormalities at the optic cup stage, including transdifferentiation of the RPE into retina (Tang *et al.*, 2010). COUPTFs can directly regulate expression of Otx2. Thus, expression of the RPE key determinants Otx2 and Mitf is regulated by several mechanisms that may act in parallel and may reinforce each other (Fig. 3.4).

Perhaps surprisingly, ectopic activation of the Wnt/β-catenin pathway in the presumptive retina is not sufficient to induce RPE cell fate. Instead, it acts in the peripheral eye to maintain undifferentiated progenitor cells and to promote differentiation into ciliary body and iris epithelium (Cho and Cepko, 2006; Kubo *et al.*, 2003, 2005; Liu *et al.*, 2007). The reason behind the insufficiency of Wnt/β-catenin to induce RPE may lie in our observation that overexpression of Otx2 and Wnt/β-catenin signaling can promote ectopic Mitf expression in the developing chick retina (Westenskow *et al.*, 2010). Thus, Otx2 may be a required cofactor that confers competence to respond to RPE promoting signals such as Wnt/β-catenin signaling.

6. Concluding Remarks

In summary, several intrinsic and extracellular factors control different aspect of eye organogenesis. Though a lot of progress has been made demonstrating how these signals are connected in a network, in many cases, we do not know whether this regulation is direct, or how these interactions work at the molecular level. We also have still little insight into the actual output of these interactions. Finally, some processes during early eye development are still a mystery. For example, it is not clear what kind of mechanism ultimately drives invagination of the distal optic vesicle, or whether additional factors are required to ensure specification of the retina in the optic vesicle. Thus, future work will need to address these and other questions.

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CHAPTER FOUR

Two Themes on the Assembly of the *Drosophila* Eye

Sujin Bao

Contents

Abstract

Cells are sequentially recruited during formation of the *Drosophila* compound eye. A few simple rules are reiteratively utilized to control successive steps of eye assembly. Two themes emerge: the interplay between cell signaling and competence determines diversity of cell types and selective cell adhesion determines spatial patterns of cells. Cell signaling through competence creates signaling relays, which sequentially trigger differentiation of all cell types. Selective cell adhesion, on the other hand, provides forces to drive cells into

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energy-favored spatial configurations. Organ formation is nevertheless a complex process. The complexity lies in the spatial, temporal, and quantitative precision of gene expression. Many challenging questions remain.

1. Introduction

Making cells different from one another and assigning them to the right places are central to organ formation. Recent advances in understanding organogenesis using model organisms have highlighted a few simple rules that govern formation of complex organs. This has been well illustrated in the *Drosophila* visual system, the compound eye. Two themes emerge: the interplay between cell signaling and cell competence generates diversity of cell types and selective cell adhesion determines diversity of cellular patterns.

The *Drosophila* compound eye is comprised of ~750 repetitive unit eyes or ommatidia arranged in a crystalline hexagonal array. The eye derives from the eye-imaginal disc which appears as a group of approximately 20 cells invaginated in the late embryo (Garcia-Bellido and Merriam, 1969). The eye disc grows and proliferates throughout the first and second larval instars. Cell differentiation begins in the posterior margin of the eye disc in the third larval instar and sweeps across the disc in a wave toward the anterior edge. The wave of differentiation is marked by the morphogenetic furrow, an indentation that arises from apical constriction of the eye epithelium (Ready, 1989). Within the furrow, the first photoreceptor neuron R8 is selected at evenly spaced positions. Behind the furrow, five-cell ommatidial preclusters emerge: photoreceptor R2 and R5 are recruited, followed by photoreceptors R3 and R4. Cells in-between preclusters undergo the second mitotic wave, an additional round of synchronized cell division. The second mitotic wave generates a pool of precursor cells, from which all remaining cell types arise. Photoreceptors R1 and R6 are recruited next, followed by R7 and nonneuronal cone cells. At an early pupal stage, bristle mother cells, also known as sensory organ precursors (SOPs), undergo cell division, giving rise to four daughter cells for assembling each bristle group. Shortly, primary pigment cells (1°s) are selected. While 1°s are differentiating, interommatidial cells (IOCs) undergo cell rearrangement that eventually gives rise to a one-cell wide hexagonal lattice, comprised of secondary and tertiary pigment cells with bristle complexes at alternate vertices (Fig. 4.1).

Advances made in genetics and developmental biology in the past two decades have brought two old concepts, "positional information" and "differential adhesion," back into our attention. It has been postulated for a century that positional information each cell receives determines the cell

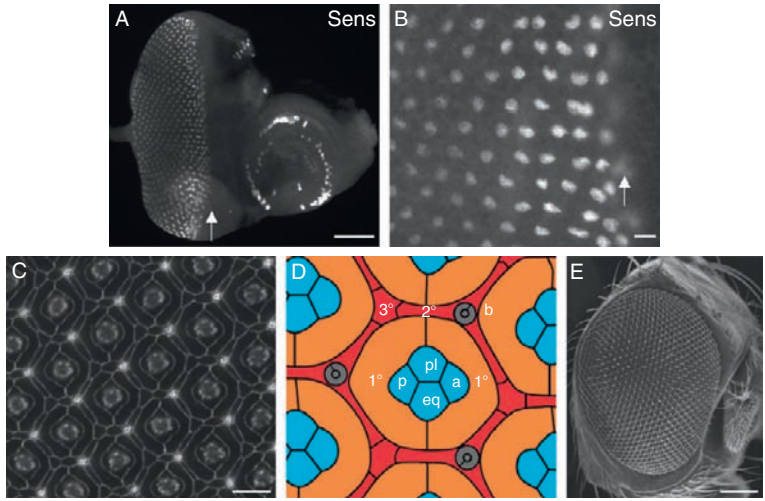

Figure 4.1 The *Drosophila* eye. (A) The R8 photoreceptor neurons marked by Senseless (Sens) are evenly spaced in the eye disc. A third instar larval eye disc was stained with an anti-Senseless antibody. Scale bar, 100 μm. A high magnification view is shown in (B). Bar, 10 μm. The morphogenetic furrow is indicated by an arrow. (C) Ommatidia are separated by interommatidial cells (IOCs) and form a precise hexagonal array in the pupal eye. An eye at 40 h APF was stained with an anti-E-cadherin antibody. Bar, 10 μm. Cell types visible on the apical surface are indicated in (D). Anterior (a), posterior (p), polar (pl), and equatorial (eq) cone cells are marked in blue. 1°, 2°, and 3° are primary (yellow), secondary (pink), and tertiary pigment cells (pink), respectively. b, bristle. (E) Regular spacing of ommatidia is maintained in the adult eye. A scanning electron microscopic (SEM) micrograph shows an adult eye. Ommatidia form a precise hexagonal array. Bar, 100 μm. Anterior is to the right in this and all subsequent figures.

identity (Wolpert, 1996). What is the molecular nature of positional information? How do cells interpret and respond to this information? Similarly, it has been noted that living cells tend to aggregate into a configuration mimicking nonliving objects that follow the basic principle of physics (Thompson, 1917). Studies of reaggregation of dissociated embryonic cells have culminated in the differential adhesion hypothesis (DAH): differential cell adhesion determines the relative position of cells within the embryos (Steinberg, 1970). How does cell adhesion generate and maintain diverse spatial configurations of cells seen in developing organs? Studies using model organisms have made substantial contributions to our understanding of animal development. The power of fly genetics along with accessibility to manipulation at single-cell resolution has made the *Drosophila* compound eye a unique model in addressing these questions. Studies of the *Drosophila* eye in the past two decades have uncovered some basic principles governing organ formation.

2. Interplay Between Cell Signaling and Competence Generates Diversity of Cell Types

Cell signaling mediated by a small number of signaling pathways is required for multiple cell fate decisions in the *Drosophila* eye (Freeman, 1997; Nagaraj and Banerjee, 2004; Roignant and Treisman, 2009; Voas and Rebay, 2004). These signaling pathways do not seem to be specific since they are utilized to select a variety of cell types, raising the question of how cell type specificity is generated. Specification of cell fate also depends on competence, the ability of a cell to respond to inductive signals (Waddington, 1940). Competence is not a passive property of cells. Instead, it is actively achieved by a complex of reactions between substances that form an unstable mixture, which at certain times an inducer can push to one equilibrium or another (Waddington, 1940). We now understand that substances that determine competence include intrinsic factors that control the response of the cell to stimuli by transcriptional and posttranscriptional mechanisms. Advances made in the *Drosophila* eye in the past two decades support the notion that the interplay between cell signaling and competence determines cell type specificity. Competence determines how a cell responds to a signal at a given time; cells with different competence can respond to the same signaling differently. Cell signaling, on the other hand, can alter competence. As a result, competence evolves as development proceeds. In the eye, the interplay between competence and cell signaling creates three major signaling relays: the signaling relay mediated by Hedgehog (Hh) in progression of the morphogenetic furrow, by the epidermal growth factor receptor (EGFR) in sequential recruitment of non-R8 photoreceptors, and by Notch in recruitment of support cells. In all these cases, a competent cell first receives signals from neighboring differentiating cells and becomes specified and then determined (Fig. 4.2). In return, a differentiating cell influences fate decisions of neighboring competent cells by sending signals. The signaling relays trigger differentiation of all cell types (Fig. 4.2).

2.1. Initiation of the morphogenetic furrow

The eye field is set up by a group of retinal determination genes during the first and second instars (Kumar, 2009; Pappu and Mardon, 2004; Silver and Rebay, 2005). In the third instar lava, the morphogenetic furrow is initiated in the posterior margin of the eye-imaginal disc and sweeps across the eye disc toward the anterior margin in a wave. Emergence of the furrow is a landmark event in eye development that marks the beginning of photoreceptor differentiation.

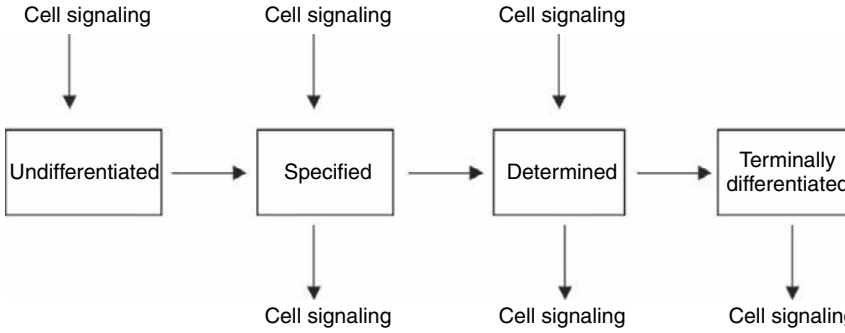

Figure 4.2 A signaling relay during organ formation. During differentiation, cells in a developing organ are first specified and then determined. Some cells also undergo terminal differentiation before maturation. During this process, naïve cells first receive and then send signals, which creates a signaling relay. Following the definition of cell fate specification and determination (Wolpert *et al.*, 1998), a cell is specified if it can differentiate into a particular fate in a neutral environment. By contrast, a cell is determined if it can differentiate into a particular fate even when grafted into a new location within the developing organ.

Initiation of the morphogenetic furrow requires combinatorial cell signaling. Several signaling molecules have been implicated in this process including Hh, Decapentaplegic (Dpp), Unpaired (Upd), Delta (Dl), Spitz and Wingless (Wg), and ecdysone (Roignant and Treisman, 2009). Ecdysone is the major growth and molting hormone of *Drosophila*. Dl is a membrane-bound signaling ligand and all others secreted signaling proteins that activate different signaling pathways through the corresponding receptors. If a positive regulator and a negative regulator are simply referred to as an activator and a repressor, respectively, these signaling molecules can be divided into two groups: the activator group and inhibitor group. Hh, Dpp, Upd, Dl, Spitz, and ecdysone are in the activator group, while Wg belongs to the inhibitor group. Within the activator group, Hh plays a central role in the furrow initiation. Hh is expressed at the center of the posterior margin in the second instar eye disc (Borod and Heberlein, 1998; Cho *et al.*, 2000; Dominguez and Hafen, 1997; Royet and Finkelstein, 1997). In the absence of Hh, the furrow fails to form and the subsequent neural differentiation is blocked (Dominguez and Hafen, 1997; Heberlein *et al.*, 1993). As a result, no eye develops. Conversely, ectopic expression of Hh in the anterior region of the eye disc can initiate an ectopic furrow (Heberlein *et al.*, 1995). Dpp, a target of Hh, is essential for furrow initiation (Borod and Heberlein, 1998; Heberlein *et al.*, 1993; Royet and Finkelstein, 1997). Dpp is expressed along the posterior and lateral margins of the second instar eye disc (Blackman *et al.*, 1991; Ma *et al.*, 1993; Treisman and Rubin, 1995). Initiation of the furrow also requires ecdysone (Niwa *et al.*, 2004). Ecdysone is present throughout *Drosophila* development (Ashburner, 1989). In contrast, Wg functions as an

inhibitor for furrow initiation. *wg* is expressed in the lateral margins complementary to Hh and Dpp. Loss of *wg* leads to an ectopic furrow (Ma and Moses, 1995; Treisman and Rubin, 1995). Ectopic Wg signaling blocks photoreceptor differentiation (Baonza and Freeman, 2002; Royet and Finkelstein, 1997). Signaling activators and inhibitors antagonize each other during furrow initiation. For example, Dpp antagonizes Wg and vice versa (Ma and Moses, 1995; Royet and Finkelstein, 1997; Treisman and Rubin, 1995; Wiersdorff *et al.*, 1996). Upd promotes furrow initiation by inhibiting *wg* expression (Ekas *et al.*, 2006; Tsai *et al.*, 2007).

Initiation of the furrow also depends on competence. Signaling activators such as Hh and Dpp are present in the posterior margin of the eye disc in second and early third instar larvae. However, the furrow is not initiated until mid-third instar, indicating presence of these signaling molecules *per se* is not sufficient to trigger formation of a furrow. In contrast, in the third instar, ectopic expression of Hh in cells anterior to the furrow initiates a new furrow (Heberlein *et al.*, 1995), indicating that cells at this stage acquire competence to generate a furrow in response to Hh signaling, and competence is not only restricted to the posterior margin of the eye disc. What intrinsic factors determine cell competence? If we broaden the definition of activator and inhibitor to cover intrinsic factors, both activators and inhibitors are present in the eye field at the time when the furrow emerges. Transcription factors Homothorax (Hth) and Extradenticle (Exd) are two inhibitors. Both *hth* and *exd* genes are transcribed in undifferentiated cells anterior to the furrow. Mutant clones for either *hth* or *exd* in the anterior region of the eye disc transform this region into an ectopic eye (Gonzalez-Crespo and Morata, 1995; Pai *et al.*, 1998), indicating that Hth and Exd prevent cells from neural differentiation. The activator group include four transcription factors: Eyes absent (Eya), Sine oculis (So), Dachshund (Dac), and Eye gone (Eyg). Genes coding for these proteins are transcribed at the posterior margin in the eye disc in-between early and late second instars (Bonini *et al.*, 1993; Cheyette *et al.*, 1994; Jang *et al.*, 2003; Mardon *et al.*, 1994). The furrow fails to initiate in mutant clones for either of these genes (Jang *et al.*, 2003; Mardon *et al.*, 1994; Pignoni *et al.*, 1997). Conversely, ectopic expression of Eya, Dac, or Eyg leads to ectopic eye structures in other organs (Bonini *et al.*, 1997; Jang *et al.*, 2003; Shen and Mardon, 1997). Coexpression of Eya with either Dac or So has a much stronger effect than expression of either factor alone (Chen *et al.*, 1997, 1999; Pignoni *et al.*, 1997). Intrinsic activators and repressors antagonize each other during furrow initiation. For example, Hth and Exd block transcription of *eya* and *dac* (Bessa *et al.*, 2002). As discussed below, activators Eya and Eyg can also repress *hth* indirectly via Dpp signaling.

Cells signaling and competence regulate one another. On one hand, cell signaling alters competence. For example, Hh in part via Dpp signaling activates *eya*, *so*, and *dac* and represses *hth* expression (Bessa *et al.*, 2002; Chen

et al., 1999; Curtiss and Mlodzik, 2000). Hh signaling also activates *eya* via a Dpp-independent mechanism (Fu and Baker, 2003; Pappu *et al.*, 2003). Notch signaling is activated at the dorsal/ventral midline, which induces *eyg* expression (Chao *et al.*, 2004). In contrast, Wg signaling promotes *hth* but inhibits *eya*, *so*, and *dac* expression (Baonza and Freeman, 2002; Lee and Treisman, 2001). Cell competence, in return, also controls cell signaling. *hh* is activated at the center of the posterior margin by the Odd-skipped family Zinc-finger transcription factors in the second instar (Bras-Pereira *et al.*, 2006). In addition, *hh* transcription is directly controlled by So and the ETS (E-twenty six) domain transcription factor Pointed (Pnt) (Pauli *et al.*, 2005; Rogers *et al.*, 2005). *upd* is induced by *eyg* in the posterior region of the eye disc (Chao *et al.*, 2004). Further, *eya* and *eyg* activate *dpp* but repress *wg* expression (Hazelett *et al.*, 1998). Therefore, the interplay between signaling and competence generates two major activation stripes: one along the posterior margin marked by Dpp expression and the other at the dorsal/ventral midline coincided with Notch activation. These two stripes converge at the center of the posterior margin of the third instar eye disc, which becomes the furrow initiation site. Then, what determines timing for furrow initiation? Both signaling and intrinsic activators are already present in the late second and early third instar eye disc but the furrow is not initiated until the mid-third instar, raising the possibility that emergence of the furrow is not an abrupt event triggered by a single factor. Rather, it is a gradual process, and the interplay between cell signaling and competence drives the system to reach a critical point where activation overcomes repression, leading to birth of the furrow.

2.2. Progression of the morphogenetic furrow

Once the furrow is initiated, the eye disc can be divided into four zones with distinct competence for neural differentiation (Pappu and Mardon, 2004). Cells in the undifferentiated zone remain in a ground state: cells are undifferentiated and they undergo proliferation. These cells are marked by expression of Hth and Exd, two inhibitors for photoreceptor differentiation (Fig. 4.3). Cells in preproneural zone cease proliferation. They lose inhibitors Hth and Exd but gain new inhibitors such as Hairy and Extramacrochaete (Emc) Neural differentiation still does not occur. In the proneural zone (in the furrow), the inhibitors in the preproneural state are downregulated and cell gain full competence for neural differentiation. Competent cells express Atonal, a basic helix-loop-helix (bHLH) transcription factor and an early marker for neural differentiation (Jarman *et al.*, 1994, 1995). Within the proneural zone, the first neuronal cell type, the R8 photoreceptor, is selected. In the differentiating zone, R8 photoreceptors are specified and determined, followed by differentiation of all other photoreceptor neurons and support cells.

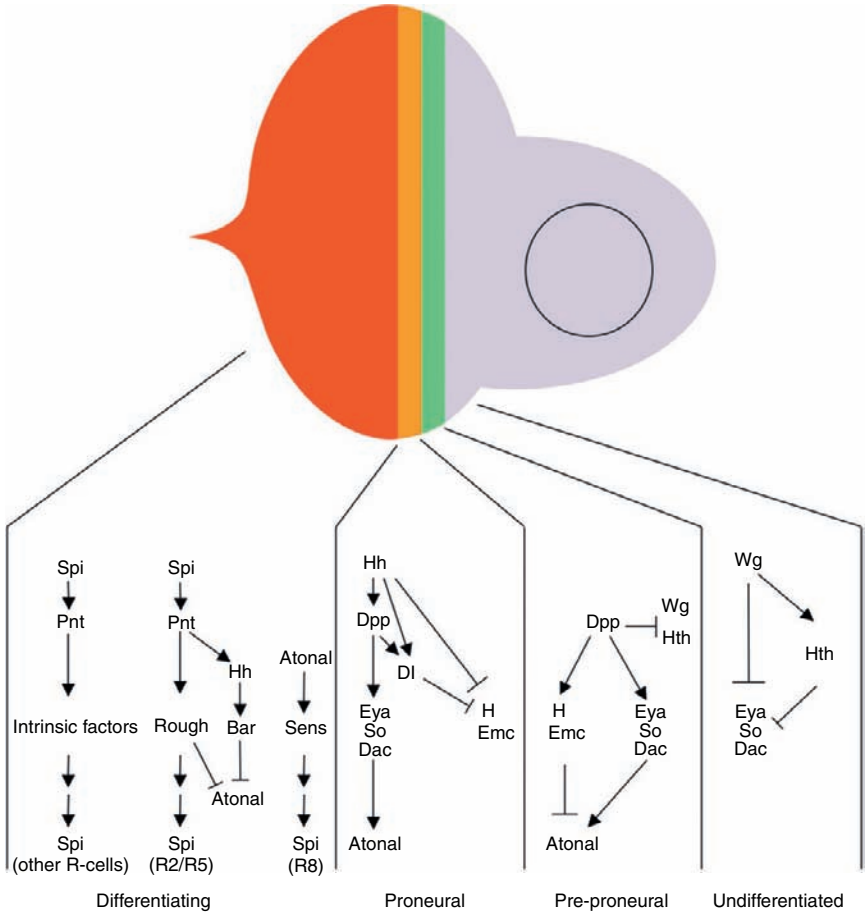

Figure 4.3 The interplay between cell signaling and competence determines the position of the morphogenetic furrow. After the furrow (yellow) is initiated, the eye disc can be roughly divided into four zones, corresponding to four different states of competence for neural differentiation. In front of the furrow, Wg signaling along with intrinsic inhibitors keeps cells in the undifferentiated state (gray). Cells receiving Dpp signaling undergo transition from the undifferentiated to pre-proneural state (green). However, these cells gain new inhibitors and Atonal is not yet expressed. Within the proneural zone (the furrow), cells receive both Dpp and Dl and gain full competence for neural differentiation marked by expression of Atonal. In the differentiating zone, Atonal promotes production of Spitz in R8s. Spitz triggers EGFR signaling, which induces Hh. EGFR and Hh via intrinsic regulators turn off Atonal. Emc, Extramacrochaete; H, Hairy; Pnt, pointed; Spi, Spitz. See text for a more detailed description of relevant genes.

A transition from one state to another is triggered by cell signaling (Fig. 4.3). Hh, a short-range signaling molecule, is a central regulator of furrow progression (Heberlein *et al.*, 1993; Ma *et al.*, 1993). Hh is produced by cells in the differentiating zone and acts on cells in the proneural zone. Hh signaling induces Dpp and Dl within and ahead of the furrow (Baonza and Freeman, 2002; Blackman *et al.*, 1991; Greenwood and Struhl, 1999). Dpp is a long-range signaling molecule that acts several cell diameter lengths ahead of the furrow. Dpp signaling downregulates Hth and Wg, inhibitors for neural differentiation, and upregulates Eya, So, and Dac (Bessa *et al.*, 2002; Chen *et al.*, 1999; Curtiss and Mlodzik, 2000; Ma and Moses, 1995; Treisman and Rubin, 1995). Dpp signaling transforms undifferentiated cells into a preproneural state. However, neural differentiation does not occur in the preproneural zone due to the presence of inhibitors such as Hairy and Emc (Brown *et al.*, 1995; Greenwood and Struhl, 1999). The last block is lifted by Dl, another target of Hh, from the proneural zone (Baker and Yu, 1997; Baonza *et al.*, 2001; Baonza and Freeman, 2005). Activation of Notch signaling in cells within the preproneural zone transforms these cells into a proneural state marked by expression of Atonal. In this transition, *Notch* promotes proneural gene expression, a role of *Notch* known as "proneural enhancement" (Baker and Yu, 1997). R8 precursors are selected within the proneural zone and prolonged expression of Atonal in R8 precursors leads to expression of Senseless (Sens), which counteracts inhibition of the R8 fate by intrinsic inhibitors such as Rough and consolidates the R8 fate (Pepple *et al.*, 2008). After the founder R8 is selected, Atonal is turned off. At least two signaling pathways via two negative regulators of Atonal are involved in repressing Atonal in the differentiating zone: EGFR and Hh induce Rough and Bar, respectively (Dokucu *et al.*, 1996; Dominguez, 1999; Lim and Choi, 2003; Lim *et al.*, 2004; Pepple *et al.*, 2008). In the differentiating zone, Atonal in differentiating R8s promotes production of Spitz, which activates EGFR in neighboring cells (Wasserman *et al*, 2000). EGFR signaling induces Hh (Rogers *et al.*, 2005), creating a relay of Hh signaling (Fig. 4.4). Interference of this relay mechanism, for example, through forced expression of either Rough, BarH1, or activated Ras1, blocks progression of the furrow (Basler *et al.*, 1990; Hayashi and Saigo, 2001; Kojima *et al.*, 1991).

2.3. Emergence of the R8 photoreceptors

Atonal is a key factor that confers the R8 potential on preproneural cells. Ectopic Atonal gives rise to extra R8 cells (Dokucu *et al.*, 1996). Conversely, in the absence of Atonal, no R8 develops and subsequent photoreceptor differentiation is blocked (Jarman *et al.*, 1994, 1995). Initially Atonal is expressed in a uniform dorsoventral stripe at the front of the morphogenetic furrow (Dokucu *et al.*, 1996; Jarman *et al.*, 1995). Shortly the stripe pattern

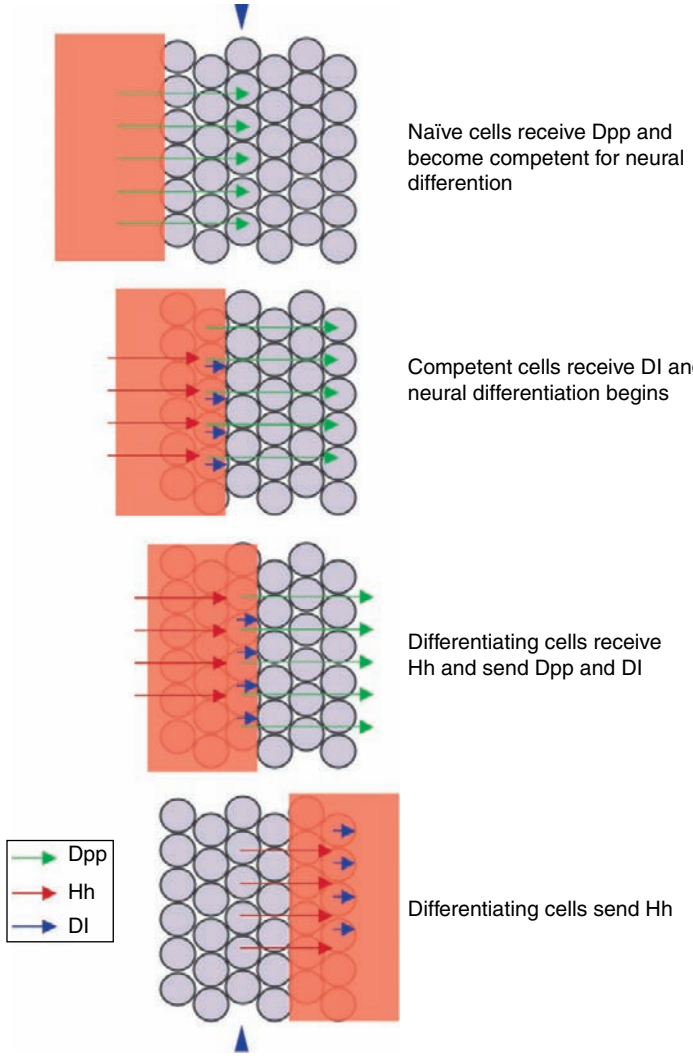

Figure 4.4 A relay of Hh signaling maintains progression of the furrow. The morphogenetic furrow is depicted by red blocks. An arrowhead points to the row of cells of which competence changes over time. Three major signaling molecules that act in different ranges are represented by arrows: Hh (red), Dpp (green) and Dl (blue).

resolves into evenly spaced clusters of approximately 15 cells known as the intermediate group. Within each intermediate group, the nuclei of 2–3 cells migrate apically, and these cells become the "R8 equivalence group" (Dokucu *et al.*, 1996). In less than 2 h, Atonal is refined to single cells, the

presumptive R8. Currently there are two models concerning the emergence order of the intermediate group and R8 equivalence group. In the first model, the intermediate group appears first, and then Atonal expression is further narrowed down to 2–3 cells in the R8 equivalence group (Lee *et al.*, 1996; Roignant and Treisman, 2009). In the second model, emergence of the R8 equivalence group precedes the intermediate group or the proneural cluster, and therefore each R8 equivalence group establishes an intermediate group (Spencer *et al.*, 1998). To date, there is a lack of clear experimental evidence to distinguish these models. Nevertheless, both models are compatible with dynamic Atonal expression at current resolution, which can be broadly divided into two steps correlated with two distinct underlying mechanisms (Baker and Zitron, 1995). In the first step, a uniform stripe is broken up into evenly spaced clusters. Breaking up the uniform stripe of Atonal marks the beginning of ommatidial patterning and lays the foundation for the hexagonal array of ommatidia seen in the adult eye. This step is controlled by a combination of nonautonomous center-outward inhibitory signals and a positive autoregulatory feedback loop. In the second step, Atonal expression within each cluster resolves into a single cell. The second step is controlled by lateral inhibition mediated by Notch (Baker and Zitron, 1995).

Scabrous (Sca) is a center-outward inhibitory signal. Sca is expressed in a subset of cells within each intermediate group and is maintained at high levels later in R8 precursors (Baker *et al.*, 1990, 1996; Lee *et al.*, 1996). In *sca* mutants, spacing of intermediate groups becomes irregular (Baker and Zitron, 1995; Baker *et al.*, 1990; Lee *et al.*, 1996). Interestingly, misexpression of *sca* also disrupts intermediate group spacing (Ellis *et al.*, 1994). Sca expression depends on Atonal and ceases shortly after *atonal* expression is shut off after the furrow (Lee *et al.*, 1996). Therefore, Atonal expression within intermediate groups promotes expression of Sca, which in turn represses Atonal outside the groups through a nonautonomous feedback loop. How Sca functions in mediating intermediate group spacing has remained unclear. Sca is capable of binding Notch (Li *et al.*, 2003; Powell *et al.*, 2001). However, intermediate groups are spaced normally in *Notch* mutants (Baker and Zitron, 1995; Lee *et al.*, 1996), suggesting that additional mechanisms are also involved in mediating the inhibitory function of Sca.

EGFR signaling has been suggested to provide another center-outward inhibitory signal. The role of EGFR in intermediate group spacing has been debated. While some evidence suggests that EGFR plays no role in intermediate group spacing (Kumar *et al.*, 1998; Rodrigues *et al.*, 2005), other observations suggest that EGFR signaling is required for R8 spacing (Baonza *et al.*, 2001; Spencer *et al.*, 1998; Wasserman *et al.*, 2000; Yang and Baker, 2001). In the absence of EGFR and downstream components of the Ras pathway, R8 spacing becomes irregular (Baonza *et al.*, 2001; Spencer *et al.*, 1998; Yang and Baker, 2001). In *EGFR* mutants or *rhomboid-1* and *roughoid* double mutants, Atonal expression is expanded

and intermediate group spacing is altered (Wasserman *et al.*, 2000). Further, removal of both *EGFR* and *sca* leads to more severe spacing defects (Baonza *et al.*, 2001). These observations support the notion that the EGFR-dependent signal acts in parallel with Sca in control of intermediate group spacing by repressing Atonal in surrounding cells. They also raise the question of what factors downstream of EGFR mediate nonautonomous inhibition of Atonal in-between the intermediate groups. The factor is most likely secreted from the intermediate groups and diffuses into the surrounding cells. It is unlikely Sca since Sca expression remains intact in EGFR mutants (Baonza *et al.*, 2001; Yang and Baker, 2001). Argos has been postulated as a candidate (Spencer *et al.*, 1998). However, in argos mutant clones, intermediate group spacing is unaffected (Baonza *et al.*, 2001). The nature of this secreted factor has remained unclear.

Breaking up the uniform stripe of Atonal is enhanced by a positive feedback loop that ensures Atonal expression is maintained within the clusters. Atonal activates its own expression within the intermediate groups (Sun *et al.*, 1998). Positive autoregulatory loop is mediated by a 5′-enhancer of the *atonal* gene, which is also sensitive to repression by Notch signaling (Sun *et al.*, 1998). By utilizing this regulatory sequence open for both positive and negative regulation simultaneously, the system rapidly amplifies the differences in Atonal levels between Notch-responsive and Notch-insensitive cells.

Restricting Atonal expression within intermediate groups to single R8 precursors is controlled by lateral inhibition mediated by Notch. Removal of the Notch activity gives rise to extra R8 photoreceptors (Baker and Zitron, 1995; Baker *et al.*, 1996; Cagan and Ready, 1989b; Ligoxygakis *et al.*, 1998). Daughterless, a negative regulator of Atonal, is a target of Notch signaling (Lim *et al.*, 2008). Lateral inhibition is broadly utilized to select a small number of cells from a large pool (Bray, 2006). Dl is expressed in all cells in the intermediate group (Baker and Yu, 1998), raising the question of how biased Notch signaling is implemented among equipotent cells within each intermediate group. One possibility is that Sca provides a bias. Sca is a secreted by cells within the intermediate group and Sca levels reach the highest in single R8 precursors (Baker and Zitron, 1995; Baker *et al.*, 1990). Sca is capable of binding Notch (Powell *et al.*, 2001). Sca and the endosomal protein GP150 are colocalized with Notch in late endosomes, suggesting the possibility that Sca promotes Notch signaling in non-R8 cells by preventing downregulation of the Notch receptor (Li *et al.*, 2003). How Sca promotes N signaling in surrounding cells but not in Sca producing cells remains an open question.

2.4. Cell fate decisions within the ommatidial precluster

All cells within the R8 equivalence group are competent to become R8 (Dokucu *et al.*, 1996). As described above, selection of a single R8 within this group is controlled by lateral inhibition mediated by Notch signaling. Asymmetric Notch signaling leads to asymmetric Atonal expression: Atonal

continues to be expressed in the R8 precursor but is lost in other cells (Baker *et al.*, 1996; Dokucu *et al.*, 1996). Atonal is a key intrinsic factor that confers R8 competence. First, Atonal promotes expression of Sens, an activator of the R8 fate (Frankfort *et al.*, 2001). Sens consolidates the R8 fate by repressing Rough expression in the R8 precursor (Pepple *et al.*, 2008). Rough is an inhibitor of the R8 fate (Dokucu *et al.*, 1996; Heberlein *et al.*, 1991). Ectopic R8 neurons develop in the absence of Rough while ectopic expression of Rough inhibits R8 selection (Dokucu *et al.*, 1996; Heberlein *et al.*, 1991; Pepple *et al.*, 2008). Second, Atonal promotes expression of Rhomboid-1 (Baonza *et al.*, 2001), which processes the EGFR ligand Spitz. Production of active Spitz enables the R8 precursor to send EGFR signaling. Activation of EGFR in the R2/R5 precursors promotes Rough expression (Dominguez *et al.*, 1998; Hayashi and Saigo, 2001). Rough inhibits the R8 fate by antagonizing Sens (Pepple *et al.*, 2008). Therefore, an initial small difference in cell competence induced by Notch signaling results in a cascade of cellular responses, which culminates in differential expression of activators and inhibitors, leading to different cell fates (Fig. 4.5). In this case, a bias for one fate versus the other is initiated by cell signaling.

Production of Spitz in the R8 precursors also marks the beginning of the second signaling relay that is mediated by EGFR. Cell fate specification of all non-R8 photoreceptors and some of the support cells requires EGFR (Flores *et al.*, 2000; Freeman, 1996; Nagaraj and Banerjee, 2007). R2 and R5 precursors first receive EGFR signaling from R8 and then produce the ligand Spitz, which activates EGFR in neighboring cells as described above. On one side of the ommatidial cluster, R3 and R4 precursors receive EGFR signaling from R2 and R5 and transmit the signal to cone cells precursors (Freeman, 1997; Roignant and Treisman, 2009). On the other side of the ommatidia cluster, R1 and R6 precursors receive EGFR signaling from R2, R5, and R8 and transmit the signal to R7 precursors, which will be discussed in more detail later. Therefore, a signaling relay mediated by EGFR triggers differentiation of all non-R8 photoreceptors (Fig. 4.5).

Neither quality nor intensity of EGFR signaling *per se* is known to provide specificity for cell fate decisions within the ommatidial precluster. On the contrary, it is known that each cell type within the precluster responds to EGFR signaling differently and different abilities of these cells to respond to the seemingly same signaling are correlated with differential expression of intrinsic factors. For example, Rough and Seven-up (Svp), two critical determinants of cell fate, are differentially expressed in precursors for photoreceptors. R2 and R5 express Rough at a high level but not Svp (Mlodzik *et al.*, 1990). In contrast, R1/R6 precursors express Svp but not Rough (Mlodzik *et al.*, 1990). R3/R4 precursors are the ones that express both. In addition to its function in preventing extra R8 as described above, Rough also prevents R2 and R5 from adopting R3/R4 or R1/R6 fates (Heberlein *et al.*, 1991). Conversely, misexpression of Rough transforms R7 into R1/R6 (Basler *et al.*, 1990; Kimmel *et al.*, 1990). Svp, a

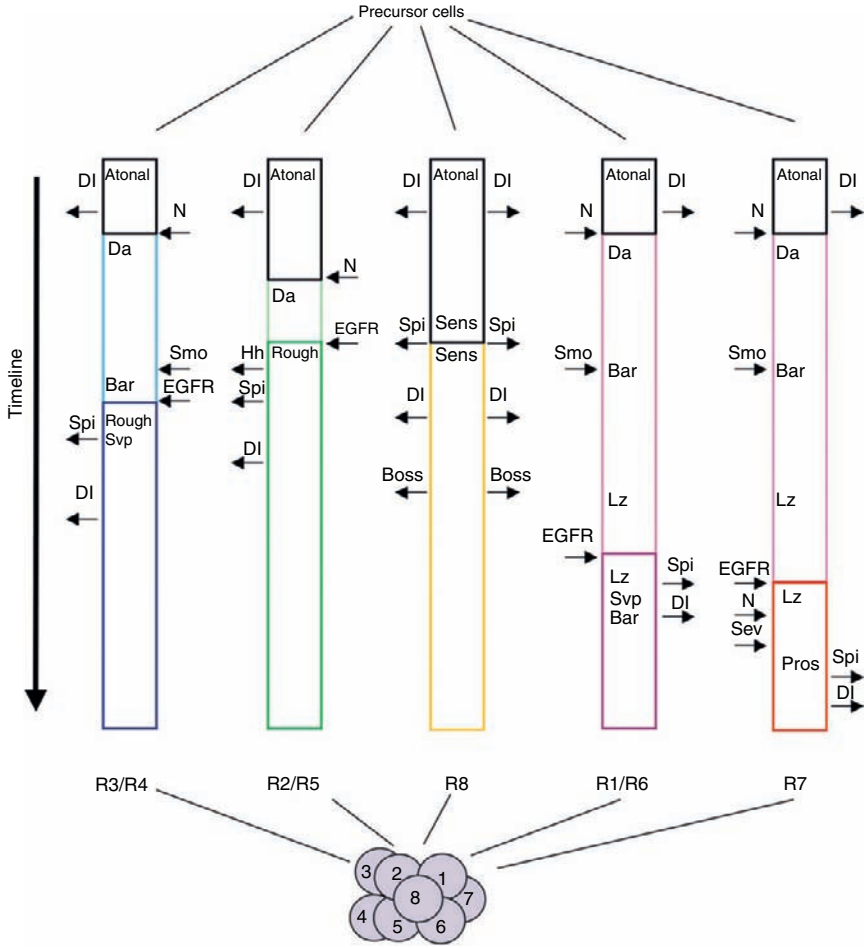

Figure 4.5 The interplay between cell signaling and competence generates diversity of photoreceptor neurons. Initially, precursor cells are equipotent and they all express Atonal. Early differences are generated by Scabrous, Spitz and Dl signals that cells receive. For simplicity, only Notch signaling is depicted. Without Notch input, cells retain Atonal, which biases the R8 fate (yellow). With Notch input, cells lose Atonal and gain high levels of Daughterless, and they are primed to adopt non-R8 fates. EGFR signaling triggers differentiation of all non-R8 neurons. Among them, cells receiving Notch early are directed to either R3/R4 or R1/R6/R7 fates while those receiving Notch later become R2/R5 (green). At the same time, intrinsic factors also bias cell fate choices: cells without Lz become R3/R4 (blue) while those with Lz favor R1/R6/R7 fates (magenta). Cells receiving additional Notch and Sevenless inputs besides EGFR signaling further become R7 (red). Hh contributes to this selection process by inducing Bar, a potent inhibitor of Atonal. The transition from an equipotent to a differentiating state is drawn in a lighter color. Da, Daughterless. Dl, Delta; Lz, Lozenge; N, Notch; Spi, Spitz; Svp, Seven-up; Smo, Smoothened. See text for detail.

nuclear hormone receptor, is required for maintaining R3 and R4 fates (Mlodzik *et al.*, 1990). Loss of Svp leads to transformation of R3/R4 and R1/R6 into R7 (Mlodzik *et al.*, 1990), indicating Svp prevents these cells from adopting the R7 fate. Similar to Rough, misexpression of Svp also transforms R7 into R1/R6 (Hiromi *et al.*, 1993). These observations indicate that Rough and Svp function together to prevent R3 and R4 from adopting the R7 fate. Therefore, different cell fates are determined by differential expression of intrinsic factors. How do these cells acquire different competence? Differential exposure to Notch signaling may provide an explanation. A few hours earlier, precursor cells for the ommatidial precluster sequentially receive Notch signaling. R3 and R4 precursors receive Notch signaling earlier and possibly longer than R2 and R5 precursors. One direct consequence of Notch signaling is loss of Atonal in these cells. Initial differences in receiving Notch signaling are amplified through a cascade of cellular responses, leading to different competence during photoreceptor recruitment (Fig. 4.5).

A pair of R3 and R4 photoreceptors are unique in that they are further made distinct from each other by cell signaling. Establishment of asymmetry between R3 and R4 depends on the Notch and Frizzled pathways (Cooper and Bray, 1999; Fanto and Mlodzik, 1999). Asymmetric signaling between R3 and R4 is essential for the ommatidia chirality and subsequent ommatidial rotation. Genetic control of the planar cell polarity has been reviewed extensively (Adler, 2002; Axelrod, 2009; Strutt and Strutt, 2009; Wu and Mlodzik, 2009).

Differentiation is a process. After photoreceptors R8, R2, R5, R3, and R4 together with the rest of photoreceptors recruited later are specified and determined, these cells undergo terminal differentiation, coupled with marked morphological changes and differential expression of opsin genes in these cells (see Chapter 5). Terminal differentiation takes 2 days after all cells are recruited to ommatidial clusters and the hexagonal pattern is in place in the early pupal eye. Interestingly, a subset of transcription factors and signaling pathways utilized for cell fate specification in the larval eye-imaginal disc are also involved in terminal differentiation of photoreceptors (Morante *et al.*, 2007; Wernet and Desplan, 2004), suggesting that the interplay between cell signaling and competence operates throughout the entire differentiation process.

2.5. Cell fate decisions within the R7 equivalence group

The R7 equivalence group includes R1, R6, and R7 photoreceptors and nonneuronal cone cells. The precursor cells for the R7 equivalence group are generated in the second mitotic wave and they are capable of switching fate from one to another under certain genetic conditions.

Both quality and quantity of cell signaling provide a bias for cell fate decision within the R7 equivalence group. EGFR signaling triggers differentiation of R1 and R6 (Dominguez *et al.*, 1998; Freeman, 1996; Xu and Rubin, 1993). Cells that receive both EGFR and Notch signaling from R1 and R6 together with Sevenless signaling from R8 choose the R7 fate. Those receiving both EGFR and Notch but not Sevenless signaling become cone cells (Flores *et al.*, 2000; Tomlinson and Struhl, 2001; Xu *et al.*, 2000). The role of cell signaling in cell fate decisions within the R7 equivalent group highlights several basic aspects of cell fate decisions within equipotent cells. First, quality of signaling provides a bias. Precursor cells for R1/R6 versus R7 and cone cells are equipotent and they are exposed to the same EGFR and Notch signaling. EGFR signaling *per se* does not provide a bias. Cells with the additional Notch input choose the R7/cone cell fate while those without choose the R1/R6 fate. Here the addition of Notch signaling provides a qualitative difference in signaling received by these equivalent cells (Fig. 4.5). Second, the quantitative level of signaling also provides a bias. Both R7 and cone cells precursors receive EGFR and Notch signaling. Different from cone cells, R7 precursors receive an additional Sevenless signaling emanating from R8 cells (Kramer *et al.*, 1991; Reinke and Zipursky, 1988). Similar to EGFR, Sevenless elicits a signaling cascade through the Ras pathway (Simon *et al.*, 1991). The combination of both EGFR and Sevenless signaling increases the intensity of the Ras signaling. Third, the birthplace decides a cell fate. Both R7 and cone cells precursor cells are competent to become either R7 or cone cells. The R8 photoreceptors express Bride-of-Sevenless (Boss), the ligand for Sevenless (Kramer *et al.*, 1991). As a result, cells adjacent to R8 are exposed to Boss and become R7 while equivalent cells that do not have this access become cone cells (Fig. 4.5). For an individual cell, therefore, environmental factors such as quality and quantity of cell signaling received—in part due to birthplace—bias the cell fate choices.

Selective responses to cell signaling depend on competence. A repertoire of transcription factors including transcription activators and repressors along with factors that control signaling reception and transduction all contribute to competence. Among these factors, Lozenge (Lz), a Runt-domain transcription activator, is key in conferring competence on these cells (Fig. 4.5). Lz is initially expressed in all undifferentiated cells posterior to the morphogenetic furrow except in precursors for the five-cell precluster (Flores *et al.*, 1998). Lz expression is maintained in precursors for the R7 equivalence group. Lz suppresses identities of cells within the five-cell precluster and is required for specifying fates within the R7 equivalence group. For example, misexpression of Lz in R3 and R4 leads to transformation of these cells to R7 (Daga *et al.*, 1996; Flores *et al.*, 1998; Hayashi and Saigo, 2001). Conversely, in *lz* mutant, cells within the R7 equivalence group lose their normal identities and express Svp, a marker for R3 and R4 (Daga *et al.*, 1996). It is known that expression of Lz requires transcription

factors Glass and So (Moses and Rubin, 1991; Yan *et al.*, 2003). However, what signals down-regulate Lz in precursors for the five-cell precluster has remained unclear. While Lz confers competence on all cells, the balance between transcription activators and repressors distinguishes one cell from another within the R7 equivalence group. Pnt is an ETS domain transcription activator (Brunner *et al.*, 1994; O'Neill *et al.*, 1994). Tram-track88 (Ttk88) and Yan are two major repressors involved in cell fate decisions within the R7 equivalence group. Ttk88 is expressed in all undifferentiated cells posterior to the furrow and it functions by promoting deacetylation of histones (Hong *et al.*, 1997; Lai *et al.*, 1996). Yan, an ETS domain transcription repressor, is expressed in all undifferentiated cells in and posterior to the furrow (Lai and Rubin, 1992). In the absence of Ttk88, cone cells are transformed to R7 (Lai *et al.*, 1996). Conversely, high levels of Ttk88 result in a fate switch from R1, R6, and R7 to cone cells (Chang *et al.*, 1995; Dickson *et al.*, 1995). Similarly, in *yan* mutants, the cone cell number is reduced and R7 number increased, suggesting a fate switch from cone cell to R7 (Lai and Rubin, 1992). Built upon this basic scheme are contributions from some additional intrinsic factors. For example, the HLH transcription factor Emc maintains the R7 fate while Roughened eye, a zinc finger transcription factor, promotes the R1/R6 fate (Bhattacharya and Baker, 2009; del Alamo and Mlodzik, 2008).

Cell signaling alters competence. There are two known mechanisms by which cell signaling alters competence. The first mechanism operates by modifying the sensitivity of a cell to signals. EGFR signaling promotes Dl expression in photoreceptors (Tsuda *et al.*, 2002). Earlier expression of Dl in R1 and R6 than in R7 precursors makes the R1/R6 precursors insensitive to Notch signaling through *cis*-inhibition (Miller *et al.*, 2009). By contrast, due to later onset of Dl expression, R7 precursors are not protected by *cis*-inhibition and therefore sensitive to Notch signaling. Here one signal modifies competence of the cell to receive another signal. The second mechanism involves control of the activity or level of transcription factors by cell signaling. Notch signaling promotes Emc expression in R7 precursors (Bhattacharya and Baker, 2009). EGFR signaling through the Ras pathway inhibits Yan and activates Pnt (Brunner *et al.*, 1994; O'Neill *et al.*, 1994; Rebay and Rubin, 1995). However, due to presence of the repressor Ttk88, activation of EGFR signaling alone is not enough for transcription of genes such as *prospero* needed to adopt the R7 fate (Kauffmann *et al.*, 1996). An extra boost of Ras signaling mediated by Sevenless leads to degradation of Ttk88 through the ubiquitination pathway mediated by Sina, Phyllopod, and Ebi (Xu *et al.*, 2000). As a result, in R7 precursors, Ras signaling alters cell competence through removal of a transcription repressor. A similar mechanism also functions to promote Dl expression in photoreceptors by EGFR. In photoreceptors, EGFR signaling releases Su(H)-mediated repression of Dl expression by inactivating the Su(H)

and its corepressor complex (Tsuda *et al.*, 2002). Therefore, cell signaling can alter competence via several independent mechanisms.

The interplay between cell signaling and competence creates the third signaling relay that is mediated by Notch. The third signaling relay generates two subtypes of support cells: cone cells and 1°s. Photoreceptors first produce Dl, which activates Notch in neighboring precursors for cone cells (Tsuda *et al.*, 2002). Differentiating cone cells produce Dl, which activates Notch in precursors for 1°s (Nagaraj and Banerjee, 2007). EGFR plays an essential role in this relay by promoting Dl expression (Nagaraj and Banerjee, 2007; Tsuda *et al.*, 2002). How activation of Notch is linked to production of Spitz, an EGFR ligand, is not clear.

2.6. Specification of primary pigment cells

Specification of 1°s requires Notch (Cagan and Ready, 1989b). Although EGFR signaling is not required for specifying 1°s (Nagaraj and Banerjee, 2007), it is required for the third signaling relay mediated by Notch. Expression of Dl in cone cells depends on EGFR (Nagaraj and Banerjee, 2007). Cone cells then signal to surrounding cells. Those that receive a high level of Notch signaling become 1°s and enwrap cone cells (Nagaraj and Banerjee, 2007). Those cells that receive a low level of Notch signaling lose contacts with cone cells and remain in the IOC pool.

Specification of 1°s also depends on the *Bar* locus that contains two genes *BarH1* and *BarH2*, coding for two homeobox transcription factors (Higashijima *et al.*, 1992). *BarH1* and *BarH2* are expressed in 1°s and R1/R6 photoreceptors (Higashijima *et al.*, 1992). Misexpression of BarH1 in cone cells transforms these cells into 1°s (Hayashi *et al.*, 1998), indicating BarH1 confers competence on 1° precursors. Expression of *BarH1* and *BarH2* depends on Spa (Fu and Noll, 1997). *spa* is transcribed in cone cells and 1°s, and transcription of *spa* depends on Lz (Flores *et al.*, 2000; Fu and Noll, 1997). Lz is expressed in all cells except in R8, R2/R5, and R3/R4, and lz transcription is controlled by *glass* and *so* (Yan *et al.*, 2003). Glass is a transcription factor expressed in all cells posterior to the furrow (Flores *et al.*, 1998; Moses and Rubin, 1991). *so*, a retinal determination gene, is broadly expressed in cells anterior and posterior to the furrow (Cheyette *et al.*, 1994). Therefore, from broadly expressed So and Glass to spatially restricted Bar, these transcription factors form a transcription hierarchy. How expression of these transcription factors becomes restricted is not clear. Since their expression coincides with cell fate decisions, it is reasonable to predict that progressively restricted expression of these transcription factors is the consequence of the interplay between cell signaling and competence. To support this notion, transcription of *spa* in cone cells is controlled by EGFR and Notch signaling together with the competence-conferring transcription factor Lz (Flores *et al.*, 2000).

2.7. Survival versus death decisions within the interommatidial cells

The second mitotic wave generates an excessive number of precursor cells for further cell recruitment. After photoreceptors, cone cells and 1°s are recruited, excess IOCs are removed by apoptosis. Interestingly, life versus death decisions do not affect global patterning in the eye. In mutants that completely block apoptosis in the eye, ommatidia are still arranged in a nearly perfect hexagonal array (Fig. 4.6B). Cell death, nevertheless, tightens the interommatidial lattice. By initially generating an excessive number of cells, the system ensures that it has sufficient materials available to build an organ. By removing unwanted cells, the system builds a functional organ using the least resource.

Not surprisingly, the intrinsic death machinery including activators and inhibitors of apoptosis is present in the eye (Fig. 4.6A and see also Bao and

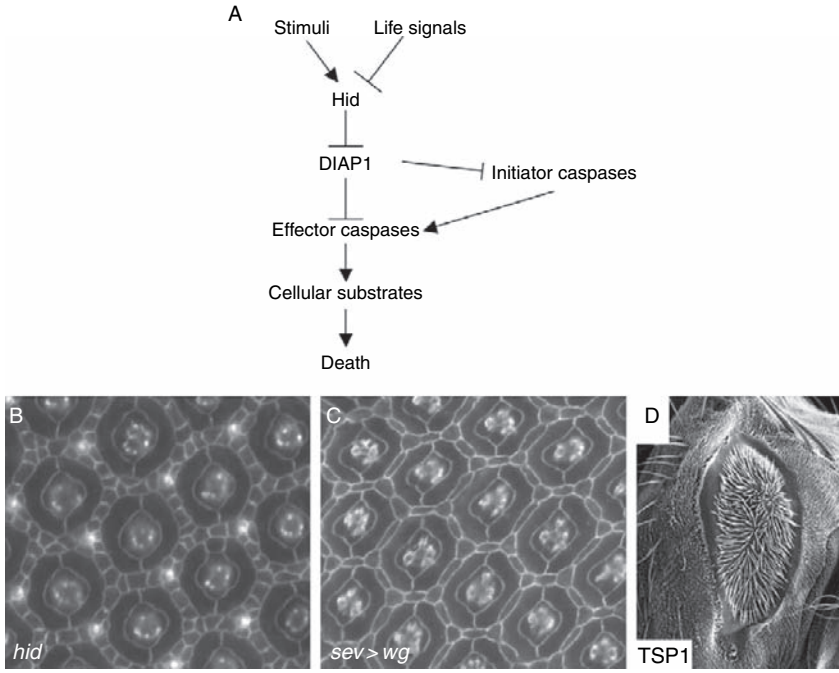

Figure 4.6 Cell number, bristles and hexagons. (A) Components of the intrinsic death machinery utilized in the eye. Modified with permission from Bao and Cagan (2003). (B) Cell death is completely blocked in *hid* mutants. The hexagonal array is nevertheless unaffected. (C) Misexpression of Wg blocks bristle development. The hexagonal array of ommatidia is maintained. (D) Bristle and retinal cells follow different differentiation programs. Removal of EGFR activities by using a temperature sensitive allele TSP1 leads to loss of retinal cells. Nonetheless, bristles still develop. Panel D is adapted with permission from Kumar and Moses (2001).

Cagan, 2003). DIAP1, a member of the inhibitor of apoptosis (IAP) family, is ubiquitiously expressed in the eye (Hays *et al.*, 2002). DIAP1 binds both initiator and effector caspases and prevents their activation, whereby DIAP1 inhibits cell death (Vaux and Silke, 2005). Cell death in the eye requires the death activator Hid (Grether *et al.*, 1995; Yu *et al.*, 2002). Hid promotes apoptosis by interfering with interactions between DIAP1 and caspases (Goyal *et al.*, 2000; Meier *et al.*, 2000; Wang *et al.*, 1999). Built upon this basic scheme are some additional mechanisms that fine tune the balance between life and death responses. For example, Morgue, a ubiquitin conjugase, promotes cell death by targeting DIAP1 to degradation through the ubiquitin pathway (Hays *et al.*, 2002). Echinus, a protein with homology to ubiquitin-specific proteases, is also required for removal of excess IOCs (Copeland *et al.*, 2007).

Life and death decisions in the developing eye require a balance between pro-life and pro-death signals. In the eye, Notch signaling is proapoptotic and EGFR promotes survival (Cagan and Ready, 1989b; Miller and Cagan, 1998; Sawamoto *et al.*, 1998). Studies using cell ablation indicate cone cells and 1°s provide a pro-life signal to antagonize the Notch-mediated pro-death signal, and this pro-life signal is mediated by EGFR (Miller and Cagan, 1998). Further, it has been shown that EGFR promotes survival by inactivating Hid protein and repressing *hid* transcription (Bergmann *et al.*, 1998; Kurada and White, 1998). Besides these pro-life and pro-death signals, the third signal comes from Roughest (Rst), a cell adhesion molecule of the Irre-cell recognition module (IRM) family (Fischbach *et al.*, 2009). Rst is essential for both cell sorting and cell death that sculpts the interommatidial lattice in the pupal eye (Reiter *et al.*, 1996; Wolff and Ready, 1991). Rst is expressed in IOCs and the dual function of Rst leads to a two-step model for cell death at this stage: cell sorting proceeds first, followed by cell death; cell sorting is required for cell death (Reiter *et al.*, 1996). All IOCs seem to be exposed to the same pro-life and pro-death signals, raising the question of how a death or life decision is made for each individual cell. Studies using live imaging indicate that cells are prone to death in a certain area in the eye—"the death zone"—suggesting that the birthplaces decides the final fate (Monserrate and Brachmann, 2007). On the other hand, studies using the similar technique indicate that precursors for secondary pigment cells compete with each other before settling the final niche (Larson *et al.*, 2008). Then why is one cell more competitive than the others. Rst may provide a clue. Rst is expressed in IOCs while its binding partner Hibris (Hbs) is expressed in 1°s and IOCs prefer to adhere to 1°s (Bao and Cagan, 2005). Preferential adhesion leads to cell competition. An increased level of Rst in a single cell makes the cell super-competitive while decreasing Rst in a single IOC makes the cell underrepresented (Bao and Cagan, 2005). These observations lead to an adhesion-based death model: The differences in their abilities to adhere to 1°s mediated by differential levels of adhesion molecules such as Rst

determine their different capacities in competition, which in turn determine the final fate, life versus death. Further validation of this adhesion-based death model requires quantification of levels of Rst or equivalent adhesion molecules in individual cells.

2.8. Cell fate decisions within the bristle group

In the *Drosophila* eye, about 800 mechanosensory bristle groups are evenly distributed at alternate vertices of hexagons (Fig. 4.1C–E). Each bristle group is comprised of four cells: the glial cell, socket cell, shaft cell and sensory neuron. Four cells within a bristle group derive from a single ganglion mother cell, called SOP (Cagan and Ready, 1989a). Interestingly, division of the SOP cell begins at the center and spreads outwards toward the periphery of the eye (Cagan and Ready, 1989a). Specification of post-mitotic cells within the bristle group occurs before 1°s are selected (Cagan and Ready, 1989a). Daughter cells undergo significant cell shape changes, leading to assembly of these four cells in an onion-like configuration. The glial cell enwraps the neuron from the side, while the shaft cell reaches around and enwraps the glial cell and neuron from the top. The socket cell then enwraps the inner three cells (Cagan and Ready, 1989a).

Both bristle and retinal cells derive from the same pool of precursor cells of epithelial origin. Selection of both groups of cells requires retinal determination genes. In the absence of *eyeless*, for example, both retinal cells and bristle cells fail to develop (Quiring *et al.*, 1994). Conversely, when a retinal determination gene is misexpressed in other discs, ectopic eyes form along with bristles (Halder *et al.*, 1995). However, bristle and retinal cells seem to adopt very different developmental programs. Disruption of either program does not affect the other. For example, expression of *wg* using a sev-Gal4 driver eliminates bristles, giving rise to a bald eye (Brunner *et al.*, 1999). Nevertheless, the hexagonal array of ommatidia is unaffected (Fig. 4.6C). Vice versa, ablation of retinal cells does not affect bristle cells. For example, loss of EGFR or misexpression of the death activator Hid leads to loss of retinal cells but leaves bristle cells intact (Fig. 4.6D and see also Freeman, 1996; Grether *et al.*, 1995; Kumar and Moses, 2001). These observations suggest that SOPs are selected and set aside early during eye development but cell division and differentiation of bristle cells do not occur until the early pupal stage. So far it is not clear what triggers cell division within the bristle group.

Selection of SOPs in the eye requires proneural genes *achaete* and *scute*. Achaete and Scute are members of the Achaete–Scute Complex (Posakony, 1994). Both Achaete and Scute are expressed in SOPs in the eye and loss of Scute leads to a bald eye (Frankfort *et al.*, 2004). Conversely, ectopic expression of *scute* results in ectopic bristles. These observations indicate that Achaete and Scute confer competence on SOPs in the eye. Downstream of

the Achate–Scute Complex, Sens is the primary target (Frankfort *et al.*, 2004). In *sens* mutants most bristles are lost, and expression of *sens* in *scute* mutants is sufficient to produce bristles (Frankfort *et al.*, 2004).

Bristle development requires Notch signaling. When Notch activities are reduced in the early pupal eye (8–16 h APF, 20 °C), extra bristles develop (Cagan and Ready, 1989b). In contrast, reduction of Notch activities later (14–22 h APF, 20 °C) leads to bald eyes. Interestingly, when Notch is inactivated between 8 and 24 h APF that covers both of these periods, eyes are still bald (Cagan and Ready, 1989b), indicating that Notch activity between 14 and 22 h APF is essential for bristle development. It is not clear whether Notch is required for differentiation of bristle cells, for bristle survival or for close cell–cell interactions within each bristle group.

3. Selective Adhesion Determines Spatial Patterns of Cells

To make an organ functional, different cell types have to be arranged in specific configurations. How are spatial configurations of cells generated and maintained within developing organs?

Studies based on dissociation and reaggregation of animal cells have demonstrated that cell adhesion is a mechanism underlying organ assembly. Sponge cells dissociated by squeezing a sponge through fine meshes can reunite and reconstruct into functional sponges (Wilson, 1907). Reaggregation of dissociated amphibian embryonic cells leads to a resorting of cells with their proper associative neighbors and often in their normal relative positions (Holtfreter, 1939). Similar reaggregation studies have led to the "differential adhesion hypothesis" (DAH) (Steinberg, 1963, 1970), which proposes that sorting out of intermixed embryonic cells and envelopment of one embryonic tissue by another are driven by tissue interfacial free energies arising from cell adhesion. Based on DAH, differential adhesion drives different cell populations to segregate from each other and organize them into specific patterns by minimizing surface free energy. In fact, in cultured cells, it has been shown that differences in numbers of identical cell adhesion molecules are sufficient to cause cell sorting and tissue spreading (Steinberg and Takeichi, 1994). But how does cell adhesion drive cell sorting *in vivo*?

Studies in model organisms indicate that differential adhesion mediated by different levels of cadherins can drive cell sorting *in vivo*. For example, positioning of the oocyte in the *Drosophila* ovary and aggregation of blastomeres in the mouse embryo are controlled by cadherins that act through homophilic adhesion (De Vries *et al.*, 2004; Godt and Tepass, 1998; Gonzalez-Reyes and St Johnston, 1998). A similar mechanism in the *Drosophila* eye acts locally to regulate aggregation of support ("cone") cells into a four-cell cluster

(Hayashi and Carthew, 2004). On the other hand, to mediate formation of more complex forms and shapes, more adhesion elements are needed. As an example, to sort multiple rows of IOCs into a single line in the *Drosophila* eye, two heterophilic-interacting adhesion molecules Hibris (Hbs) and Roughest (Rst) are required (Bao and Cagan, 2005).

The importance of cell adhesion in organ formation highlights the necessity for temporal and spatial regulation of cell adhesion. Cell–cell signaling and dynamics of the cytoskeleton are central regulators of cell adhesion. Cell signaling along with competence determines cell fates by regulating gene expression. Not surprisingly, genes coding for adhesion molecules are also subject to regulation by cell signaling and competence via a transcriptional mechanism. Recent studies have also increasingly implicated the cytoskeleton as an important regulator of cell adhesion. The dynamics of the cytoskeleton can influence cell adhesion by several independent posttranscriptional mechanisms (Gumbiner, 2005; Kaksonen *et al.*, 2006; Mege *et al.*, 2006; Wirtz-Peitz and Zallen, 2009). On the other hand, cell adhesion in return can modulate cell signaling (Perez-Moreno *et al.*, 2003). In addition, cell adhesion is also implicated in regulation of the dynamics of the cytoskeleton (Kobielak and Fuchs, 2004). Therefore, cell adhesion and cell signaling as well as the cytoskeleton can regulate one another.

Accessibility of the fly eye for live imaging along with the power of fly genetics has made the fly eye a unique model for understanding the role of cell adhesion in organ assembly. In the *Drosophila* eye, cells are sequentially recruited to the ommatidial cluster. What makes cells adopt certain configurations within the ommatidial cluster? What makes cells stay in clusters? How are ommatidia aligned unanimously in one orientation? How is ommatidial rotation controlled to achieve precise chirality? How is ommatidial spacing maintained? Recent studies in the *Drosophila* eye have provided some new insights. This chapter will focus on morphogenetic events that take place in the pupal eye. Spatial organization of photoreceptors occurs in the larva and the function of cell adhesion in this process is less well understood. Genetic control of ommatidial rotation in the larva has been reviewed in depth (Adler, 2002; Axelrod, 2009; Strutt and Strutt, 2009; Wu and Mlodzik, 2009).

3.1. Homotypic adhesion and packing of cone cells

In the pupal eye, four cone cells pack together in a four-leaf-clover configuration in the middle of ommatidia. Arrangement of cone cells is reminiscent of soap bubbles in water (Hayashi and Carthew, 2004). Further, when the number of cone cells is altered by genetic manipulation, packing of cone cells remains analogous to soap bubbles of the equivalent number (Fig. 4.7). These observations provide strong evidence that spatial organization of cone cells follows a mechanism that minimizes the overall surface area. Cone cells are recruited to ommatidia earlier than pigment cells. What

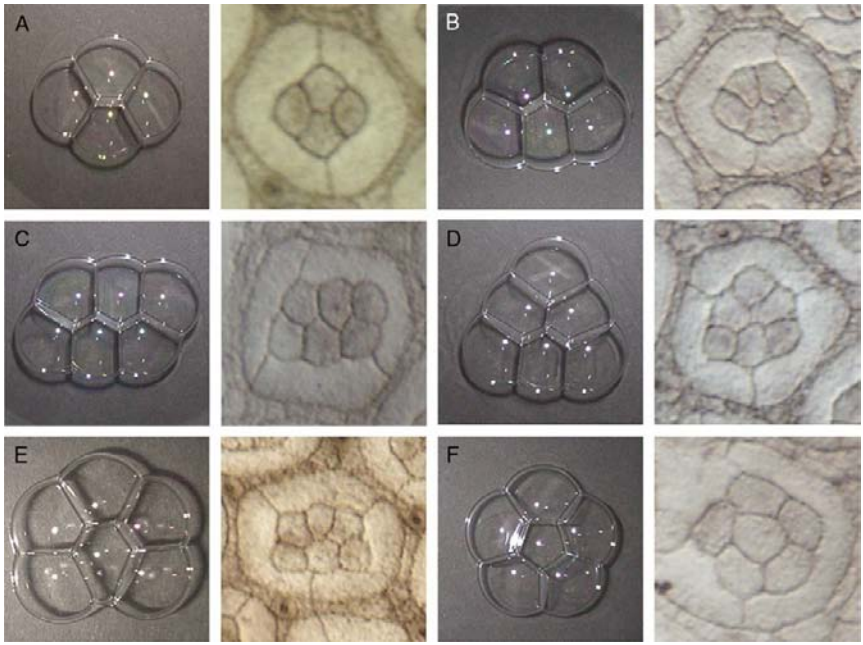

Figure 4.7 Configurations of cone cells in the Drosophila eye resemble patterns of soap bubbles in water. Patterns of four (A), five (B) and six (C-F) soap bubbles (left) are compared with those of cone cells of the same number (right). Pupal eyes from Roi/+ flies at 40 h APF were stained with cobalt sulfide (Images courtesy of R. W. Carthew).

makes cone cells more adhesive to each other? Packing of cone cells requires E- and N-cadherin. Upon removal of both E- and N-cadherin in a single cone cell, the cell detaches from the cone cell group (Hayashi and Carthew, 2004). Misexpression of N- but not E-cadherin in single 1°s leads to marked repositioning of cone cells. Consistently, N- but not E-cadherin is differentially expressed: N-cadherin is expressed in cone cells but not in pigment cells while E-cadherin is ubiquitously expressed (Hayashi and Carthew, 2004). Both N- and E-cadherin form homophilic interactions (Hynes and Zhao, 2000). These observations demonstrate that quantitative differences in homophilic-interacting adhesion molecules are sufficient to drive cell sorting *in vivo*.

The roles of cadherins in packing cone cells also raise several new questions. First, E-cadherin, similar to N-cadherin, also mediates homophilic interactions (Hynes and Zhao, 2000; Gumbiner, 2005). Why does not overexpression of E-cadherin in pigment cells alter cone cell arrangement as N-cadherin does? Second, clearly, N-cadherin plays a role in patterning cone cells. However, removal of N-cadherin in the whole eye does not alter cone cell configuration (Hayashi and Carthew, 2004). In the

N-cadherin mutant fly, differences in cadherin expression should be eliminated among these cells. What mediates differential adhesion between cone cells and pigment cells in N-cadherin mutants? Third, in the wild-type eye, four cone cells are arranged in a specific spatial relationship with a unanimous orientation. For example, the anterior and posterior cone cells are always separated by the polar and equatorial cone cells (Fig. 4.1D). What mechanism controls the asymmetry of cone cell configuration across the eye? These questions might not be easily answered solely by cadherin-based adhesion. Recent studies indicate that the adhesion molecule Hbs plays a role in preventing contacts between anterior and posterior cone cells (Grillo-Hill and Wolff, 2009), adding a new element to cone cell patterning. How Hbs functions in this process is yet to be determined.

3.2. Preferential adhesion and sorting of interommatidial cells

Following selection of 1°s, a large number of IOCs remain in multiple rows with irregular cell shapes and they undergo cell rearrangement. Within a day, these cells sort from multiple rows into a single line and unwanted cells removed by apoptosis (Cagan and Ready, 1989a).

Sorting of IOCs requires adhesion molecules Rst and Hbs (Bao and Cagan, 2005). Rst and Hbs are members of the IRM family within the Ig superfamily (Fischbach *et al.*, 2009). IRM adhesion molecules are conserved from *Caenorhabditis elegans* to flies and humans. In *Drosophila*, there are four IRM proteins identified to date: Rst, Hbs, Sticks and stones (Sns), and Kin of irre (Kirre, also known as Dumbfounded or Duf). Rst and Kirre are orthologs of mammalian Neph1 while Hbs and Sns are orthologs of Nephrin (Fischbach *et al.*, 2009).

In rst^{CT} mutant eyes, multiple rows of IOCs fail to sort into single line (Wolff and Ready, 1991). *rst* transcript is detected in IOCs (Ramos *et al.*, 1993). Hbs, a binding partner of Rst is expressed in 1°s and cone cells (Bao and Cagan, 2005). Therefore, Rst and Hbs are expressed in complementary cell types (Fig. 4.8A–D). During IOC sorting, Rst protein is preferentially localized at the border between 1°s and IOCs, suggesting that interactions between Hbs and Rst are stronger than homophilic interactions between Rst. Genetic manipulations indicate IOCs prefer to adhere to 1°s than to IOCs themselves, a situation referred to as preferential adhesion (Bao and Cagan, 2005). As a result, IOC–1° contacts are energy-favored while IOC–IOC contacts are energy-disfavored. Sorting of IOCs from multirows into a single file reflects minimization of surface free energy (Bao and Cagan, 2005). At this stage of eye development, sorting of IOCs is not regulated by quantitative differences in homophilic-interacting cadherins. Rather, it is controlled by heterophilic-interacting IRM adhesion molecules that mediate preferential adhesion.

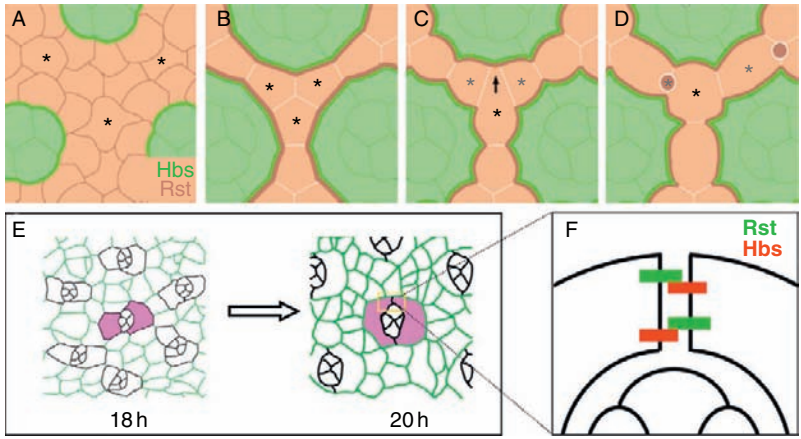

Figure 4.8 Preferential adhesion in spatial organization of pigment cells. (A) At the onset of cell rearrangement, multiple rows of interommatidial cells (IOCs, orange) are scattered between ommatidia (green). Three candidates for the tertiary pigment cell are marked by asterisks. (B) After 1°s are specified, adhesion molecules Hbs and Rst are expressed in complementary cell types: Hbs is expressed in 1°s and Rst in IOCs. Due to heterophilic interactions between Rst and Hbs, IOCs prefer to adhere to 1°s. This cell behavior is referred to as preferential adhesion. As a result, IOC–IOC contacts are reduced and multiple rows of IOCs sort into a single line. Note that IOC-IOC contacts are not yet minimized and three tertiary candidates are still competing for the tertiary niche. (C) After one IOC establishes junctions with three 1°s, the other competing IOCs join other IOCs to compete for survival. (D) A single secondary pigment cell is selected. Other IOCs are removed by cell death. IOC-IOC contacts are now minimized. (E) Emergence of 1°s. Tracing of pupal eyes is shown. Developing 1°s are highlighted in magenta and IOCs in green. (F) At an earlier stage, developing 1°s express both Hbs and Rst. Heterophilic interactions between Hbs and Rst promote formation of junctions between the two emerging 1°s. Panels A–D are adapted with permission from Bao and Cagan (2005).

The preferential adhesion model, on the other hand, raises a new question. If IOC–IOC contacts are energy-disfavored based on the model, why are IOC–IOC contacts not further reduced so that ommatidia come in direct contacts with each other? A clue comes from four members of the IRM family. Based on their homology to mammalian proteins, the fly IRM adhesion molecules can be subdivided into two groups: Rst and Kirre in the Neph1 group, and Hbs and Sns in the Nephrin group. Proteins from the Nephrin group are expressed in 1°s, and those from the Neph1 group in IOCs (Bao *et al.*, 2010). IRM proteins form strong intergroup interactions and weak intragroup interactions. Similar to Rst that mediates preferential adhesion of IOCs to 1°s through preferred interactions with Hbs (Bao and Cagan, 2005), proteins in the Nephrin group also mediate preferential adhesion of ommatidia to IOCs through preferred intergroup interactions (Bao *et al.*, 2010). As a result, ommatidia and IOCs form mutual preferential

adhesion: preferential adhesion of IOCs to ommatidia minimizes IOC–IOC contacts; preferential adhesion of ommatidia to IOCs minimizes ommatidia–ommatidia contacts, which provides a mechanism for maintaining separation of ommatidia.

3.3. Dynamic adhesion and emergence of primary pigment cells

In the early pupal eye, 1°s are specified following cone cells. At 18 h APF, two cells adjacent to anterior–posterior cone cells are selected as 1° precursors. These cells start to spread around cone cells. Within 2 h, two 1° precursors touch each other apically (Fig. 4.8E). Soon the apical contacts expand more basally, giving rise to two pieces of shields zippering down from apical to basal that enwrap cone cells (Cagan and Ready, 1989a).

Generation of the zippered shields of 1°s requires both Rst and Hbs (Bao and Cagan, unpublished). Rst expression is known to be dynamic (Reiter *et al.*, 1996). In the early pupal eye, Rst protein is found at high levels in undifferentiated cells and low in differentiating cells (Reiter *et al.*, 1996). In contrast, Hbs protein is found at low levels in undifferentiated cells and high in differentiating cells (Bao and Cagan, unpublished). As a result, both Rst and Hbs proteins are present in the developing 1°s. Interestingly, both proteins always colocalize at the border between adjacent cells (Bao and Cagan, 2010). Rst is known to bind Hbs *in trans* and interactions between Rst and Hbs promote formation of junctions (Bao and Cagan, 2005). Colocalization of Rst and Hbs in the same developing 1° cells along with their preferred interactions *in trans* suggests that heterophilic interactions between Hbs and Rst promote formation of junctions between two emerging 1°s, which may provide a molecular basis for zippering of the two cells (Fig. 4.8F).

3.4. Cell adhesion and cell signaling

Similar to cell fate specification, cell adhesion is also under control of cell signaling and competence. In the *Drosophila* eye-imaginal disc, EGFR together with Atonal is required for elevated levels of E-cadherin within the ommatidial cluster (Brown *et al.*, 2006). In the pupal eye, Notch signaling differentially regulates *IRM* genes. In developing 1°s, Notch suppresses *rst* while activating *hbs* transcription (Bao and Cagan, unpublished). As a result, in the emerging 1°s, *rst* transcription is repressed while *hbs* is activated, leading to differential expression of *hbs* and *rst* in 1°s versus in IOCs, respectively. Besides Notch, other signaling also contributes to fine tuning adhesion in the eye. Reduction of Dpp signaling, for example, leads to transient ommatidia–ommatidia contacts without affecting cell fate

(Cordero *et al.*, 2007), suggesting a defect in preferential adhesion when Dpp signaling is compromised. Activation of EGFR signaling by misexpressing secreted Spitz in a single IOC leads to extra IOCs surrounding the target cell (Monserrate and Brachmann, 2007), reminiscent of effects by misexpressing IRM adhesion molecules Hbs and Sns. How Dpp and EGFR pathways are involved in regulating cell adhesion still remains unclear.

Cell adhesion can also regulate cell signaling. It is known that cell adhesion can regulate signaling in vertebrates. For example, cadherins are required for membrane localization of Eph receptors and activation of EGFR and VEGFR-2 *in vitro* and *in vivo* in mammals (Carmeliet *et al.*, 1999; Orsulic and Kemler, 2000; Pece and Gutkind, 2000). In *Drosophila* epithelia, interactions between surface signaling receptors and ligands often take place at the adherens junctions (Woods and Bryant, 1993). In the *Drosophila* eye, the cadherin–catenin complex is required for membrane localization of several transmembrane proteins including signaling receptors (Bao, unpublished). How cell adhesion regulates cell signaling in the eye is not well understood.

3.5. Cell adhesion and the cytoskeleton

In this chapter, cell adhesion is used to define adhesive interactions that occur on the cell surface. Cell adhesion promotes cell–cell contact (Fig. 4.9A). On the other hand, individual cells have the propensity to adopt the spherical shape, a cellular property referred to as cortical tension (Lecuit and Lenne, 2007). Cortical tension reduces cell–cell contact (Fig. 4.9A). In the *Drosophila* pupal eye, preferential adhesion mediated by adhesion molecules Hbs and Rst promotes 1°–IOC contacts (Bao and Cagan, 2005). A single IOC receiving an elevated level of Rst has an expanded apical profile at the expense of its neighbors (Fig. 4.9B–B′). Conversely, single IOCs with reduced Rst tend to have smaller apical profiles and are underrepresented (Bao and Cagan, 2005). Manipulation of E-cadherin does not affect cell shape (Bao and Cagan, 2005), indicating cell shape change is a result of specific adhesive interactions. Cortical tension, on the other hand, depends on the actin cytoskeleton (Sheetz, 2001). Actin regulators are known to play a role in control of cortical tension. If we use activator and inhibitor again to describe a positive regulator and negative regulator of cortical tension, respectively, these regulators can be divided into two groups: the activator and inhibitor groups. In the *Drosophila* eye, Rho1, Rho Kinase (Rok), and nonmuscle myosin II are in the activator group. The small GTPase Rho1, a member of the Rho family, is a potent regulator of the actin cytoskeleton (Hall, 2005). Rok, the ortholog of mammalian Rho kinase ROCK1/2, is a target of Rho1 (Leung *et al.*, 1995; Matsui *et al.*, 1996). Rok directly phosphorylates myosin II and inhibits the myosin II phosphatase, whereby Rok activates myosin II (Amano *et al.*, 1996). In the eye, loss of either of these factors

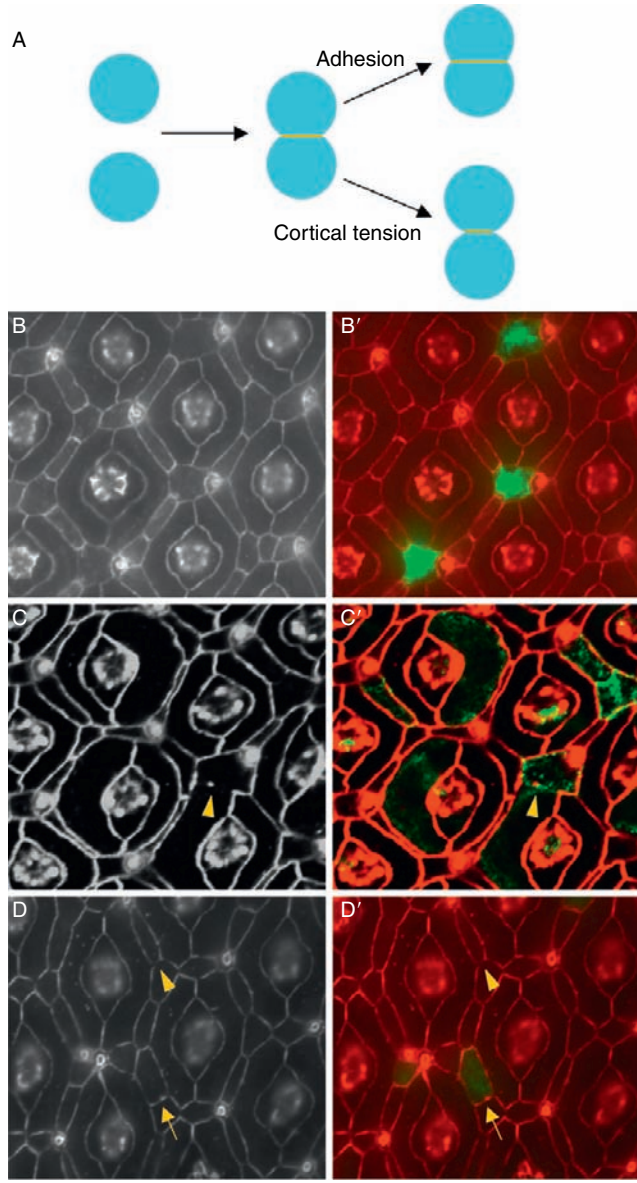

Figure 4.9 Cell adhesion and cortical tension control cell shape. Genetically manipulated cells are marked by GFP (green). Pupal eyes were stained with an anti-E-cadherin antibody (red) to visualize cell morphology. The single E-cadherin channel is shown in B–D and merged views in B′–D′. (A) Cortical tension counteracts adhesion. When two cells (light blue) are brought into contact, adhesion promotes and cortical tension reduces cell–cell contact. Size of cell–cell contact is correlated with formation of junctions (orange line). Modified from Lecuit and Lenne (2007). (B–B′) Preferential

blocks apical constriction (Corrigall *et al.*, 2007; Escudero *et al.*, 2007; Warner and Longmore, 2009b). In the pupal eye, for example, in the absence of Rho1 F-actin levels are reduced and IOCs have expanded apical profiles (Fig. 4.9C and see also Warner and Longmore, 2009a). Built upon this basic Rho1-Rok-myosin axis are some additional regulators that fine tune cortical tension. For instance, Diaphanous, the fly ortholog of mammalian formin and an effector of Rho1, also positively regulates cortical tension (Corrigall *et al.*, 2007; Escudero *et al.*, 2007; Warner and Longmore, 2009b). The inhibitor group includes Twinstar/cofilin, Slingshot, Cindr/CD2AP, and the Cdc42-Par6-aPKC polarity complex. Twinstar is the *Drosophila* ortholog of cofilin that depolymerizes actin filaments and Slingshot is a phosphatase that reactivates cofilin (Chen *et al.*, 2001; Niwa *et al.*, 2002). Cindr, the fly ortholog of CD2AP, is in complex with capping proteins CPα and CPβ and regulates actin organization (Johnson *et al.*, 2008). The small GTPase Cdc42 is another member of the Rho family (Hall, 2005). Par6 and aPKC are regulators of apico-basal polarity (Wodarz and Nathke, 2007). Depletion or removal of either factor leads to apical constriction (Johnson *et al.*, 2008; Warner and Longmore, 2009a). Cell shape changes mediated by these actin regulators are most likely independent of selective adhesion. Rst, for example, is not reduced upon depletion of Cindr (Johnson *et al.*, 2008), suggesting that apical constriction seen in these manipulated cells is not the result of reduced selective adhesion.

Adhesion molecules are often directly or indirectly linked to the cytoskeleton, and cell adhesion and dynamics of the cytoskeleton can regulate each other. Evidence accumulated to date suggests that the cytoskeleton can regulates the activity of adhesion complexes by at least three mechanisms. The first mechanism involves regulation of stability of adhesion complexes through physical interactions. Adhesion molecules are often connected to the cytoskeleton and the functions of adhesion molecules depend on the linkage to the cytoskeleton (Mege *et al.*, 2006). For example, the assembly of the cadherin–catenin complex requires the cortical actin cytoskeleton (Quinlan and Hyatt, 1999). In the *Drosophila* pupal eye, membrane localization of Rst depends on E-cadherin (Grzeschik and Knust, 2005), and localization of both Rst and E-cadherin depends on α-catenin (Seppa *et al.*, 2008), indicating the importance of linkage to the cytoskeleton for stability

adhesion promotes cell–cell contact. Single IOCs (green) that receive extra Rst expand apical profiles. As a result, 1°-IOC contacts are increased. (C–C′) Loss of Rho1 leads to expansion of apical profiles. Arrowheads highlight a single-cell mutant for *rho1* (green). (D–D′) Heterophilic interactions between Hbs and Rst promote formation of cell junctions. Normally the E-cadherin level is low in-between IOCs at 36 h APF (arrowheads). Upon forced expression of Hbs (green) in single IOCs, robust junctions (arrows) form in-between IOCs. Panels C–C′, image courtesy of S. Warner and G. Longmore.

of the adhesion complexes. The second mechanism involves regulation of stability of adhesion complexes through protein trafficking. It is known that the Arp2/3 complex, which promotes the nucleation of the actin filament, is directly involved in endocytosis (Kaksonen *et al.*, 2006; Wirtz-Peitz and Zallen, 2009). In *Drosophila*, Cdc42 along with Par6 and aPKC controls stability of E-cadherin by regulating Arp2/3-mediated endocytosis (Georgiou *et al.*, 2008; Harris and Tepass, 2008; Leibfried *et al.*, 2008). In the *Drosophila* eye, Rho1 maintains adherens junctions by inhibiting Cdc42/Par6-dependent E-cadherin endocytosis, and removal of Rho1 in two adjacent IOCs in the pupal eye disrupts adherens junctions (Warner and Longmore, 2009b). The third mechanism involves regulation of conformation of adhesion molecules. It has been proposed that signaling mediated by regulators of the cytoskeleton can induce conformational changes in adhesion molecules in an inside-out fashion (Gumbiner, 2005). To date, very little is understood about regulation of cell adhesion by this inside-out mechanism in the *Drosophila* eye. Further, much attention has been given to the actin cytoskeleton. Evidence suggests that other types of the cytoskeleton also play a role in regulating cell adhesion. Rst, for example, is discontinuous on the membrane in the absence of the spectrin-based cytoskeleton (Lee *et al.*, 2010).

Cell adhesion, on the other hand, can also regulate the cytoskeleton. α-catenin, a component of the cadherin–catenin complex, can directly regulate the assembly of the actin filament (Drees *et al.*, 2005; Yamada *et al.*, 2005). Loss of Armadillo, the fly ortholog of *β*-catenin, leads to disruption of polarity of the actin cytoskeleton (Cox *et al.*, 1996). The cadherin–catenin complex is an essential component of adherens junctions that are closely linked to the underlying actin belt (Kobielak and Fuchs, 2004). The presence of all components of the cadherin–catenin complex *per se*, however, is not sufficient to assemble adhesion junctions and the actin belt. In the *Drosophila* pupal eye, IOCs express all components of the cadherin–catenin complex but IOCs form very few adherens junctions with neighboring IOCs during cell rearrangement (Bao and Cagan, 2005). Forced expression of Hbs in an IOC leads to robust adherens junctions (Fig. 4.9D; see also Bao and Cagan, 2005), indicating that selective adhesion plays an instructive role in assembling adherens junctions. Since adherens junctions and the underlying actin belt are closely linked, this observation also suggests that selective adhesion can instruct the assembly of the actin cytoskeleton in an outside-in fashion.

In summary, evidence from the *Drosophila* eye and other model systems has revealed that selective adhesion, when temporally and spatially regulated, determines unique cellular patterns. Cell adhesion is regulated by cell signaling by transcriptional mechanisms. Cell adhesion, on the other hand, can also modulate cell signaling. Both cell adhesion and the cytoskeleton can control cell shape although the underlying mechanisms may be different.

Nevertheless, cell adhesion and the cytoskeleton can regulate each other by posttranscriptional mechanisms. Therefore, cell adhesion in conjunction with cell signaling and the cytoskeleton may provide a mechanism for generation of a variety of cell shapes and diverse cellular patterns on a global scale with remarkable precision.

4. Conclusions and Perspectives

Studies of the fly eye have revealed both simplicity and complexity of organ formation. The simplicity lies in a few simple rules being reiteratively utilized to control successive steps of organ formation. Two themes emerge from the fly eye: the interplay between cell signaling and competence determines diversity of cell types and selective cell adhesion determines diversity of cellular patterns. The interplay between cell signaling and competence creates three signaling relays, which sequentially trigger differentiation of all cell types. Selective cell adhesion, when temporally and spatially regulated, can provide physical forces to assemble cells into diverse spatial patterns. The complexity lies in the qualitative and quantitative precision of gene expression. Any organ system is built on cells and each cell is a system. Understanding the intrinsic properties of the cell is key to understanding the precision of organ formation.

A unique cell fate depends on both competence of the cell and signaling the cell receives. The concept of competence was put forward over six decades ago (Waddington, 1940). To date it becomes clear that competence depends on intrinsic factors that determine the response of the cell to stimuli by both transcriptional and posttranscriptional mechanisms. However, we are still unable to clearly define competence. There are two major obstacles that hinder our efforts. First, we have only incomplete knowledge of intrinsic factors that determine competence. Studies in the past century have uncovered some critical factors. However, they are perhaps still the tip of the iceberg. Functions of a gene can be easily masked by functional redundancy and may not be uncovered by the traditional genetic screens. Epigenetic regulation adds one more layer of complexity to the system. Second, competence itself is dynamic. A competent cell in a developing organ is in an unstable state and biochemical reactions with the cell evolve over time even without the outside influence (Waddington, 1940). The environment of a cell, through cell signaling, further alters the dynamics of these reactions. The dynamic nature of competence may not be readily revealed in fixed tissues. Competence is most likely composed of actions and reactions of multiple factors and the number of interactions among genes and proteins at any given time point can be quite large. Integration of these interactions requires systems biology and computational biology.

Despite these challenges, understanding competence is central to understanding cells in a developmental context. In addition, a parallel between cells in a developing organ and those in certain complex pathological conditions such as cancer suggests that cells in those complex diseases may not be accurately reflected by changes in the activity of one or a few "pathways." Rather, they should be viewed and treated as cells with altered "competence" in an altered environment.

As predicted by D'Arcy Thompson, biological processes follow universal physical principles (Thompson, 1917). Evidence accumulated to date clearly indicates that surface mechanics plays a role in the self-assembly of tissues and organs. We are only beginning to understand the physical properties underlying morphogenetic processes in the eye. Cell–cell adhesion mediated by known adhesion molecules can explain how simple spatial patterns of cells are generated and maintained. We still do not understand how complex patterns such as organization of photoreceptors in clusters are generated and maintained. Further, cell adhesion has to be dynamically regulated. How does cell signaling and competence control temporal and spatial expression patterns of adhesion molecules? In developing organs, cells are constantly changing positions. How do cells accomplish rapid assembly and disassembly of junctions in response to stimuli? How do changes in the cytoskeleton rapidly affect adhesive interactions on the surface? Answers to these questions will be instrumental for further understanding the molecular basis of organ formation.

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CHAPTER FIVE

Building a Fly Eye: Terminal Differentiation Events of the Retina, Corneal Lens, and Pigmented Epithelia

Mark Charlton-Perkins *and* Tiffany A. Cook

Contents

Abstract

In the past, vast differences in ocular structure, development, and physiology throughout the animal kingdom led to the widely accepted notion that eyes are polyphyletic, that is, they have independently arisen multiple times during evolution. Despite the dissimilarity between vertebrate and invertebrate eyes, it is becoming increasingly evident that the development of the eye in both groups shares more similarity at the genetic level than was previously assumed, forcing a reexamination of eye evolution. Understanding the molecular underpinnings of cell type specification during *Drosophila* eye development has been

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a focus of research for many labs over the past 25 years, and many of these findings are nicely reviewed in Chapters 1 and 4. A somewhat less explored area of research, however, considers how these cells, once specified, develop into functional ocular structures. This review aims to summarize the current knowledge related to the terminal differentiation events of the retina, corneal lens, and pigmented epithelia in the fly eye. In addition, we discuss emerging evidence that the different functional components of the fly eye share developmental pathways and functions with the vertebrate eye.

Abbreviations

CC	cone cell
IOB	interommatidial bristle
IOC	interommatidial cell
IPR	inner photoreceptor
MF	morphogenetic furrow
OPR	outer photoreceptor
PPC	primary pigment cell
PR	photoreceptor
Pros	Prospero
Rh	Rhodopsin
RPE	retinal pigment epithelia
Sens	Senseless
SPC	secondary pigment cell
TPC	tertiary pigment cell

1. Overview

The adult compound eye of the fruit fly, *Drosophila melanogaster*, is composed of a repeated array of ~800 individual unit eye, called ommatidia. Each adult ommatidium consists of approximately 20 cells (Fig. 5.1). Eight of these cells are photoreceptor (PR) neurons, photosensitive cells that project directly to the brain to transmit visual input. Immediately atop the PRs are six nonneuronal cells—four cone cells (CCs) and two primary pigment cells (PPCs)—that together secrete the corneal lens and an underlying crystalline structure known as the pseudocone. Approximately six secondary pigment cells (SPCs) and tertiary pigment cells (TPCs), also called interommatidial cells (IOCs), are then shared to form a boundary between

ommatidia to limit light scattering. Finally, a mechanosensory bristle (inter-ommatidial bristle, IOB) is present at every other apex of each ommatidium (Cagan and Ready, 1989a).

Drosophila undergoes a series of metamorphic processes before eclosing as an adult fly, 10 days after hatching. During each developmental stage, the eye undergoes discreet molecular and cellular changes. As an embryo, the organism sets aside small sets of cells that eventually produce adult external structures, such as the eye, wings, and legs. The cells specified to become ocular tissue are reserved in the larvae as part of the eye-antennal imaginal disc (see Fig. 5.2A and Chapter 1), a flat epithelial sheet that proliferates while the organism feeds and grows via three larval stages. At the end of the third and last larval stage, an epithelia-to-neuronal transition occurs at the anterior portion of the disc, marked by a physical change in the structure of the eye disc known as the morphogenetic furrow (MF). The MF migrates posterior to anterior through the eye disc, leaving behind cell clusters that ultimately mature into the highly regular lattice of ommatidia that forms the adult compound eye (Fig. 5.2A and B; Cagan and Ready, 1989a; Tomlinson and Ready, 1987b; Wolff and Ready, 1993).

Neuronal specification is the initial step of ommatidia formation and involves a stereotypical recruitment of the eight PR cells, R1–R8, through the reiterative use of EGF and Notch signaling (Brennan and Moses, 2000; Doroquez and Rebay, 2006; also see Chapter 4). The R8 cell arises first, followed by pairwise recruitment of R2/5, R3/4, and R1/6, and ending with R7 recruitment. Next, four nonneuronal CCs (also known as Semper cells) are recruited, and these cells are the last to be added during larval development. During early pupation, two PPCs then join each ommatidial cluster and fully enwrap the CCs (Fig. 5.2C–F). The light-isolating pigmented IOCs and the IOB are also recruited at this time and adopt a highly regular organization at the apical surface of the pupal retina (Fig. 5.2C and E–H; Cagan and Ready, 1989a; Ready *et al.*, 1976; Waddington and Perry, 1960; Wolff and Ready, 1993).

All of the initial specification and patterning of the PRs, CCs, PPCs, IOCs, and IOBs occurs within a flat epithelial sheet and is complete within the first half of pupation. It is only during the last half of pupation that this flat retinal surface reshapes into the complex three-dimensional adult eye (Fig 5.2). During this latter half of development, the PRs extend their light-gathering apical surface, establish appropriate connections in the brain, and express the necessary proteins for phototransduction. In addition, the pseudocone and corneal lens are secreted, and pigmentation is established. Below, we highlight some of the molecular events that are known to drive these terminal differentiation steps. As will become obvious from this discussion, PR differentiation has been well studied, whereas studies on corneal lens and pigmented epithelia development remain considerably less explored.

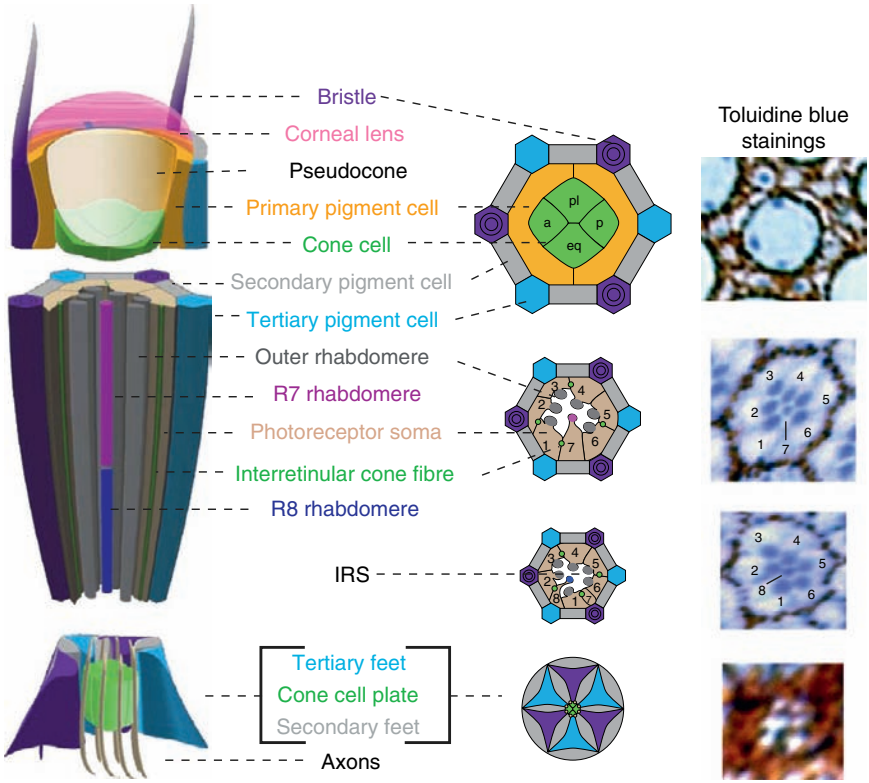


Figure 5.1 Structure of an adult *Drosophila* ommatidium. Schematic of different regions of an adult ommatidium: the corneal lens region (top), the neural retina (middle), and the retinal floor (bottom). Corresponding regions from toluidine blue-stained semi-thin sections of an ommatidium are provided at the right. Color scheme is as follows: photoreceptor (PR) cell bodies, beige; PR rhabdomeres, dark gray cylinders (outer PRs), dark magenta cylinder (R7), or dark blue cylinder (R8); cone cells, green; primary pigment cells, yellow; secondary pigment cells, gray; tertiary pigment cells, turquoise; mechanosensory interommatidial bristle, purple hexagon; eye unit, longitudinal. The cone cells and primary pigment cells secrete the corneal lens (translucent pink) and a gelatinous pseudocone (translucent white). Each cone cell also extends an "interretinular fiber" between the photoreceptors, eventually expanding just proximal to the rhabdomeres to create a CC feet "plate" at the base of the retina. Based on the position within the ommatidia, the four cone cells are referred to as the apical (a), posterior (p), polar (pl), and equatorial (eq) cone cells. Secondary and tertiary pigment cells and the bristle form a characteristic hexagon around each ommatidia, with the pigment granules easily observed in the toluidine blue stainings as reddish-brown (pteridine-containing) and black (xanthommatin-containing) vesicular-like structures. The apical surfaces of the secondary and tertiary pigment cells are tightly restricted, but the basal surfaces of these cells expand at the base of the retina to form a fenestrated membrane through which the axons project into the brain. The six outer photoreceptor rhabdomeres (gray from cells R1 through R6) form a trapezoid at the top of the eye and extend the length of the retina, enwrapping the IPR rhabdomeres—the R7 rhabdomere

2. The Retina: *Drosophila* PR Differentiation

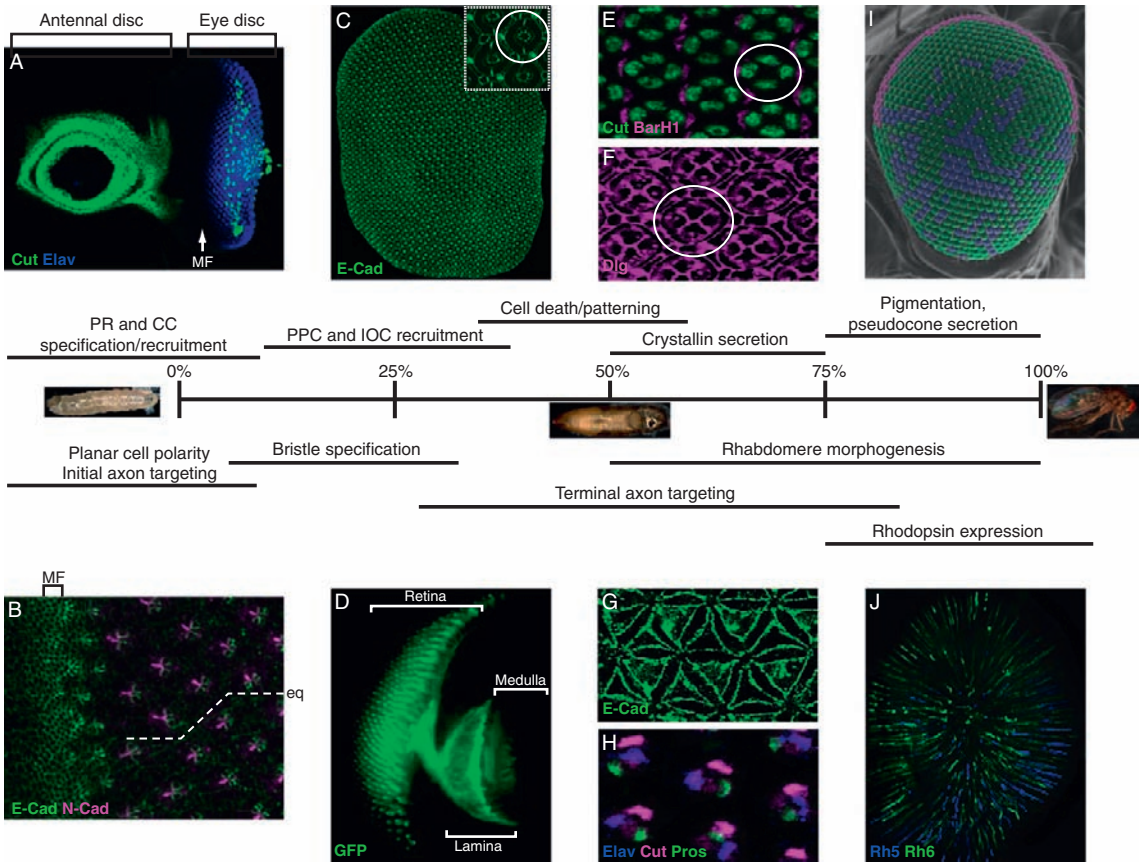
2.1. General overview of fly PR subtypes

Historically, two classes of PRs have been defined in the fly eye, called outer photoreceptors (OPRs) and inner photoreceptors (IPRs). Functionally, these classes largely correspond to vertebrate rod and cone PRs, respectively. As such, OPRs and IPRs differ in several respects, including their position within the ommatidium, cell shape, rhodopsin gene expression, axonal projections, and physiological function.

Similar to the ciliary-based outer segments of vertebrate PRs, an expanded apical membrane compartment, known as a rhabdomere, houses the light-sensitive Rhodopsin proteins and the phototransduction machinery in fly PRs. In the fly, however, this compartment is not ciliary based, but instead, is comprised of organized microvilli that form a long cylindrical structure.

Six of the eight PRs found within an ommatidium, the R1 through R6 cells, represent the rod-like OPRs, and each of these cells develops a large rhabdomere that spans the depth of the retina. Together, their rhabdomeres form an asymmetric trapezoid whose chirality is determined by the planar cell polarity pathway active within the R3 and R4 cells (Adler, 2002; Mlodzik, 1999; Strutt, 2008). At the equator of the eye, the chirality of the trapezoid changes, allowing mirror symmetry of the eye (Fig. 5.2B). OPRs are highly sensitive to a broad spectrum of wavelengths of light and are important for motion detection and vision under dim light conditions (Hardie, 1985; Meinertzhagen and Hanson, 1993). The remaining two PRs, R7 and R8, represent the cone-like IPRs. The rhabdomeres of these cells are shorter and more slender than OPRs, and function under bright light conditions for color discrimination, R7 cells detecting UV wavelengths (345–375 nm), and the majority of R8 cells being sensitive to blue (437 nm) or green (508 nm) wavelengths (Fig. 5.2J; Feiler *et al.*, 1992; Hardie, 1985; Salcedo *et al.*, 1999; Yamaguchi *et al.*, 2010). An exceptional subset of one to two rows of ommatidia is present in the dorsal

(Magenta) extends through the top two-thirds of the retina and the R8 rhabdomere (Blue) occupies the bottom third. In addition, the cell body of the R7 is positioned between the R1 and R6 cell, whereas the R8 cell body is located between the R1 and R2 cell, seen by cross section (middle diagrams and thin sections). The interhabdomeric space (white) that is important for preventing rhabdomere fusion is also seen. The entire central portion of the ommatidia is encapsulated by the cone cells—distally, with the rhabdomeres attached by "hemidesmosome-like" contacts, and proximally, with the rhabdomeres attached to the cone cell feet just below the end of the rhabdomere.


Antennal disc
Eye disc
A
Cut Elav
MF
C
E-Cad
E
Cut BarH1
F
Dlg
I
PR and CC specification/recruitment
PPC and IOC recruitment
Cell death/patterning
Crystallin secretion
Pigmentation, pseudocone secretion
0%
25%
50%
75%
100%
Planar cell polarity
Initial axon targeting
Bristle specification
Rhabdomere morphogenesis
Terminal axon targeting
Rhodopsin expression
MF
B
eq
E-Cad N-Cad
D
Retina
Medulla
Lamina
GFP
G
E-Cad
H
Elav Cut Pros
J
Rh5 Rh6

half of the eye in which the IPRs are not involved in color discrimination, but instead are involved in detecting the vector of polarized light important for navigation (Fig. 5.2I; Hardie, 1985; Labhart and Meyer, 1999; Wernet *et al.*, 2003). These ommatidia are referred to as Dorsal Rim Area (DRA) ommatidia (see Fig 5.2).

2.2. Terminal differentiation of fly PRs

Fly PR development occurs in two major steps: PR cell specification and terminal differentiation (Mollereau *et al.*, 2001). PR cell specification occurs during the latest stages of larval development, and has been a topic of extensive study (for a review, see Chapter 4). PR terminal differentiation occurs during pupal development when PRs form their rhabdomeres, establish proper axonal projections into the brain, and begin expressing the rhodopsin genes that will in part determine their adult function (see Fig. 5.2 timeline). Below, we briefly review some of these events and several of the molecular players that promote these processes.

Figure 5.2 Time course of *Drosophila* eye development. A summary of various developmental processes that occur during *Drosophila* pupal eye development (0–100%). Prior to pupation, in late third instar larva, the antennal/eye disc (A) is easily recognized by strong Cut expression (green) in the antennal portion (anterior, left), and clusters of Elav-positive photoreceptor clusters (blue) in the eye portion (posterior, right) corresponding to individual ommatidial units. Cut-positive cells are also present in the eye-imaginal disc, which represent subretinal glia and CCs precursors. Nonstained cells anterior to the morphogenetic furrow (MF) are retinal progenitors that are still proliferating (see Chapters 1 and 4 for further description). (B) The constricted apical surface of cells within the MF is obvious with E-Cadherin staining (green). In addition, the boundary between the R3 and R4 cell, marked by intense N-cadherin staining (purple), reveals the rotation of the ommatidia relative to the equator that is important for establishing the chiral trapezoid of photoreceptors observed in the adult retina. (C) E-cadherin staining (green) of a whole retina isolated from pupa at ~50% pupation shows the highly regular organization of ommatidia. Inset: A single ommatidium is circled. (D) Photoreceptor-driven Moesin::GFP at 50% pupation shows outer PR axons projecting to the lamina and IPR axons projecting to the medulla. (E) Cut (green) and BarH1(Magenta) specifically recognize the four CC and two primary pigment cell (PPC) nuclei at 50% pupation. (F) Discs Large (Dlg, Purple) highlights the apical contacts of the CCs, PPCs and interommatidial cells in 50% pupal retinas. (G) E-cadherin (green) of the basal surface of the retina shows the petal-shaped distribution of the IOC feet. (H) The bristle cell lineage is composed of four cells which express the transcription factors Cut and Pros, and the neural factor Elav. These nuclei are present at the base of the retina during their development, and eventually move more apically. (I) A scanning electron micrograph of an adult eye pseudocolored to represent the distribution of the pale (blue), yellow(green) and Dorsal Rim Area (DRA; magenta) ommatidia in the eye. (J) Whole mounted adult retina immunostained with Rhodopsin 5 (blue) and Rhodopsin 6 (green) in R8 rhabdomeres. Note the enrichment of Rh6 in the dorsal portion of the retina, corresponding to the dy ommatidia (see text for more detail).

2.2.1. Rhabdomere development

The rhabdomere is an elongated apical structure of tightly packed and highly organized microvilli which is supported by a stalk membrane and *zonula adherens* (Fig. 5.3; Izaddoost *et al.*, 2002; Longley and Ready, 1995; Pellikka *et al.*, 2002). Although the rhabdomere is the apical surface of the PR, it extends perpendicular to the cell body. This orientation results from the CCs rising above the PRs during early pupation, causing the PR apical membranes to turn 90° and appose one another (Cagan and Ready, 1989a; Longley and Ready, 1995). At ~55% pupation, microvillus projections begin to emerge and delineate the apical membrane surface into two functional units—the rhabdomere and the stalk (Cagan and Ready, 1989a; Longley and Ready, 1995). Simultaneously, an extracellular matrix forms in the interrhabdomeric space that surrounds each developing rhabdomere and contributes to the exact spacing and positioning of each rhabdomere within an ommatidium (Husain *et al.*, 2006; Zelhof *et al.*, 2006). This process continues rather rapidly, and by 78% pupation, the interrhabdomeric space is well established, the microvilli have elongated, and the rhabdomeres have an elliptical cross section that becomes progressively more round throughout development (Cagan and Ready, 1989a; Longley and Ready, 1995) see also see Supplemental Movie in Sang and Ready (2002). By adulthood, the OPR rhabdomeres have expanded from an initial length of ~1 μm to occupy the full depth of the retina of ~100 μm. The R7 rhabdomeres only occupy the distal two-thirds of the retina while the R8 rhabdomeres fill the proximal one-third (Hardie, 1985).

Rhodopsin contributes over 50% of a membrane proteins of the rhabdomere, and is required for the building and maintenance of the rhabdomere structure (Kumar and Ready, 1995). In fact, almost all proteins involved in the phototransduction pathway are similarly required to maintain photoreceptor integrity (Wang and Montell, 2007), indicating that like vertebrate PRs, form and function are tightly linked. Perhaps not surprisingly, transcription factors that regulate the expression of different components of the phototransduction machinery are also important for regulating rhabdomere morphogenesis. Two such factors are the homeodomain transcription factors Orthodenticle (Otd) and Pph13/Hazy (Mishra *et al.*, 2010; Ranade *et al.*, 2008; Tahayato *et al.*, 2003; Vandendries *et al.*, 1996; Zelhof *et al.*, 2003). Interestingly, Otd and Pph13 individually regulate subsets of rhodopsins and phototransduction-encoding genes, and mutations in either factor cause rhabdomere defects; however, PRs lacking both factors fail to form any rhabdomeric structure, providing evidence that these factors control two independent PR morphogenetic pathways (Mishra *et al.*, 2010). How these pathways are integrated, however, remains to be determined.

Besides the phototransduction machinery, a number of actin-binding proteins are critical for building the microvilli-rich rhabdomeric membrane,

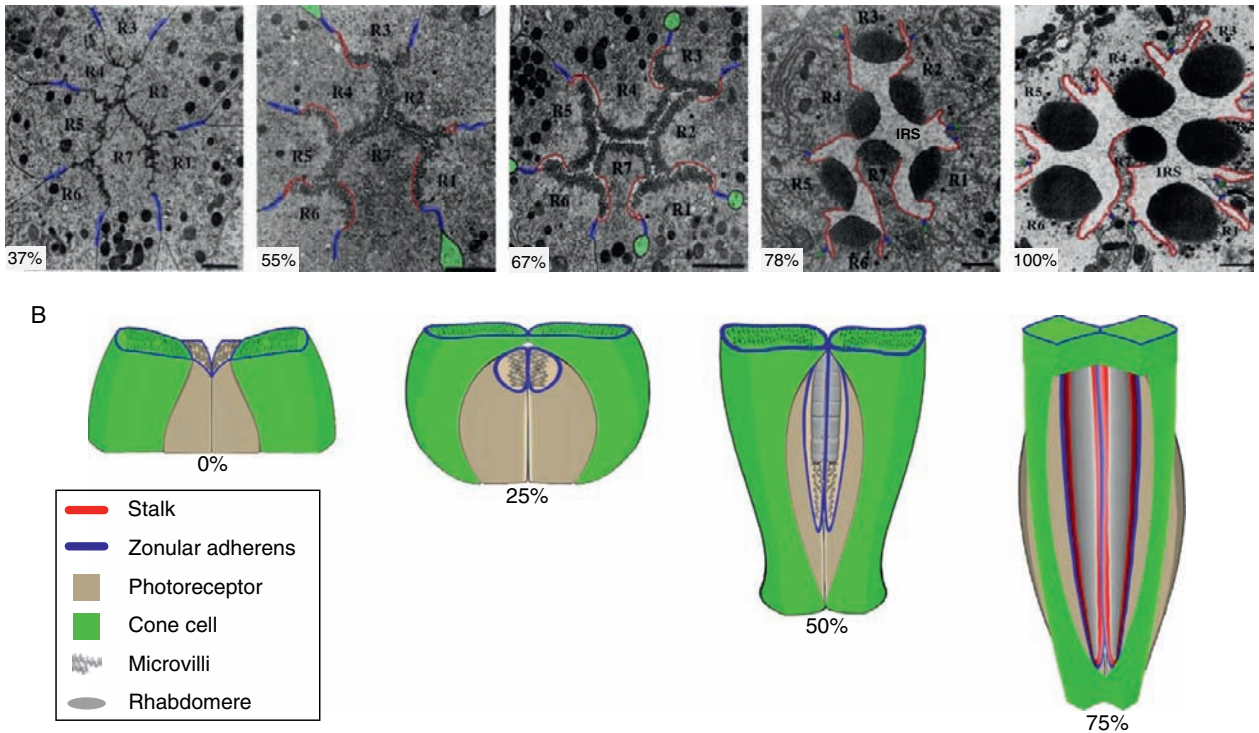


Figure 5.3 Rhabdomere morphogenesis. (A) Coronal TEMs showing the apical membrane elaborations of photoreceptors R1 through R7 at different stages of development. The zonula adherens are marked with blue, the stalk region is highlighted in red, and the interrhabdomeric space (IRS) is the clear space between rhabdomeres that are obvious by 78% pupation (modified from Longley and Ready, 1995, with permission from Elsevier). Some of the interretinular fibers from cone cells, found directly adjacent to the *zonula adherens* are highlighted in green. (B) Diagram of the 90° turn of the photoreceptor apical surfaces during early pupation and elongation of the rhabdomeres (gray), the stalk region (red), and *zonula adherens* (blue) at later stages of development. Only two cone cells (green) and two photoreceptors are shown for clarity.

including Amphiphysin, WASp, Rac1, Moesin, Myo-II, MyoIII, and MyoV (Baumann, 2004; Chang and Ready, 2000; Deretic *et al.*, 2004; Hicks *et al.*, 1996; Li *et al.*, 2007; Zelhof and Hardy, 2004; Zelhof *et al.*, 2001). Moreover, molecules present in the stalk region, the *zonula adherens*, and the interrhabdomeric space play critical roles in rhabdomere elongation and maintenance. The stalk region expresses apical complex proteins such as Crumbs, Dpatj, and Par-6, and mutations in these factors lead to shortened and/or bifurcated rhabdomeres (Bachmann *et al.*, 2008; Izaddoost *et al.*, 2002; Nam and Choi, 2003, 2006; Nam *et al.*, 2007; Pellikka *et al.*, 2002; Richard *et al.*, 2006a). The *zonula adherens* also recruit members of the Par protein complex (e.g., Par3), and mutations in these factors disturb distal, but not proximal, rhabdomere formation (Pinal *et al.*, 2006). In contrast, components of the interrhabdomere space, including spacemaker (also known as eyes shut), prominin, and chaoptin, are important for maintaining distinct rhabdomeres between PRs, with mutations leading to the coalescence of rhabdomeres, a phenotype reminiscent of the fused rhabdoms commonly found in other invertebrate compound eyes (Husain *et al.*, 2006; Van Vactor *et al.*, 1988; Zelhof *et al.*, 2006). Finally, factors associated with microtubule-based vesicle transport are critical for rhabdomere formation, including the small GTPases Rab1, Rab6, and Rab11 (Satoh *et al.*, 1997, 2005; Shetty *et al.*, 1998) as well as the Dynein/Dynactin complex (Fan, 2004; Fan and Ready, 1997; Tai *et al.*, 1999), likely by transporting membrane-associated proteins such as Rhodopsin and TRP channels to the rhabdomeres, as well as regulating the endocytic recycling of these factors. As will be discussed later, many of these same proteins are also important for the formation and maintenance of vertebrate PRs, suggesting that studies of fly PR morphogenesis will be an important resource for understanding events related to retinal degeneration in vertebrates.

Because of the large rhabdomeres of OPRs and their preponderance in an ommatidium, much of what is understood about rhabdomere morphogenesis derives from studies of R1–R6 cells. While many of the same factors are also important in IPR morphogenesis, IPRs do exhibit differences that raise questions as to whether these cells require distinct regulatory pathways for their differentiation. For example, how is the smaller diameter of IPR rhabdomeres achieved, how is the length of their shorter elongation controlled, and how is the R7 rhabdomere positioned distally to the R8 rhabdomere? Similarly, how do the IPRs in DRA ommatidia (see below) acquire the same diameter as OPRs, and how do these cells form the distinct untwisted organization of their microvilli required for light polarization sensitivity, in contrast to all other rhabdomeres? Several factors involved in OPR morphogenesis are different in IPRs. For instance, Myo-II is critical for OPR rhabdomere formation, yet its expression in IPRs is weaker (Baumann, 2004)—could this account for the smaller size of their

rhabdomere? In addition, several transcription factors originally identified for their ability to regulate IPR rhodopsin gene expression also control distinct aspects of IPR-specific morphogenetic processes. The zinc finger transcription factor, Senseless (Sens), and the homeodomain protein Otd, for example, are important to preserve the proximal position of the R8 cell (Tahayato *et al.*, 2003; Xie *et al.*, 2007), while the TALE homeodomain transcription factor Homothorax is critical for mediating all aspects of DRA ommatidia development, including their unique rhabdomere structure (Wernet *et al.*, 2003). The target genes that these factors regulate to control these events are entirely unexplored, but should be useful for uncovering additional pathways that are important during PR morphogenesis.

2.2.2. Nuclear position

During specification, PR nuclei show a stereotypical basal-to-apical position as they are recruited: the nuclei from previously recruited cells are forced basally, so that eventually, the latest "born" cell nuclei are most apically position, and the oldest "born" cells have more basally located nuclei (Fig 5.7). One exception to this rule occurs with the R3/R4 nuclei, which maintain apical contacts even after the R1/R6 cells have been recruited (Tomlinson and Ready, 1987b); however, the functional consequence of this difference is currently unknown.

In the adult eye, the PR nuclei also occupy characteristic positions: the OPR nuclei are positioned most distally, the R7 nucleus lies slightly below these, and the R8 nucleus occupies the most proximal portion of the retina. This means that the rhabdomeres of R1–R7 project proximally from their nuclei, whereas the rhabdomere of the R8 projects distally from its nucleus, suggesting this cell adopts a distinct cell polarity. Consistent with cell polarity being involved in proper nucleus localization, microtubule- and actin-associated proteins such as Glued/Dynactin and Klarischt/Marbles/Laminin A affect nuclear position of most PRs, even in the imaginal disc (Fan and Ready, 1997; Fischer-Vize and Mosley, 1994; Whited *et al.*, 2004). PR-specific factors also can control nuclear position. Prospero (Pros), for instance, is expressed in the R7 cell, and Pros mutants develop IPRs whose rhabdomeres still retain their distal R7 position, but whose nuclei are proximally located, a characteristic unique to R8s (Cook *et al.*, 2003). Likewise, overexpressing the R8-specific transcription factor Sens in R7 cells fails to change the position of the R7 rhabdomere, but does lead to a proximally positioned nucleus (Xie *et al.*, 2007). Since Pros and Sens also influence other aspects of R7 versus R8 cell fates (see below), these data suggest that the nuclear position differences between the R7 and R8 are intimately linked to their cell fate choice. Why this is the case is currently not understood, but studies aimed at this question are likely to uncover additional developmental differences between these two related cell types.

2.2.3. PR projections

Axons from OPRs and IPRs project to two distinct optic ganglia beneath the retina: OPRs project to the first optic ganglion, the lamina, whereas R7 and R8 IPRs segregate to distinct layers in the second optic ganglion, the medulla (Fig. 5.4). Similar to other aspects of retinogenesis, PR axonal projection patterning occurs through two distinct processes and is distinct for OPRs versus IPRs. This topic has been extensively and elegantly reviewed recently, and thus will only be briefly summarized here (for reviews see Chiba, 2001; Mast *et al.*, 2006; Matthews *et al.*, 2008; Sanes and Zipursky, 2010; Tayler and Garrity, 2003).

PRs begin projecting their axons immediately after their specification in the eye imaginal disc. As the R8 is the first cell to be recruited during eye development, it initiates PR axonal projections into the developing optic lobes, to the top layer of the medulla, the M1 layer. As the R8 passes

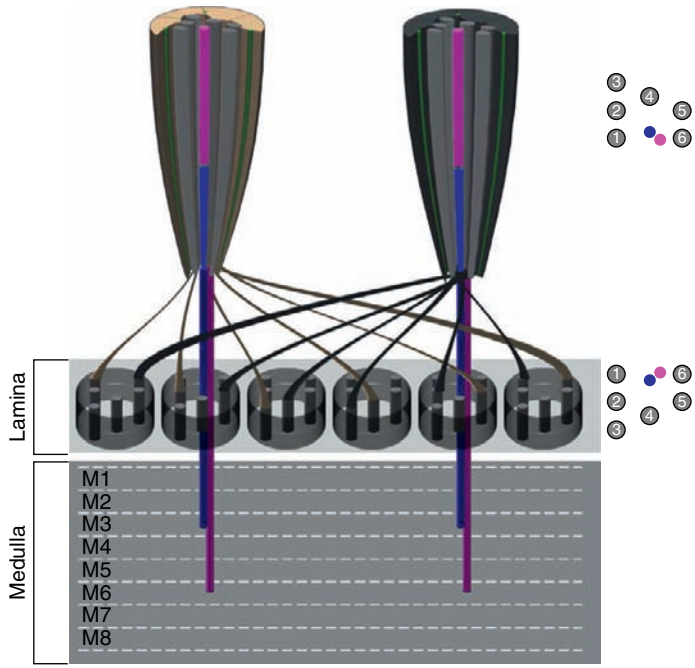


Figure 5.4 Axonal targeting differences between outer and inner photoreceptors. Diagram representing two ommatidia sharing lamina cartridges. The axons from the six outer PRs from each ommatidium turn 180° and project to six different cartridges present in the lamina neuropil present directly underneath the retina. R1–R6 positions within the lamina represent a mirror image of the outer photoreceptor arrangement found in the retina. The R7 (magenta) and R8 (blue) axons bypass the lamina and project to layers M3 and M6 respectively in the adult medulla.

through the lamina, it activates the proliferation and differentiation of lamina neurons, which then recruit additional glia (Dearborn and Kunes, 2004; Perez and Steller, 1996; Winberg *et al.*, 1992). The OPRs follow the R8 axon fascicles into the lamina, where they encounter two rows of glia, called the lamina plexus, that prevent OPRs from projecting beyond this point (Poeck *et al.*, 2001). The R7 cell then projects its axon through the lamina and terminates in a layer slightly below the R8 projection. Interestingly, projections to the medulla appear to be a default choice, because many factors important for R1–R6 projections cause misprojections into this optic neuropil, and play permissive rather than instructive roles (Cafferty *et al.*, 2004; Garrity *et al.*, 1996; Hing *et al.*, 1999; Kaminker *et al.*, 2002; Newsome *et al.*, 2000; Ruan *et al.*, 2002; Suh *et al.*, 2002).

These initial axonal projections are maintained until approximately 30% pupation, and afterward, undergo further refinement. At this time, the OPR axons from individual ommatidia begin to establish lateral contacts with other ommatidia in a process known as neural superposition (for reviews see Hardie, 1985; Meinertzhagen, 1975). This is an important process in Drosophila, because the rhabdomeres of different OPRs within a single ommatidium point to different directions whereas OPRs from adjacent ommatidia do converge on the same point, due to the curvature of the eye. Thus, to integrate the visual input from photoreceptors in separate ommatidia that converge on the same point, OPR axons twist 180° and project outward into six different "lamina cartridges," maintaining a spatial pattern that replicates their position within the ommatidia (Fig. 5.4). For instance, the R1 PR axon projects to an R1 position within one cartridge, while the R2 projects to the R2 position in different cartridge. This convergence of visual information across six ommatidia leads to increased sensitivity and providing input important for motion detection. Interestingly, just like during the establishment of rhabdomere polarity in the retina, the R3/R4 PRs also determine the orientation of projections during neural superposition (Clandinin and Zipursky, 2000). Moreover, the atypical cadherin molecule Flamingo that controls the Frizzled-dependent asymmetric localization of the R3/R4 rhabdomeres is also critical for directing OPR axon growth cones to the correct cartridges (Lee *et al.*, 2003; Usui *et al.*, 1999). These data highlight the coordinated use of the same factors to establish proper positioning and function for PRs during fly retinogenesis.

Unlike the OPRs, the R7 and R8 within an ommatidium share the same light path, and since their axons pass directly through the lamina layer into the medulla, they provide a perfect retinotopic map of the eye. Immediately after reaching the medulla, R7 projects slightly deeper than R8. At ~17% pupation, the medulla begins to laminate, which allows further separation of the R7 and R8 axonal terminals. By 35% pupation, R7 has reached the M3 layer in the medulla while R8 remains in the M1 layer. At approximately 50% pupation, both the R7 and R8 axons project deeper into the medulla, with

R7 reaching its final destination in the M6 layer and R8 terminating at the M3 layer (Fig. 5.4). Similar to Flamingo functioning redundantly to control OPR patterning and axonal projections, factors important for controlling several aspects of R7 versus R8 PR patterning in the retina are also important for their proper targeting in the medulla. For instance, the transcription factor Sens not only regulates R8 specification, R8 cell position, and rhodopsin expression, but it is critical for M6-specific targeting of the R8 axon (Frankfort *et al.*, 2001; Morey *et al.*, 2008; Xie *et al.*, 2007). Likewise, the homeodomain protein Pros, involved in R7-specific rhodopsin expression and nuclear position, is partly responsible for the targeting of R7 cells to the M3 layer (Cook *et al.*, 2003; Kauffmann *et al.*, 1996; Morey *et al.*, 2008). Interestingly, mutation of either Sens or Pros lead to a reciprocal switch in R7 vs R8 projections—that is, *sens* mutants project to the R7 layer, while *pros* mutants project to the R8 layer (Morey *et al.*, 2008), arguing that IPRs share factors that mediate medulla projections, with Sens and Pros further refining this projection pattern to distinct layers. To date, such a molecule has not yet been identified, but interesting candidates for this are Runt and Spalt, two transcription factors whose expression is restricted to the R7 and R8 shortly after their neural specification in the eye imaginal disc. Indeed, misexpressing Runt in OPRs does lead to mistargeting to the medulla. However, both Runt and Spalt loss of function IPRs maintain their appropriate targeting in the medulla, suggesting that other factors must also be involved or that these factors function redundantly (Kaminker *et al.*, 2002; Mollereau *et al.*, 2001).

2.2.4. Rhodopsin gene expression

OPRs are important for motion detection, whereas IPRs are important for color discrimination under bright light conditions (Bicker and Reichert, 1978; Hardie and Kirschfeld (1983); Hardie (1979); Hu and Stark, 1980; Menne and Spatz, 1977; Yamaguchi *et al.*, 2010). In order to capture as much light information as possible, all OPRs express the same broad wavelength-sensitive Rhodopsin, Rhodopsin 1 (Rh1; O'Tousa *et al.*, 1985; Stark *et al.*, 1976; Zuker *et al.*, 1985). In contrast, IPRs express a complex pattern of Rhodopsin-encoding genes in order to maximize the range of wavelengths they detect—R7 cells express UV-sensitive opsins, Rh3 and/or Rh4, while R8 cells can express Rh3, the blue-sensitive Rh5, or the green-sensitive Rh6 (Chou *et al.*, 1996, 1999; Fortini and Rubin, 1990; Fryxell and Meyerowitz, 1987; Huber *et al.*, 1997; Mazzoni *et al.*, 2008; Mismer and Rubin, 1989; Montell *et al.*, 1987; Zuker *et al.*, 1987; Fig. 5.6). Rh gene expression begins during late (79–84%) pupation, with OPR-specific Rh1 being expressed first, and IPR-specific Rhs 3–6 being expressed shortly thereafter (Earl and Britt, 2006). Here, we will discuss the genetic pathways relevant for establishing the cell-specific expression of the *Rhodopsin*-encoding genes, as these have helped elucidate a better understanding of the genetic relationships among different color-sensitive photoreceptors.

2.2.4.1. Rhodopsin promoters are multipartite The expression of each Rhodopsin protein can be properly recapitulated by <250 bp of regulatory sequence upstream of the TATA box (Fortini and Rubin, 1990; Papatsenko *et al.*, 2001; Tahayato *et al.*, 2003). Sequence analysis of these promoters revealed a Rhodopsin Conserved Sequence I (RCSI) that is shared by all six *Drosophila* Rhodopsin promoters (Fig. 5.5). This sequence is an inverted repeat of a homeodomain-binding site separated by 3 nucleotides, and matches the P3 site previously identified as a perfect recognition sequence for a subset of paired-related homeodomain-containing proteins that include Pax6, the "master control gene" for eye development (Czerny and Busslinger, 1995; Sheng *et al.*, 1997; Wilson *et al.*, 1993; also see Chapter 1). Consistent with the possibility that Pax6 may regulate rhodopsin gene expression through this site, multimerization of the RCSI (3XP3)

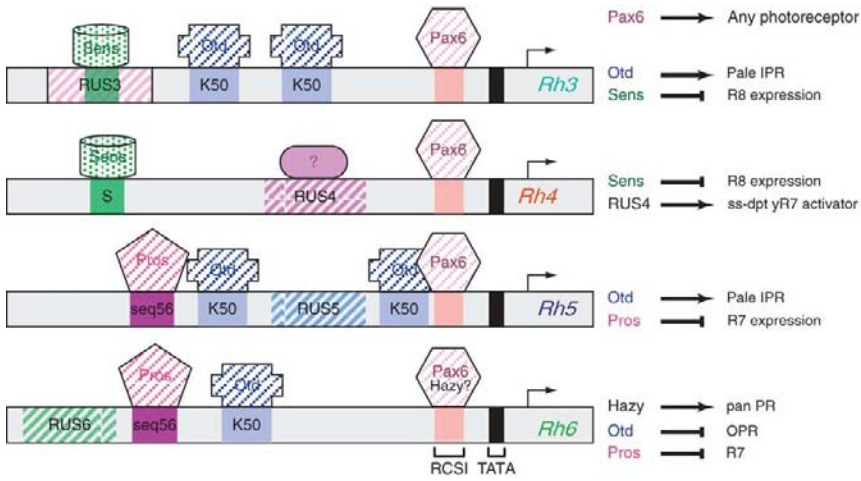


Figure 5.5 Regulatory sequences of the inner photoreceptor Rhodopsin-encoding genes. Schematic of the minimal promoters for Rh3 through Rh6 that recapitulate expression of the endogenous genes. Senseless binding sites (S) are green, Otd binding sites (K50) are light blue, Pax6/RSCI sites (Rhodopsin Conserved Sequence I) are pale pink and Pros sites (seq56) are dark magenta. Rhodopsin Unique Sequences (RUS) 3, 4, 5, and 6 are represented by striped boxes. The summary of the role of each transcription factor is highlighted to the right. Otd activates Rh3 and Rh5, the two Rhodopsins expressed the pale ommatidia, and represses Rh6 in outer photoreceptors (Tahayato *et al.*, 2003). Pros represses the R8 Rhodopsins, Rh5 and Rh6, in R7 photoreceptors (Cook *et al.*, 2003), while Sens represses the R7 Rhodopsins, Rh3 and Rh4, in R8 photoreceptors (Xie *et al.*, 2007). A transcription factor that is predicted to be activated by Spineless in yellow R7 cells to activate Rh4 is indicated by a ? on the Rh4 promoter. In addition, Hazy has recently been shown to be necessary and sufficient for Rh6 expression and bind to the RCSI, making it possible that Hazy, and not Pax6, is responsible for activating the Rh6 promoter in the fly eye (Mishra *et al.*, 2010).

is sufficient to drive PR-specific gene expression in a wide range of animals, suggesting that the RCSI is recognized by an evolutionarily conserved paired-like transcription factor present in PRs like Pax6 (Berghammer *et al.*, 1999; Gonzalez-Estevez *et al.*, 2003; Sheng *et al.*, 1997). However, whether Pax6 is the only factor responsible for this function currently remains unclear since recent studies indicate that another homeodomain factor, Hazy/Pph13, may also be critical for regulating RCSI-dependent *Rh* gene expression (Mishra *et al.*, 2010; Punzo *et al.*, 2001).

Outside the RCSI, each *Rh* promoter contains unique upstream sequences (Rhodopsin Unique Sequences, RUS) that show strong homology across multiple *Drosophila* species (Fig. 5.5), suggesting that these elements are responsible for directing gene-specific regulation (Fortini and Rubin, 1990; Papatsenko *et al.*, 2001; Tahayato *et al.*, 2003). This led to the model that Rhodopsin promoters are bipartite, with the RCSI providing generic PR specificity, and RUS elements providing subtype specificity (Fortini and Rubin, 1990). However, additional sequences have since been identified that are shared between different "classes" of rhodopsin promoters: for instance, both R7-specific rhodopsin promoters contain a conserved R8 repression element (S box, Fig. 5.5), and both R8 rhodopsin promoters share a conserved R7 repression element (seq56, Fig. 5.5; Cook *et al.*, 2003; Tahayato *et al.*, 2003; Xie *et al.*, 2007). This suggests that a more complex combinatorial regulation leads to the diverse patterns of IPR rhodopsin gene expression. While the factors that recognize these shared sequences have largely been identified, and are discussed in more detail below, the factors that recognize the RUS sequences remain surprisingly elusive.

2.2.4.2. OPR versus IPR decisions

The Spalt (Sal) genes define IPR cell fate. Based on the fact that PR specification begins with the R8 and culminates with the R7, mature R7 and R8 cells were believed to arise from genetically distinct cell types. However, using a genetic screen for PR-restricted enhancer traps in the adult eye, Mollereau *et al.* (2000, 2001) identified a zinc finger transcription factor complex (*spalt* genes SalM and SalR), that is specifically enriched in R7 and R8 PRs, suggesting for the first time that these cells may share genetic components. Consistent with the possibility that Spalt regulates the fate of both cells, when the *sal* complex is genetically removed, ommatidia develop eight to nine OPRs and no IPRs, while overexpressing SalM in OPRs is sufficient to convert all PRs into IPRs (Domingos *et al.*, 2004; Mollereau *et al.*, 2001) These findings led to the discovery that PRs in the eye imaginal disc are bipotential, requiring Sal expression in the R7 and R8 to define IPR versus OPR cell fates. These studies also revealed that PR development occurs in at least two developmental steps: recruitment in the imaginal disc, and IPR versus OPR cell fate

choices later during development. Currently, the mechanisms by which the *sal* genes achieve their function remain unclear.

2.2.4.3. R7 versus R8 cell fate decisions: Pros and Sens coordinate Rhodopsin expression, axonal targeting, and cell morphology Mature R7 and R8 IPRs differ in numerous respects, including rhabdomere position, nucleus location, axonal targeting, and opsin gene expression. Thus, the finding that Sal controlled IPR versus OPR decisions led to questions of how the R7 and R8 cell then later distinguish themselves from a common IPR precursor. Many answers to this question came from studies focused on understanding how the IPR *Rhodopsin* genes themselves are regulated. Pros, for instance, was identified in a yeast one-hybrid screen for its ability to bind to a conserved sequence in the Rh5 and Rh6 promoters, and was subsequently shown to be expressed in R7 cells to specifically repress the expression of these R8-specific opsins, as well as prevent R8-specific nuclear position and axonal projections (Cook *et al.*, 2003; Kauffmann *et al.*, 1996; Morey *et al.*, 2008). In contrast, the transcription factor Sens is expressed in R8 cells, binds to and represses R7-specific opsin promoters through a common S-box sequence, and prevents R7-specific rhabdomere position and axon targeting (Cook *et al.*, 2003; Morey *et al.*, 2008; Xie *et al.*, 2007). Sens also contributes to positively activating both R8-specific opsins, likely as a non-DNA-binding coactivator (Xie *et al.*, 2007). Together, these data suggest that Sal specifies a "generic" or default IPR that can express all IPR opsins, has an R8-like nuclear position, and has an R7-like rhabdomere position. Subsequently, Pros in the R7 and Sens in the R8 then repress the characteristics that are incompatible with their proper function in the adult eye. While their ability to regulate Rhodopsin gene targets is clear, how Pros and Sens control other aspects of R7 versus R8 cell fates, and whether other factors also participate in refining IPR differences remains an open question.

2.2.4.4. Ommatidial subtype specification: Hth, IroC, Otd, Ss, Melt, and Wts Four distinct subtypes of ommatidia, called pale (p), yellow (y), dorsal yellow (dy), and dorsal rim area (DRA) ommatidia, are present in the adult eye and are defined based on which rhodopsins are expressed the R7 and R8 cells (Fig. 5.6B). DRA ommatidia are the least abundant of the subtypes, and these are restricted to one to two rows of ommatidia along the dorsal half of the eye. Unlike the other three subtypes, DRA ommatidia are not involved in color discrimination, but instead are involved in discerning the vector of polarized light to aid during navigation (Hardie, 1985; Labhart and Meyer, 1999; Wernet *et al.*, 2003; Wunderer and Smola, 1982). This is facilitated by the fact that both IPRs express the same UV Rhodopsin, Rhodopsin 3, and because the membranes of the two IPR rhabdomeres

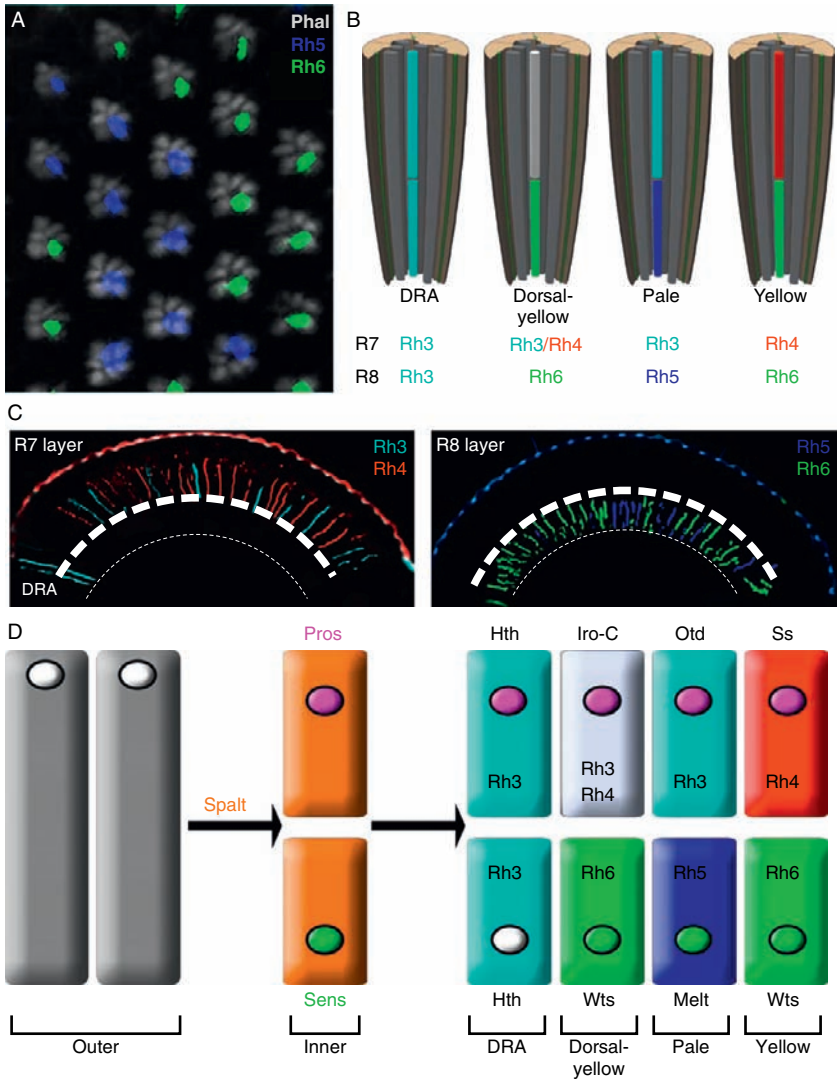


Figure 5.6 Ommatidial subtypes express different inner photoreceptor Rhodopsins. (A) A whole-mount staining of an adult retina stained with phalloidin (gray) shows the trapezoidal arrangement of the actin-rich rhabdomeres of the six outer photoreceptors and the random distribution of the pale and yellow ommatidia are revealed by immunostaining for Rh5 (blue) and Rh6 (green) that are expressed in the central R8 cells. (B) Diagram of the Dorsal Rim Area (DRA), dorsal yellow, pale, and yellow subsets found in the *Drosophila* eye, defined by the Rhodopsins expressed in the R7 and R8 inner photoreceptors. All outer photoreceptors express the same Rhodopin, Rhodopsin 1. (C) Transverse sections of adult eyes, dorsal left, stained with R7 Rhodopsins (left), Rh3 (cyan) and Rh4 (red), or R8 Rhodopsins (right), Rh5 (blue) and Rh6 (green). Note that two rows of ommatidia at the dorsal side of the eye express Rh3 in the R7 and

form two crossed-over polarizing filters (Labhart and Meyer, 1999; Wernet *et al.*, 2003; Wunderer and Smola, 1982).The TALE homeoprotein, Homothorax (Hth), is necessary and sufficient to induce all known DRA characteristics (Wernet *et al.*, 2003); however, the responsible mechanisms and the target genes utilized by Hth to accomplish this function remains unexplored.

Distinction of p and y ommatidia was originally observed by the presence of the random distribution of a screening pigment in ~70% of ommatidia that appeared yellow under white light illumination versus the pale appearance in the remaining 30% of ommatidia (Kirschfeld *et al.*, 1978). Later molecular analysis of Rh gene expression in *Drosophila* noted that the 30:70 ratio corresponded to the ratio of R7 cells expressing Rh3 and Rh4, (Fortini and Rubin, 1990) and R8 cells expressing Rh5 and Rh6, respectively (Chou *et al.*, 1996; Papatsenko *et al.*, 1997). Indeed, ~30% of ommatidia ("pale" ommatidia) express coupled Rh3:Rh5 expression in the R7 and R8, respectively, while the remaining ~70% of ommatidia ("yellow" ommatidia) express coupled Rh4:Rh6 in the R7 and R8 together with an additional screening pigment that gives the yellow color under white illumination (Chou *et al.*, 1996, 1999; Mazzoni *et al.*, 2008; Papatsenko *et al.*, 1997; Stark and Thomas, 2004; Fig. 5.6A–C). Interestingly, Mazzoni *et al.* (2008) recently noted that a subset of "yellow" ommatidia that are restricted to the dorsal third of the eye coexpress Rh3 and Rh4 in the R7, but still express Rh6 in the underlying R8. Thus, these dorsal-restricted ommatidia are referred to as dorsal yellow (dy) ommatidia. These are a particularly curious subset of ommatidia, as they do not adhere to the normal "one sensory receptor per sensory cell" paradigm commonly adopted in sensory systems to avoid overlapping signals (Mazzoni *et al.*, 2004), and are not distributed throughout the eye, but instead are regionally localized. Molecularly, the Iroquois complex of transcription factors (Iro-C) specify the dy ommatidia, consistent with the fact that Iro-C factors are repeatedly used during other dorsal–ventral patterning events in the fly eye (Cavodeassi *et al.*, 2000; Mazzoni *et al.*, 2008; Singh and Choi, 2003). Functionally, these ommatidia are likely to recognize a broader spectrum of wavelengths in the UV (Feiler *et al.*, 1992), and are positioned to a region of the eye that is most commonly found facing the sky. Behaviorally, how the fly takes advantage of this subtype, however awaits exploration although it has been proposed that it serves to detect the solar orientation. Yamaguchi *et al.* (2010) recently established a useful method for testing the contribution

R8 layers, representing the DRA ommatidia. Rh3 and Rh4 expression in the dy ommatidia are weaker than in the remainder of the eye. (D) Schematic representing the factors that direct inner photoreceptor identity, differentiation, and rhodopsin expression. The relative position of the nuclei that would be in the cell body for the different cell types are also indicated. See text for detail.

of different IPRs to wavelength discrimination in *Drosophila*, which could be applied to address this exciting question in the near future.

Over the past few years, several factors have been identified that are necessary for creating the *Drosophila* retinal mosaic (summarized in Fig. 5.6D). These studies indicate that the p versus y decision is first made in R7 cells, and requires the stochastic activation of the transcription factor Spineless in yR7s (Ss; Wernet *et al.*, 2006). Spineless is necessary and sufficient to activate Rh4 if expressed in IPRs or OPRs, and Ss-negative R7s (pR7s) express Rh3 by default (Wernet *et al.*, 2006). However, mutation of a potential binding site for Ss in the Rh4 promoter does not affect reporter expression *in vivo*, and Ss is not able to regulate Rh4 promoter activity *in vitro* (T. Cook, unpublished results), indicating that Ss is likely to activate another factor to directly control Rh4 expression. Once the p versus y decision in R7s is made, pR7s sends an inductive signal to the underlying R8 (pR8s) to activate Rh5. In the absence of this signal, such as in eyes lacking all R7 cells, R8 cells express Rh6 by default (Chou *et al.*, 1999). Therefore, although the pale fate in R7s is the default decision, the default decision in R8 cells is the yellow fate. Currently, the "pale" signaling molecule in pR7s remains unknown, but what is clear is that the activation of both pale opsins, Rh3 and Rh5, is directly controlled through K50 homeodomain binding sites by the transcription factor Otd (Fig. 5.5; Tahayato *et al.*, 2003). Since Otd is expressed in all PRs, this suggests that a pale-specific coactivator is critical for this function.

Although the R7-dependent pale signaling pathway is not known, some of the signaling molecules that are required for mediating Rh5 versus Rh6 expression in the receiving R8 cell have been identified. These include the membrane-associated pleckstrin homology-containing protein Melted and the serine/threonine cytoplasmic kinase Warts (Wts, *a.k.a.* Lats; Mikeladze-Dvali *et al.*, 2005). Melt expression is necessary and sufficient to induce Rh5 expression and to repress Wts expression in pR8s, whereas Wts is necessary and sufficient to induce Rh6 expression and repress Melt expression in yR8s. The bistable repression loop between Melt and Wts thereby ensures the mutually exclusive expression of Rh5 and Rh6 in different R8 subtypes. Consistent with Rh6 being the default R8 opsin, however, Wts appears to mediate the final output of the loop, while Melt is primarily involved in preventing Wts expression in pR8s. Since neither Melt nor Wts are DNA-binding factors, current work is focused on identifying the transcriptional mediators of the Melt/Wts pathway. This is a particularly interesting question, because Melt and Wts are most recognized for their roles in two independent growth regulatory pathways—the TOR and Hippo pathways, respectively (Harvey and Tapon, 2007; Hergovich and Hemmings, 2009; Reis and Hariharan, 2005; Teleman *et al.*, 2005; Yin and Pan, 2007). Thus, further clarification of the role of these proteins in fly PR specification may have

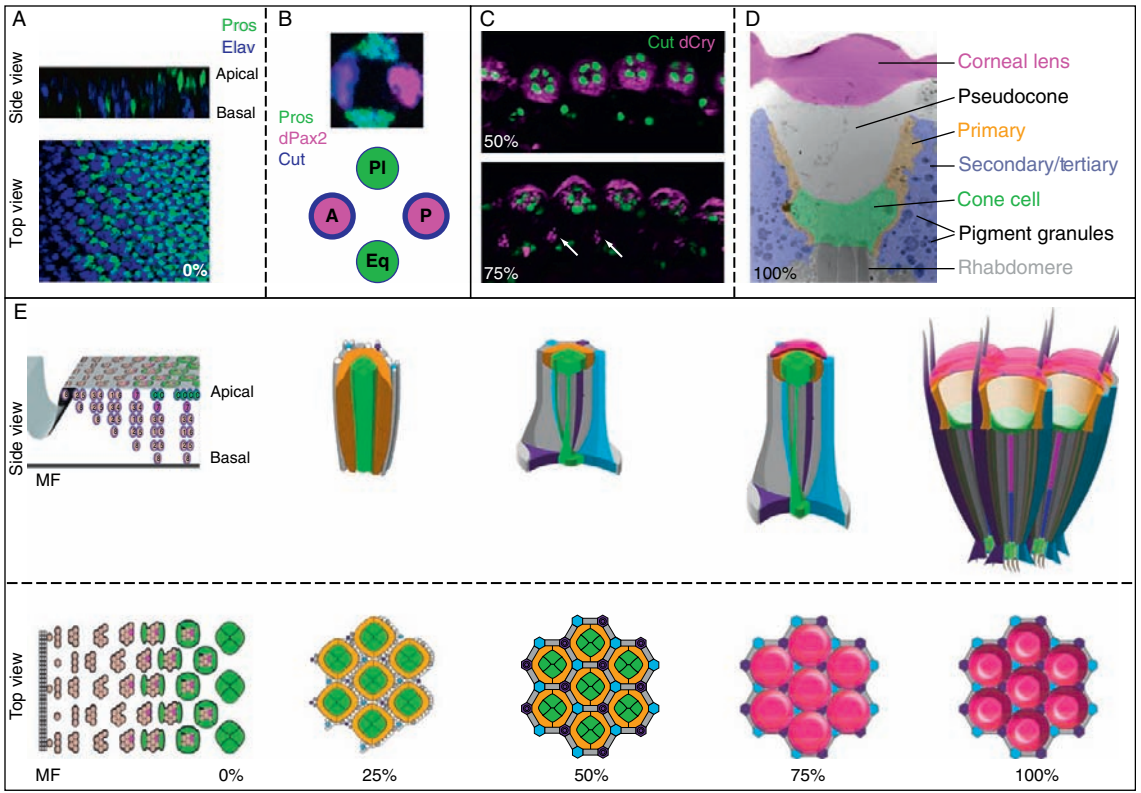
far-reaching implications in other fields of biology. Other questions that remain unanswered relate to how the initial stochastic decision for p versus y cell fate is made in the R7 layer, and what signaling pathway transmits this decision to the underlying R8 cell.

3. The Corneal Lens

In comparison with PR differentiation, much less is known regarding corneal lens formation in the *Drosophila* eye. This is somewhat surprising, because the lens is the most obvious structure in the fly eye for anatomical observation (Fig. 5.8A). The fly dioptic system comprises two distinct components: the corneal lens, a convex lamellated structure containing electron-dense microfibrils, and the underlying pseudocone, a fluid filled cavity seen by TEM cross section (Fig. 5.7D; Tomlinson, 1988; Youssef and Gardner, 1975). Early studies demonstrated that the corneal lens has a refractive index of 1.49, which determines a focal length that closely matches the distance from the corneal surface to the tip of the rhabdomeres (Youssef and Gardner, 1975). The pseudocone, however, has a lower refractive index of 1.3, suggesting that it may only have limited focusing power. Indeed, whether the pseudocone serves to focus light, similar to a lens, or merely creates the necessary distance between the cornea and the PRs remains unclear. Regardless, these data indicate that, like many land-dwelling animals, the *Drosophila* corneal surface is likely to be largely responsible for focusing light on the retina.

3.1. Cone and PPC recruitment and patterning

The CCs are the first nonneuronal cells to be recruited in the eye imaginal disc, and this occurs immediately after PR specification is complete (Fig. 5.7A). In fact, CCs are derived from a common precursor pool of 5 cells, known as the R7 equivalence group, which gives rise to both the R7 PR and the four CCs (Dickson *et al.*, 1992; Tomlinson *et al.*, 1987). Cells within the R7 equivalence group express the Sevenless tyrosine kinase receptor, the EGF receptor, and the Notch receptor (Cagan and Ready, 1989b; Fortini *et al.*, 1993; Jennings *et al.*, 1994; Rebay *et al.*, 1993; Tomlinson and Struhl, 2001; Tomlinson *et al.*, 1987). Each of these cells require EGF and Notch signaling to form. However, only one of these cells differentiates into the R7 neuron due to the fact that only a single cell comes in direct contact with the Sevenless ligand, membrane-bound Boss, which is expressed on the previously specified R8 precursor. Since the Sevenless receptor signals through the same Ras/MAPK pathway as the EGF receptor, this Boss-receiving cell receives higher Ras signaling, and becomes specified as a neuron, while the remaining 4 cells adopt the default fate,


A
Pros
Elav
Side view
Apical
Basal
Top view
0%
B
Pros
dPax2
Cut
Pl
A
P
Eq
C
Cut dCry
50%
75%
D
Corneal lens
Pseudocone
Primary
Secondary/tertiary
Cone cell
Pigment granules
Rhabdomere
100%
E
Side view
Apical
Basal
MF
Top view
MF
0%
25%
50%
75%
100%

that of cone (or Semper) cell (Cagan *et al.*, 1992; Hart *et al.*, 1990; Kramer *et al.*, 1991; Reinke and Zipursky, 1988; Van Vactor *et al.*, 1991). Over-activating Sevenless receptor signaling or overexpressing activated Ras in CC precursors can transform them into ectopic R7 PRs, and removing Sev signaling from the eye causes a failure in R7 differentiation but maintains the normal complement of four CCs (Basler *et al.*, 1991; Dickson *et al.*, 1992; Tomlinson and Ready, 1986). Together, these data led to the model that cells within the R7 equivalence group are all similarly capable of becoming R7 or CCs, and that this fate choice merely requires Sev-activated signaling. While these findings have been critical for defining the components of the Ras signaling pathway, the molecular mechanisms that mediate the dose-dependent neural (R7) versus nonneural (CC) fate decision remain unclear. Interestingly, however, not all cells within the R7 equivalence group respond the same to different mutants affecting R7/CC fate decisions instead, only one to two cells are generally affected (Basler *et al.*, 1991; Bhattacharya and Baker, 2009; Dickson *et al.*, 1992; Flores *et al.*, 2000; Hayashi *et al.*, 1998; Lai and Rubin, 1992; Matsuo *et al.*, 1997; Tsuda *et al.*, 2002). These data suggest that cells within the R7 equivalence group are actually not equivalent and that some bias toward R7 or CC fate exists in among these cells. Consistent with this idea, we have recently found that differential expression of two transcription factors, Pros and dPax2, in

Figure 5.7 Events leading to *Drosophila* corneal lens formation. (A) A third instar imaginal disc, stained with Elav (blue) to mark specified photoreceptors and the transcription factor Prospero (green), to mark the R7 photoreceptor and the cone cell precursors. The side view shows that the nuclei of cell move from a basal to apical position as they are recruited. (B) A high magnification of the cone cell layer from a single ommatidium shows that distinct subpopulations of cells that express different levels of Prospero (green), dPax2 (magenta), and Cut (blue) exist. This also is represented diagrammatically, with high Pros expression in equatorial (eq) and polar(pl) CCs, and high dPax2/Cut expression in anterior (a) and posterior (p) CCs. (C) Drosocrystallin (magenta) begins to be made in CCs, marked with Cut (green) at 50% pupation and is secreted from the cells by 75%. Drosocrystallin is also expressed at lower levels in the interommatidial bristle lineage (arrows). (D) A transmission electron micrograph of an adult ommatidium, pseudocolored to highlight the striated corneal lens (magenta), the clear pseudocone (gray), the primary pigment cells (PPCs, yellow), the cone cells (CCs, green), and the secondary/tertiary pigment cells (IOCs, purple). Note the abundant, large pigment granules in the IOCs, that the PPCs outline the CCs and pseudocone, and that the CCs lie between the pseudocone and the tips of the photoreceptor rhabdomeres. (E) Top and side view schemata of lens development, beginning from the imaginal disc through different stages of pupation using the same color scheme as in Fig. 5.1. The apical surface contacts change between the a/pCCs and eq/pl CC during pupation, patterning, and pruning of the IOCs occur prior to 30% pupation, and the corneal lens is secreted by ~75%. Afterward, the pseudocone is secreted and pushes the cone cells away from corneal lens.

different CC precursors are important for establishing this bias (Fig. 5.7B), and that concurrent regulation of these factors is necessary to completely convert cells within the R7 equivalence group into R7 or CC fates (Charlton-Perkins and Cook, submitted).

Even though CCs are specified from the same precursor pool, they are recruited pairwise: first, the anterior and posterior CCs (aCC and pCC), followed, one or two ommatidial rows later, by the equatorial and polar CCs (eqCC and plCC; Tomlinson, 1988; Tomlinson and Ready, 1987a; Wolff and Ready, 1993). These two sets of CCs have also been referred to as primary and accessory CCs, respectively (Tomlinson and Ready, 1987a). Once recruited, the apical surfaces of the a/p CC contact each other, pushing aside the eq/pl CC surfaces. Soon after pupation, however, the apical contacts switch to the eq/pl CCs, as these cells rise apically above the a/p CCs. At ~18% pupation, the CCs then recruit two PPCs via Notch signaling, which ascend along the a/p CCs surfaces, wrap around the CC cluster, and meet in the middle of the pl/eq CCs. The PPCs remain anchored to the retinal floor until the retina begins to elongate, at which time they detach and fully wrap the CC bodies. Thus, the PPCs are the only cells in the retina that are not attached to the retinal floor (Cagan and Ready, 1989a). After the CCs and PPCs are recruited, both cell types provide EGF and Notch signals that are required for proper patterning of the remaining IOCs (Cagan and Ready, 1989b; Flores *et al.*, 2000; Freeman, 1996; Miller and Cagan, 1998; Nagaraj and Banerjee, 2007; Voas and Rebay, 2004; Wech and Nagel, 2005; Yu *et al.*, 2002; also see Chapter 4).

3.2. Lens terminal differentiation

The events that ultimately form a functional corneal lens can be separated into two developmental stages: one at ~50% pupation during which a "wispy lens material" is secreted that will later comprise the outermost corneal lens structure, and a later stage at ~75% pupation during which a gelatinous substance is secreted into the pseudocone (Cagan and Ready, 1989a). Together, the PPCs and CCs secrete the majority of the corneal lens, but SPCs also appear to contribute a darker material that is present at the tapered ends of the cornea between ommatidia. CCs, however, seem to solely contribute to the pseudocone (Cagan and Ready, 1989a; Waddington and Perry, 1960). Thus, it is likely that CCs switch developmental processes to contribute to the corneal lens versus the pseudocone. Because the corneal lens is a hard structure that is continuous with the cuticle of the fly head, as the CCs secrete the pseudocone, the CC cell bodies are compressed into a thin layer against the apical surfaces of the PRs. The PPCs, in contrast, surround the walls of the pseudocone (Fig. 5.7D).

The contents of the corneal lens and pseudocone remain largely unknown. Indeed, the only protein identified to date, named Drosocrystallin, was purified from a large-scale extraction of isolated corneal lens almost two decades ago (Komori *et al.*, 1992). Biochemical analysis of Drosocrystallin revealed that it is calcium-binding glycoprotein, and sequence analysis suggests that it may be a member of a large class of cuticular proteins in insects (Janssens and Gehring, 1999; Komori *et al.*, 1992). More recent immunohistochemistry analysis has demonstrated that Drosocrystallin is also present in mechanosensory organs, including the IOB (Dziedzic *et al.*, 2009). Thus, like other developmental systems, *Drosophila* may have co-opted a gene product involved in other cellular processes to be expressed in the lens simply because of its ability to form a clear crystalline material when expressed at high concentrations (Piatigorsky, 2003). Figure 5.7C shows that Drosocrystallin is almost exclusively synthesized in CCs at 50% pupation, and is secreted into the corneal lens structure at ~75% pupation. Drosocrystallin expression is also observed in the IOB lineage by this time (Fig. 5.7C, arrows). In the mature cornea, this protein distributes into fine lines that correspond to the striations that are observed in the structure by TEM (Fig. 5.7D; Komori *et al.*, 1992). Two other abundant calcium-binding proteins were isolated at the same time as Drosocrystallin, but their identity remains unspecified.

With regard to proteins present in the pseudocone, even less is known. Two antibodies that specifically recognize this structure have been described (Edwards and Meyer, 1990; Fujita *et al.*, 1982), but neither reagent remains available and the protein products recognized by these antibodies were not determined. However, one of these antibodies, 3G6, was originally identified as a glial cell marker in grasshoppers, and was only later shown to recognize crystalline cones from a variety of insects (Edwards and Meyer, 1990). Curiously, this observation supports a hypothesis that has been suggested several times by other investigators: that CCs may exhibit some glial-like features. This conjecture is partially based on the fact that CCs express Cut, dPax2, and Pros, transcription factors that are regularly associated with glia in other parts of the fly nervous system. In addition, PR morphology is severely disrupted in mutants that affect CC development (Banerjee *et al.*, 2008; Daga *et al.*, 1996; Fu and Noll, 1997; Siddall *et al.*, 2003; Yan *et al.*, 2003). Consistent with CCs serving as potential glia for PRs, CCs do fully enwrap the PRs in the retina: distally, the CCs form a "rhabdomere cap" that holds the most apical portion of the cell, interretinular fibers intercalate along the length of the PRs, and a bulbous cluster of the CC end feet wrap the basal portion of the rhabdomeres and cell body (Banerjee *et al.*, 2008; Cagan and Ready, 1989a) (Figs. 5.1, 5.3). Thus, although it remains to be shown definitively, it is possible that CCs not only contribute to lens formation but may also function to maintain retinal integrity.

Many questions remain regarding the developmental regulation of CC and PPC differentiation and what the functional consequences of their development may be. Some insight into this has come from the analysis of homeodomain-containing transcription factors that are expressed in these cell types: BarH1/2, dPax2, Pros, and Cut (Blochlinger *et al.*, 1993; Fu and Noll, 1997; Higashijima *et al.*, 1992; Kauffmann *et al.*, 1996). BarH1/2 is restricted to PPCs and the bristle lineage in the pupal eye, and loss of Bar function leads to fusion of some ommatidia and the appearance of a hole in the center of the corneal lens, previously described as a "blueberry" phenotype (Higashijima *et al.*, 1992; Fig. 5.8F). A somewhat similar phenotype is observed in the most affected regions of spa^{pol} mutants, an eye-specific allele of dPax2, named for its sparkling (spa), polished (pol) appearance by light microscopy (Rickenbacher, 1954; Fig. 5.8B and G). dPax2 is expressed in both CCs and PPCs, but its primary phenotype appears to be loss of BarH1-positive PPCs (Fu and Noll, 1997). Recent studies, however, also suggest that dPax2 regulates Drosocrystallin expression (Dziedzic *et al.*, 2009), which is largely synthesized in CCs (see above). Since lenses still form in spa^{pol} mutants, these data indicate that Drosocrystallin is not required for the formation of the crystallin lens structure *per se*.

Both Pros and dPax2 are transcriptional targets of the same pathways required for CC development (Flores *et al.*, 2000; Hayashi *et al.*, 2008; Xu *et al.*, 2000). However, Pros is only expressed in CC nuclei during early CC recruitment, is turned off during mid-pupation, and its expression is then reinitiated in the CC cytoplasm at ~70% pupation (Charlton-Perkins and Cook, submitted; Cook *et al.*, 2003; Kauffmann *et al.*, 1996). Interestingly, because Pros expression is reinitiated at the same time that pseudocone formation begins in CCs, further understanding *pros* gene regulation in the eye may provide insight into the genetic pathways involved in this latter stage of lens development. Similarly to dPax2 mutants, removal of Pros during CC development causes relatively subtle changes in lens formation (Charlton-Perkins and Cook, submitted; also see Fig. 5.8B, C, G, and H). In contrast, removing both Pros and dPax2 causes a complete loss in lens formation and no CCs form (Fig. 5.8D and I) and (Charlton-Perkins and Cook, submitted). Thus, Pros and dPax2 combinatorially participate in CC formation. Surprisingly, despite the fact that Cut is expressed in all CCs from early specification through adulthood, little is known regarding its role in CC specification (Daga *et al.*, 1996), and no role for Cut in lens genesis has been reported. However, its expression correlates strongly with properly specified CCs, suggesting that it will play an important role in CC function. Collectively, these data suggest that Pros and dPax2 regulate CC-specific targets and contribute to pseudocone and corneal lens formation, while dPax2 controls BarH1 expression in PPCs and that these cells contribute to maintaining separate ommatidia and aids in completing the formation of the corneal lens (Fig. 5.8J).

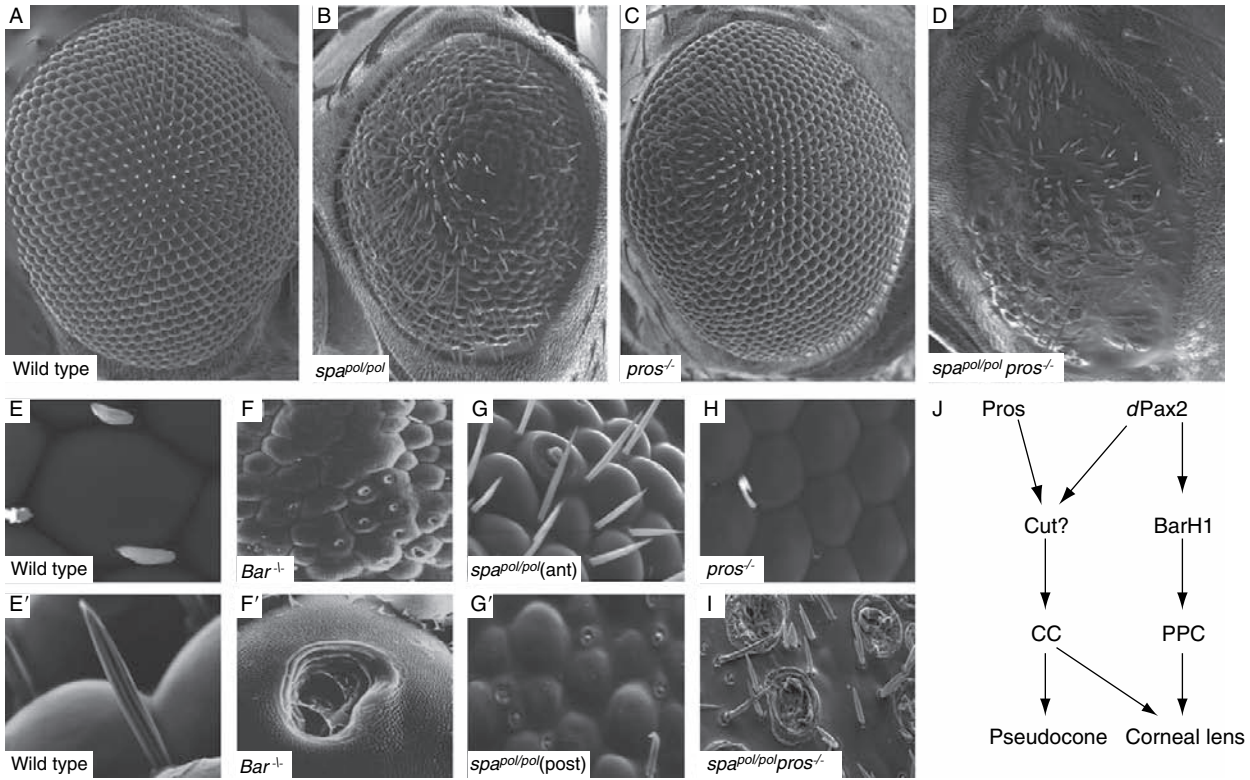


Figure 5.8 Scanning electron micrographs of adult eyes from wild type (A,E, E'), dPax2 *spa*^{pol} mutants (B,G, G'), whole eye *prospero* (*pros*) mutants (C,H), *pros* / *spa*^{pol} double mutants, and BarH1 mutants (F,F'; modified from (Higashijima *et al.*, 1992), with permission from Genes and Development). The spapol mutant is a dPax2 hypomorph that shows differences in lens phenotypes between the anterior (G) and posterior (G') portions of the eye. All images are oriented with anterior to the left. A summary of the role for these various CC and PPC expressed transcription factors is shown in J. See text for more detail.

4. SPCs and TPCs: The Fly Retinal Pigment Epithelia?

Besides the PPCs, two additional nonneuronal pigmented cell types are present in the adult fly eye, known as SPCs and TPCs. These are also often referred to as IOCs, as they are shared between ommatidia, and arise from the same pool of interommatidial precursor cells (IPCs). Morphologically, SPCs and TPCs differ by the number of cell contacts they establish in the mature retina: SPCs contact two cells, whereas TPCs contact three. In addition, SPCs are involved in the secretion of at least a portion of the corneal lens, while TPCs (but not SPCs) must establish alternating positions at the vertices of each ommatidia with the bristle cell lineage (Cagan and Ready, 1989a), suggesting that the development of these two cell types may be somewhat different. It is also intriguing that SPCs and TPCs may be the default state of cells within the eye imaginal disc, since in eyeless mutants, the few cells that do survive differentiate into IOCs, and in ommatidia localized to the eye margin, all PRs, CCs, and PPCs are induced to die, yet SPC/TPCs are retained (Lim and Tomlinson, 2006).

Both the initial recruitment and patterning of SPCs and TPCs is well documented and nicely reviewed in Chapter 4. Briefly, after PPC recruitment, any remaining unspecified cells in the eye begin vying for contacts between PPCs. Approximately 70% of these cells will form SPC/TPCs, while the remaining 30% (~2,000 cells/eye) will be eliminated by programmed cell death. The oblique SPCs are established first, based on contacts with two PPCs, followed by formation of the TPCs and horizontal SPCs, which contact three and four PPCs, respectively (Cagan and Ready, 1989a). By ~37% pupation, IOC recruitment and patterning is largely complete, although at least some cell death continues until ~62% pupation (Cagan and Ready, 1989a).

After recruitment, the apical surfaces of the SPCs/TPCs gradually tighten via a process involving the transcription factor Escargot, a member of the Snail-related family of zinc finger transcription factors (Lim and Tomlinson, 2006). In contrast, the basal surfaces of the SPC/TPCs expand to form a nice petal shaped lattice, or spokes of a wheel (Fig. 5.2H), that ultimately forms the fenestrated membrane of the retina. This membrane may functionally represent the blood–retina barrier, and is rich in stress fibers and septate junctions (Banerjee *et al.*, 2008; Longley and Ready, 1995). The molecular regulators of these complex morphogenetic changes, however fascinating, remain unexplored.

By ~62.5% pupation, the IOCs begin to generate two major types of pigmented granules. Type I granules are large and filled with the brown-colored pigment xanthommatin, also referred to as ommachrome. These

granules are present in SPCs/TPCs, PPCs, PRs, and CC feet (Cagan and Ready, 1989a; Shoup, 1966; Wolff and Ready, 1993). Type II granules are small and contain xanthommatin and drosopterin, a red pigment also known as pteridine, and these are predominantly found in SPCs/TPCs. Over 85 different eye color mutants have been identified in the past 100 years, and these have not only leant insight into how these pigment granules are formed, but have also led to a better understanding of a wide range of protein sorting processes (Lloyd *et al.*, 1998). These eye color mutants have been categorized into three functional subclasses: (1) the granule group, (2) the pigmentation synthesis group, and (3) the ABC transporter group. The "granule group" primarily encodes factors involved in protein sorting/biogenesis, and includes members of the AP3 adaptor complex, the VPS sorting complex, and several members of the Rab family of small GTPases (Kretzschmar *et al.*, 2000; Ma *et al.*, 2004; Mullins *et al.*, 1999; Ooi *et al.*, 1997; Simpson *et al.*, 1997; Warner *et al.*, 1998). The "pigment synthesis group" encodes enzymes that are involved in the processing of intracellular tryptophan required for the formation of xanthommatin and drosopterin (Summers *et al.*, 1982). Finally, the "ABC transporter group" includes complexes associated with transmembrane transport (Jones and George, 2004). Members of this group are White, Brown, and Scarlet, all part of the ABC-G subfamily of ABC transporters. They represent "halves" of a complete ABC transporter and become active by heterodimerization. White/Brown dimers transport drosopterin pigments, while White/Scarlet dimers transport xanthommatin pigments (Dreesen *et al.*, 1988; Ewart *et al.*, 1994; Pepling and Mount, 1990). Originally, these transporters were thought to be localized to the IOC cell membranes, but later studies suggest that White and Scarlet transport xanthommatin precursors directly into the pigment granules, thus using a mechanism analogous to that used for melanin transport into melanosomes (Mackenzie *et al.*, 2000; Tearle *et al.*, 1989).

Functionally, eye pigmentation is important for limiting light scattering between ommatidia. However, it is also important for maintaining PR integrity, protecting them from light-induced damage. For instance, mutations in the White gene, and other mutations that lead to white-eyed flies, causes severe rhabdomere degeneration when flies are exposed to constant light for 10 days, whereas wild-type, red-eyed flies are unaffected under identical conditions (Lee and Montell, 2004). This evidence is compelling in light of the neuroprotective function assigned to eye pigmentation in humans and vertebrate models, which show retinal degeneration acceleration in situations of retinal hypopigmentation.

Another critical role of SPC/TPCs is in the formation of the Rhodopsin chromophore 11-*cis*-retinal (Wang and Montell, 2005, 2007; Wang *et al.*, 2007). This important function of the SPC/TPCs was only recently discovered, with the majority of the vitamin A processing pathway in the fly

being expressed outside the retina. NINA-B (*neither inactivation nor afterpotential B)*, the functional ortholog of RPE-65 in Drosophila (Oberhauser *et al.*, 2008), for instance, is expressed in neurons within the brain, while NINA-D, a scavenger receptor required for dietary B-carotene absorption, is expressed in the midgut (Gu *et al.*, 2004; Wang *et al.*, 2007). Montell and coworkers reasoned, however, that since *Drosophila* Rhodopsin maturation requires chromophore binding, screening for mutants that disrupted specific aspects of Rhodopsin function may lead to the identification of additional proteins involved in the Vitamin A processing pathways. Indeed, from such a screen, the retinoid binding protein PINTA (*prolonged depolarization afterpotential is not apparent*) was identified. PINTA is specifically expressed in IOCs, placing these cells for the first time into the phototransduction pathway, and establishing that IOCs are more similar to the vertebrate retinal pigment epithelia (RPE) than previously thought. While PINTA remains the only protein involved in chromophore production that has currently been localized to IOCs, the oxidoreductase NINA-G functions downstream of PINTA (Ahmad *et al.*, 2006; Sarfare *et al.*, 2005), making it likely this factor, as well as other factors involved in chromophore production, are also expressed in these cells.

5. Comparison of the *Drosophila* Eye with the Vertebrate Eye

One of the first breakthroughs into the idea of a common origin of eye development came from studies on the shared function of Pax6 in regulating eye formation (Glaser *et al.*, 1992; Halder *et al.*, 1995; Quiring *et al.*, 1994). Since then, the majority of genes in the retinal determination cascade originally identified in *Drosophila* have been shown to have homologs in vertebrates that are comparably critical during early eye specification. These include the transcription factors Pax6, Dac, Eya, and So/Six3/Six6 (Gehring and Ikeo, 1999; Treisman, 1999; Wawersik and Maas, 2000; Wawersik *et al.*, 2000; also see Chapter 1). Patterning factors are also conserved among different eye types: Hedgehog in flies and Sonic hedgehog in vertebrates, for instance, each provides a moving wave of morphogenesis during early retinogenesis (Jarman, 2000; Wallace, 2008). In addition, proteins such as Ato/Ath5 and Pros/Prox1 have been shown to play evolutionarily conserved roles in generating different neuronal cell types during fly/vertebrate retinogenesis (Brown *et al.*, 2001; Cook, 2003; Cook *et al.*, 2003; Dyer, 2003; Dyer *et al.*, 2003; Wang *et al.*, 2001; White and Jarman, 2000). Determining what other similarities exist among visual systems across separate phyla will be an ongoing enterprise and is nicely covered in several recent reviews (Arendt, 2003; Cook and Zelhof, 2008;

Gehring, 2005; Jonasova and Kozmik, 2008; Sanes and Zipursky, 2010; Vopalensky and Kozmik, 2009). In the following section, we highlight some of the accumulating evidence suggesting that PR differentiation between vertebrates and flies share many developmental features.

5.1. The neural retina

Initially, the idea that vertebrate and invertebrate PRs are developmentally related was highly debated because of the many obvious differences between these cell types. For instance, light stimulation produces opposite electric potentials in these two PR cell types—in vertebrates they hyperpolarize via a phosphodiesterase cascade, while invertebrate PRs depolarize, using a phospholipase C cascade. The expanded PR apical surfaces used to concentrate light absorption also use different strategies—vertebrates have microtubule-based ciliary outer segments while Drosophila has actin-rich rhadomeric membranes. Vertebrate PR cells indirectly transfer visual information to the brain via retinal interneurons and ganglion cells, whereas *Drosophila* PRs are the only retina-specific cell types and project to functionally equivalent interneurons located in the underlying optic lobes (Sanes and Zipursky, 2010). Despite these marked anatomical and physiological differences, fly and vertebrate PR share striking similarities in the factors required for their morphogenesis. These include Otd/Crx (Furukawa *et al.*, 1997; Ranade *et al.*, 2008; Rivolta *et al.*, 2001; Swaroop *et al.*, 1999; Tahayato *et al.*, 2003; Vandendries *et al.*, 1996), Crumbs/CRB1 (Izaddoost *et al.*, 2002; Kowalczyk and Moses, 2002; Mehalow *et al.*, 2003; Pellikka *et al.*, 2002; Richard *et al.*, 2006b), Arrestin (Chen *et al.*, 1999; Lee and Montell, 2004; Nakazawa *et al.*, 1998), Prominin (Maw *et al.*, 2000; Yang *et al.*, 2008; Zelhof *et al.*, 2006), Spacemaker (Eyes Shut)/RP25 (Abd El-Aziz *et al.*, 2008; Collin *et al.*, 2008; Husain *et al.*, 2006; Osorio, 2007; Zelhof *et al.*, 2006), MyoIII (Hicks *et al.*, 1996; Porter and Montell, 1993; Redowicz, 2002; Walsh *et al.*, 2002), and Rab proteins (Deretic, 1998; Deretic *et al.*, 1995; Kwok *et al.*, 2008; Li *et al.*, 2007; Marzesco *et al.*, 2001; Moritz *et al.*, 2001; Shetty *et al.*, 1998). Moreover, the majority of these factors are associated with retinal degenerative diseases, further emphasizing the evolutionary importance of these factors for maintaining an intact adult visual system.

Not only does PR morphogenesis in fly and vertebrate share similar factors, but other differentiation events also make use of common regulators. For example, many of the same factors that are critical for PR axon guidance in flies are also used for patterning neuronal connections in the vertebrate retina, raising the exciting possibility that the fly eye will provide an effective paradigm for deciphering the relatively intricate axonal patterning present in the vertebrate visual system (for a more complete review of this topic, see Sanes and Zipursky, 2010). In addition, PR-specific gene regulation also involves conserved factors between vertebrates and

invertebrates. Drosophila Otd and its vertebrate orthologs Otx2 and Crx, for instance, are essential for regulating many common PR-specific target genes (Chen *et al.*, 1997; Furukawa *et al.*, 1997; Hsiau *et al.*, 2007; Koike *et al.*, 2007; Livesey *et al.*, 2000; Nishida *et al.*, 2003; Peng and Chen, 2005; Ranade *et al.*, 2008; Tahayato *et al.*, 2003). Combined, compelling evidence is beginning to emerge that suggest that similar genetic pathways are involved in building and/or maintaining multiple PR cell types. Thus, as has been postulated (Arendt, 2003; Cook and Zelhof, 2008; Erclik *et al.*, 2009; Gehring, 2005; Vopalensky and Kozmik, 2009), eyes in Urbilateria (Bilateria's last common ancestor) may have had PR cells that already expressed a number of interacting factors that have been maintained in the PR cell types found today, while structural and functional aspects have specialized to meet particular life conditions.

5.2. The cornea and lens

To date, few studies have addressed whether the genetic pathways involved in lens morphogenesis are conserved between vertebrates and invertebrates. However, like vertebrates, flies have a corneal structure that is largely responsible for focusing, and a crystalline region interposed between the cornea and the retina (the pseudocone in flies and the lens in mammals) that is likely to also contribute to the focusing power. Because a crystalline structure is necessary for a functional dioptic system, identifying proteins that regulate crystallin expression is a useful avenue for exploring conservation in lens development. Indeed, although many crystallins are recruited from ancestral proteins with distinct functions from their refractive function in the eye, their transcriptional regulation is relatively conserved (Cvekl and Duncan, 2007; Kozmik *et al.*, 2003; Piatigorsky, 2003, 2006; Tomarev and Piatigorsky, 1996)

An impressive demonstration of this conservation came from studies that revealed that the chicken δ1-crystallin enhancer can direct expression specifically in the lens-secreting cells in *Drosophila* (Blanco *et al.*, 2005). In vertebrates, this enhancer relies on binding sites for Sox2 and Pax6; similarly, Blanco and collaborators demonstrated that fly SoxN and dPax2 perform these same functions in *Drosophila*. The observation that dPax2 and Pax6 are functional homologs in this context is exciting, because both factors arise from a common ancestral factor known as PaxB (Kozmik *et al.*, 2003). Thus, these data suggests that upon the divergence of PaxB into two separate factors, Pax2 "claimed" lens function in invertebrates whereas Pax6 claimed this function in vertebrates. Besides Pax and Sox factors sharing functions during crystallin regulation, our recent findings that Pros is important during the differentiation of lens-secreting cells in *Drosophila* (Charlton-Perkins and Cook, submitted) parallels findings that vertebrate Pros, Prox1, is important for fiber cell elongation (Wigle *et al.*, 1999) and lends further support for genetic pathways being shared to form highly diverse lens structures.

In addition to transcription factors that may be functionally conserved, a number of signaling pathways may also serve overlapping functions during vertebrate and invertebrate lens genesis. For instance, high levels of FGF signaling are critical for many aspects of vertebrate lens development, and strong redundancy in this system appears to have been maintained to ensure correct signaling (Robinson, 2006; Zhao *et al.*, 2008). Similarly, as discussed earlier, proper levels of EGF signaling are essential for multiple aspects of *Drosophila* CC and PPC differentiation, and only slight variations in these levels have dramatic effects on lens development (Flores *et al.*, 2000; Fortini *et al.*, 1992; Freeman, 1996; Hayashi *et al.*, 2008; Miller and Cagan, 1998; Nagaraj and Banerjee, 2007; Tsuda *et al.*, 2002; Voas and Rebay, 2004; Wech and Nagel, 2005). Since both pathways mediate their functions through the Ras/MAPK pathway, it is possible that vertebrate lenses adopted the FGF receptor whereas the fly adopted the EGF receptor to mediate the same events, much like the diverged functions of Pax6 and dPax2 described above. Interestingly, while Notch signaling has long been known to be critical for lens morphogenesis in flies, only recently has Notch signaling only recently been recognized for its contribution to vertebrate lens development (Cagan and Ready, 1989b; Jia *et al.*, 2007; Le *et al.*, 2009; Miller and Cagan, 1998; Rowan *et al.*, 2008; Saravanamuthu *et al.*, 2009). Fortunately, in flies, only one EGF receptor and one Notch receptor are present, whereas in the mouse, knocking out three of the four FGF receptors was necessary to reveal the extent to which this signaling pathway contributes to lens formation (Zhao *et al.*, 2008). Similarly, it is likely that multiple Notch receptors and Notch ligands are going to participate in vertebrate lens formation (Bao and Cepko, 1997; Le *et al.*, 2009; Saravanamuthu *et al.*, 2009; Zecchin *et al.*, 2005). Once more, the possibility of using the fly as a genetic model for understanding lens formation and maintenance should be an advantageous approach for addressing future questions related to normal and diseased states affecting the eye anterior segment development.

5.3. The pigmented epithelia

Evidence for whether the *Drosophila* IOCs are the functional equivalent to the vertebrate RPE remains particularly sparse. The vertebrate RPE accomplishes complex and diverse functions that make it essential for visual function, including light absorption, water and ionic balancing to guarantee PR excitability, maintenance of immune privilege, nutrient uptake and delivery to PRs, cycling of retinal, and recycling of outer segments (Rosenthal *et al.*, 2005; Strauss, 2005). Moreover, malfunction of any one of these functions leads to vision failure and/or retinopathies. Interestingly, at least a subset of these functions has now been shown to be present in *Drosophila*, including light absorption and the Rhodopsin chromophore production. Likewise, both of these functions are necessary for retinal

normal function. Another similarity between vertebrate RPE cells and *Drosophila* IOCs is the use ABC transporters to generate their pigmented granules. Although the vertebrate ABCRs are members of the A subfamily of transporters, while the fly's belong to the G subfamily, the ABC-A and G groups share the strongest conservation among the other subgroups (Jones and George, 2004; Jones *et al.*, 2009). Interestingly, ABCA4 mutations are associated with human retinal degenerative diseases, and albinos are recognized for their sensitivity to light-induced retinal damage. This parallels nicely with the fact that flies lacking eye pigmentation, either through specific mutations in ABC transporters or through other depigmentation mutations, show drastic light-induced PR degeneration (Lee and Montell, 2004; Xu *et al.*, 2004; TC, unpublished observations). Finally, the recent postulation that *Drosophila* IOCs may be essential for creating the fly blood–retina barrier (Banerjee *et al.*, 2008) harkens strongly to the role of the RPE providing the first line of protection from the surrounding choroidal blood supply. Despite these parallels, it is fairly certain that not all functions of the vertebrate and invertebrate RPE are conserved. For instance, in vertebrates, the RPE is critical for phagocytosing the constantly growing PR outer segments, whereas in *Drosophila*, no convincing evidence suggests that rhabdomeres shed their membranes into the IOC compartment. Nevertheless future studies aimed at detecting other possible similarities between the fly and vertebrate pigment epithelial cells are likely to gain a deeper understanding of at least some aspects of RPE function.

6. Summary

This age of high throughput gene expression profiling is an exciting time in biology and has led to a better appreciation of the striking degree to which developmental processes have been conserved to create different body plans. The eye is no exception, and as we have attempted to summarize here, a remarkable symmetry is found between vertebrate and fly eyes. Based on the rapid progress we have recently made in this area, there is no doubt that continuing such efforts, taking full advantage of the genetic tools now available in both mouse and fly models, will identify additional shared developmental processes that generate a diversity of cell types and visual structures.

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CHAPTER SIX

Retinal Progenitor Cells, Differentiation, and Barriers to Cell Cycle Reentry

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Contents

Abstract

Neurogenesis in the retina occurs via the coordination of proliferation, cell cycle exit and differentiation of retinal progenitor cells. Until recently, it was widely assumed that once a retinal progenitor cell produced a postmitotic neuron, there was no possibility for cell-cycle re-entry. However, recent studies have shown that mature differentiated horizontal neurons with reduced Rb pathway function can re-enter the cell cycle and proliferate while maintaining their differentiated features. This chapter will explore the molecular and cellular mechanisms that help to keep differentiated retinal neurons and glia postmitotic. We propose that there are cell-type specific barriers to cell-cycle re-entry by differentiated neurons and these may include apoptosis, chromatin/epigenetics mechanisms, cellular morphology and/or metabolic demands that are distinct across cell populations. Our data suggest that differentiated neurons span a continuum of cellular properties related to their ability to re-enter the cell cycle and undergo cytokinesis while maintaining their differentiated features.

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A deeper understanding of these processes may allow us to begin to explain the cell type specificity of neuronal cell death and tumor susceptibility. For example, neurons that have more barriers to cell-cycle re-entry may be less likely to form tumors but more likely to undergo degeneration. Conversely, neurons that have fewer barriers to cell-cycle re-entry may be more likely to form tumors but less likely to undergo degeneration.

1. Introduction

Retinal progenitor cells (RPCs) are highly proliferative multipotent cells that undergo unidirectional changes in their competence to produce each of the seven major classes of retinal cell types in an evolutionarily conserved birth order (Cepko *et al.*, 1996). Appropriate expansion of RPC populations during retinal development must be coordinated with eye growth to ensure that the retina is the proper size, and defects in this coordination can lead to retinal degeneration (RD) and blindness (Martins *et al.*, 2007). Similarly, the precise timing of cell cycle exit must be coordinated with RPC competence to ensure that each of the seven classes of cell types is produced in the proper ratio (Dyer and Cepko 2001). Defects in this process could result in irreversible perturbations in the retinal circuitry and visual function. For more than a century, it has been believed that proliferation and differentiation are incompatible in the nervous system. Indeed, experimental manipulations that induce differentiated neurons to reenter the cell cycle have led to rapid cell death (Bonda *et al.*, 2009; Hoglinger *et al.*, 2007; Pelegri *et al.*, 2008; Skapek *et al.*, 2001; Zhu *et al.*, 2004).

Recently, it has been shown that the separation between proliferation and differentiation may not be as discrete as previously believed. When the function of the Rb family of proteins is reduced in the developing retina, mature differentiated horizontal neurons can reenter the cell cycle and clonally expand while maintaining their differentiated features including neurites and synapses (Ajioka *et al.*, 2007). This has led to a complete reevaluation of the view that it is impossible to induce differentiated neurons to proliferate and expand without dying. More importantly, it suggests for the first time that barrier to proliferation of differentiated neurons may not be the incompatibility of proliferation and differentiation but rather, there exist cell type-specific obstacles to cell cycle reentry. That is, why can horizontal neurons proliferate while maintaining their differentiated features but other types of neurons cannot? In this review, we will explore this question as it relates to our understanding of RPCs, neuronal differentiation and cellular reprogramming. A deeper understanding of these interconnected processes may shed light on human retinopathies that involve neuronal degeneration and deregulated proliferation.

2. Retinal Progenitor Cells

The retina is derived from the neuroectoderm of the prosencephalon of the developing neural tube. After the establishment of the three germ layers during gastrulation, the notochord induces neural tube formation of the overlying ectoderm. The neuroepithelium undergoes massive non-uniform cell proliferation, which establishes the three primary brain vesicles, the forebrain (prosencephalon), midbrain (mesencephalon), and hindbrain (rhombencephalon). In the forebrain, bone morphogenic protein (BMP) inhibition by noggin in collaboration with Wnt and FGF signaling specify the presumptive eye field (Sernagor *et al.*, 2006). Rx1 expression induces bilateral evaginations of the eye field neuroepithelium producing the optic vesicle (Mathers *et al.*, 1997). Rx induces the upregulation of eye field transcription factors (TFs) Pax6, Six3, Lhx2, and Optx2 (Zhang *et al.*, 2000). Cooperative expression of these TFs result in the invagination of the optic vesicle to produce the bilayered optic cup composed of a neuroblastic layer, which will give rise to RPCs, and the retinal pigment epithelium.

Early during development, RPCs tend to divide symmetrically to increase the pool of progenitors in the newly formed optic cup. Upregulation of the TFs Pax6, Chx10, and Sox2 may be important for sustaining the proliferative capabilities of these early RPCs, and mutations in these genes can result in proliferation defects characterized by small or absent eyes (Hill *et al.*, 1991; Mathers *et al.*, 1997; Taranova *et al.*, 2006). Following this early expansive phase of RPC proliferation, the cells begin to divide asymmetrically producing one daughter cell that differentiates and a second daughter cell that continues to divide as a progenitor cell. Finally, near the end of retinogenesis, both daughter cells tend to undergo terminal cell cycle exit when the entire pool of RPCs is depleted. Importantly, individual RPCs display a considerable amount of heterogeneity across development related to their patterns of cell division and this may indicate that there is a stochastic component of RPC proliferation or that there is considerable intrinsic heterogeneity of RPCs (Dyer and Cepko 2001). If we consider these patterns of RPC proliferation with respect to retinal cell fate specification, we can quickly appreciate the complexity of generating the precise proportions of each of the seven major classes of retinal cell types in an evolutionarily conserved birth order. For example, ganglion cells, horizontal neurons, and cone photoreceptors are born early during development when RPCs are undergoing expansive cell division. In contrast, rods, bipolars, and Müller glia are produced late during development when most of the daughter cells are undergoing terminal cell cycle exit (Lavail *et al.*, 1991; Rapaport *et al.*, 2004). There is now compelling evidence that unidirectional changes in RPC competence during development help to

guide this process to ensure that the correct proportion of each cell type is produced at the correct time during retinogenesis. However, it is not clear how the cell intrinsic programs that regulate proliferation, RPC competence, and differentiation are interconnected to ensure an efficient and accurate generation of each cell type at the appropriate stage during development (Elliott *et al.*, 2008).

3. Retinal Progenitor Cell Heterogeneity

Single cell microarray data on RPCs from different developmental time points support the competence model of cell fate specification. Early progenitors, E12.5–E16.5, express Sfrp2, Fgf3, and the TFs Foxp1, Etv1, Etv6 (Trimarchi *et al.*, 2008). At P0, these transcripts normally expressed in early progenitors are markedly absent and Crym, Car2, and Ptch-1 are upregulated. Genetic studies in *Drosophila* (Isshiki *et al.*, 2001) eye development led to the investigation of the mammalian homolog to Kruppel, Ikaros, a zinc-fingered TF expressed in early progenitors and absent in late RPCs. Ikaros knockouts show decreased ganglion, horizontal and amacrine cell neurogenesis. Overexpression of Ikaros at late stages of retinogenesis results in the production of early-born neurons and a concomitant reduction in bipolar cell genesis and a complete abrogation of Müller cell production. Ikaros expression is necessary and sufficient to confer competence to RPCs to produce ganglion, horizontal and amacrine cells (Elliott *et al.*, 2008).

Although there is an evolutionarily conserved birth order that is temporally regulated by transiently expressed factors in RPCs, a large amount of evidence suggests that individual RPCs exhibit intrinsic heterogeneity that acts in collaboration with the temporal competence state changes, and affects the pattern of cell division and the ability of subsets of RPCs to produce specific retinal cell types. Acheate-scute (AscL1) and Neurog2 are proneural bHLH TFs expressed in a subset of RPCs in the developing mammalian retina (Nelson *et al.*, 2009). AscL1 regulates the expression of Notch ligands, which are important for maintaining the pool of progenitors in the prenatal retina. Neurog2 controls expression of other bHLH TFs that are involved in the cell fate specification of retinal neurons. Similarly, FoxN4 is expressed in a spatially restricted pool of RPCs (Li *et al.*, 2004). In the absence of FoxN4, horizontal cell neurogenesis fails due to loss of Prox1 expression and amacrine cell neurogenesis is severely diminished. These data demonstrate that individual RPCs display variations in their molecular composition that influence retinogenesis.

While there is a myriad of evidence for intrinsic regulation of RPC proliferation and cell fate specification, there is also evidence that extrinsic factors may play a prominent role. FGF, TGFalpha, and EGF stimulate proliferation in RPCs (Anchan *et al.*, 1991; Lillien and Cepko 1992). Interestingly, the FGF response is more robust in early-born progenitor cells while TGFalpha and EGF are more effective in stimulating late-born progenitor mitosis supporting the notion that cell intrinsic factors interact with extrinsic factors to modulate cell division. Another example is GDF11, a diffusible TGF-beta family member, which negatively regulates RGC production by reducing Math5 expression and upregulating proneural bHLH TFs involved in cell fate specification of later born retinal cell types (Kim *et al.*, 2005). Follistatin is an antagonist to GDF11 and modulates the negative regulation of ganglion cell production. The result is an appropriate proportion of ganglion cells and a subsequent shift in RPC competence. This cue, in collaboration with other competence cues, modulates the timing of early competence states in a spatial/temporal manner.

Sonic hedge hog (SHH), another extrinsic cue involved in retinal histogenesis, is secreted by newly born retinal ganglion cells and promotes RPC proliferation by shortening the G1 and G2 phase and inhibiting cell cycle exit (Locker *et al.*, 2010; Wang *et al.*, 2005). In contrast, TGFbeta2 is expressed by neurons in the postnatal retina and induces a sharp decline in RPC proliferation (Close *et al.*, 2005). These data suggest that extrinsic factors can influence retinal histogenesis primarily by modulating the length of the cell cycle, and the interplay between multiple extrinsic signaling pathways and intrinsic control of proliferation must be coordinated with changes in RPC competence. Indeed, we favor a model in which the amount of time an RPC spends in a particular competence state may directly affect the proportion of retinal cell types produced. Intrinsic and extrinsic factors can control cell cycle length and the pattern of cell division and may thereby influence the composition of the mature retina by influencing the amount of time a given progenitor cell spends in each discrete competence state.

4. Proliferation and Differentiation in Neurons

The prevailing hypothesis explaining the incompatibility of differentiation with proliferation is that TFs expressed in progenitors and TFs expressed in differentiated cells are mutually exclusive because they specify genes that are active in proliferating cells or genes that regulate the acquisition of neuronal identity. In the retina, Chx10, Pax6, and Sox2 are highly upregulated and contribute to proliferation of RPCs during development (Sernagor *et al.*, 2006). Genetic deletion of these factors results in small eye

or absent eye phenotypes (Burmeister *et al.*, 1996; Halder *et al.*, 1995; Hill *et al.*, 1991; Taranova *et al.*, 2006). These data demonstrate the necessity of these TFs for the proliferation of RPCs. However, Chx10 is also necessary for the differentiation of bipolar neurons (Burmeister *et al.*, 1996). Similarly, Pax6, a potent mitogen factor in RPCs, is expressed in all mature amacrine cells as well as Müller glial and retinal ganglion cells (Cherry *et al.*, 2009), and Sox-2 labels a subset of mature amacrine cells (AII). These data suggest that it may take more than just distinct transcriptional programs to control the separation of differentiation and proliferation in neurons. In order to uncouple proliferation and differentiation, cells may have to overcome obstacles as a result of neuron-specific cell death pathways, metabolic barriers to cellular replication, epigenetic or genomic hurdles, or physiological barriers as a result of the elaborate morphological structures of different classes of neurons.

4.1. Neuronal cell death

Mechanisms of neuronal cell death are cell type specific and stimulus specific (Heidenreich 2003; Yuan *et al.*, 2003). The expression of and/or cell type-specific regulation of neuronal cell death pathways in postmitotic neurons are influenced by the expression of TFs that define that particular neuronal population. Overexpression of CyclinD1, a cell cycle component that promotes proliferation, in differentiated retinal neurons results in apoptosis (Skapek *et al.*, 2001). Aberrant reentry into the cell cycle is a well-characterized cellular event that can lead to neuronal death in various neurodegenerative diseases (Hoglinger *et al.*, 2007; Pelegri *et al.*, 2008). These data suggest that neuronal survival is connected to cell cycle arrest, and differential regulation of specific neuronal death pathways may underlie the horizontal cell's ability to proliferate when the Rb pathway is downregulated. What we know about the mechanisms underlying RD comes primarily from the investigation of RD mutants. Naturally occurring mutations in the phosphodiesterase 6 (Pde6) protein involved in phototransduction revealed several common mechanisms of neuronal cell death characterized by decreases in neuronal survival caused by excitotoxicity, oxidative stress, and energy depletion (Bowes *et al.*, 1990). Mice with *Pde6* mutations have elevated levels of cGMP, which activate cyclic nucleotide gated channels and promote the influx of excess Ca^{2+} (Farber and Lolley 1974). Transient calcium influx in neurons result in the production of cAMP followed by activation of PKA and finally the phosphorylation of a TF involved in neuronal survival, CREB-1. Knockout models of CREB-1 demonstrate the necessity of CREB-1 for neuronal survival by the widespread neurodegeneration exhibited by CREB-1 KO mice (Lonze *et al.*, 2002). Excessive calcium has been shown in specific models of RD to decrease pCREB-

1 (Paquet-Durand *et al.*, 2006). In contrast, other models of degeneration have shown upregulation of kinase activity of CREB regulators (Hauck *et al.*, 2006), which suggests that deregulation of CREB-1 is a component of RD. Calcium also activates nitric oxide synthase (nNOS), which produces NO that reacts with O_2 in the cell to produce peroxynitrate, a potent reactive oxygen species that damages lipids, proteins, and DNA (Komeima *et al.*, 2008). Increases in DNA damage stimulate PARP-1 to form Par polymers which induce the translocation of apoptosis inducing factor (AIF) to the nucleus. The release of AIF from the mitochondria is thought to be mediated by calpains, calcium activated proteases implicated in neuronal degeneration (Doonan *et al.*, 2005; Higuchi *et al.*, 2005). Ultimately, activation of this signaling pathway results in nuclear DNA condensation and neuronal cell death. Blocking PARP activity delays RD in RD1 mutants (Paquet-Durand *et al.*, 2007). Similarly, genetic deletion of AIF results in increased resistance of neurons to particular death inducing stimuli (Klein *et al.*, 2002). Therefore, some cell death pathways are specific for particular subsets of neurons in the retina and throughout the CNS, and this may begin to explain why some neurons respond differently from other neurons in their ability to uncouple proliferation and differentiation.

4.2. Neuronal metabolism

Neurons are both highly metabolically active and restricted in their ability to extract energy from carbon sources; therefore, they are less adaptive to low energy conditions and will undergo cell death in the form of apoptosis or autophagy. Neurons require enormous amounts of energy to sustain their electrical activity. Glucose is broken down into lactic acid by anaerobic glycolysis in Müller glia and it is then shuttled to photoreceptors (Tsacopoulos *et al.*, 1998). Lactic acid is converted into pyruvate by LDH, and oxidative phosphorylation occurs in the mitochondria of the photoreceptor. PARP-1 uses NAD^+, a powerful coenzyme in redox reactions, to repair DNA damage. However, excessive activation of PARP-1 depletes the cell of energy-producing NADH. AIF, a component of Complex I in the mitochondria, translocates from the mitochondria in response to PARP activation. This translocation disrupts the electron transport chain and contributes to the overall depletion of energy within the neuron. Cell death can also occur by sustained periods of low energy resulting in chaperone-mediated autophagy (Massey *et al.*, 2004).

An example of neurodegeneration where energy depletion is the primary cause of cell death is the secondary death of cones in RD mice, rods die as a result of direct mutations in the signal transduction pathway and cones degenerate soon after by an unknown process. mTOR is a kinase that regulates protein synthesis and ribosomal biogenesis. In cones from RD

mice, phospho-mTOR is downregulated (Punzo *et al.*, 2009). Hypoxia inducing factor 1 (HIF1alpha), a TF that upregulates enzymes involved in metabolism under low oxygen conditions and Glut-1, a glucose transporter, are upregulated in the degenerating retina. Administration of insulin delays the subsequent death of the cones by chaperone-mediated autophagy, the process by which selected cytoplasmic components are "digested" by lysosomal proteolytic enzymes such as Cathepsin D (Punzo *et al.*, 2009). Thirty percent of cytosolic proteins contain KFERQ-motifs that Hsc70 and other heat shock proteins recognize and target to lysosomes via interactions with lysosomal-associated membrane protein 2A (LAMP2A). Biogenesis associated with cell division is very demanding metabolically and it is possible that the unique metabolic demands of particular classes of neurons make them more or less susceptible to cell cycle reentry.

4.3. Chromatin and epigenetic processes

Global chromatin structure contributes to the perpetuation of cell identity and plays an indelible role in the transition of a cell from pluripotent stem cell to differentiated postmitotic neuron. Epigenetics, the study of heritable modifications of the genome, that influence gene expression without changing the DNA sequence, is concerned with the packaging of DNA around its core proteins. The core protein unit consists of an octameric disk composed of two copies of H3, H4, H2A, and H2B histone proteins wrapped around approximately 147 bp of DNA (Clapier and Cairns 2009). The positive charges of the histone residues interact with the negative phosphate backbone of the nucleic acid. Enzymes that modify the packaging of DNA (i.e., HAT, HDAC) do so by modifying the interaction of the histone residues to the phosphate backbone. Acetylation, methylation, or phosphorylation of the N-terminal histone tail can disrupt this association and leave portions of DNA open for large macromolecular complexes, chromatin remodelers, to remove, reposition, or slide histones away from promoter regions of DNA, and leave these unassociated promoter regions accessible to transcriptional machinery.

DNA methylation is essential for normal embryogenesis (Li *et al.*, 1992). DNA methyltransferases DNMT1, DNMT3A, and DNMT3B preserve the methylation pattern of the parent cell during mitosis by methylating the nonconserved strand during replication (Okano *et al.*, 1999). MeCP2 binds to methylated DNA and recruits HDACS and chromatin remodeling proteins to DNA to condense chromatin and shut down transcription (Chahrour *et al.*, 2008). This process is believed to confer cellular memory and consequently, maintains a cell's identity. In somatic nuclear transfer, DNA methylation is dramatically decreased after activation and subsequent cleavage resulting in a "reprogrammed" genome.

Not only does chromatin regulate cell identity but it also contributes to the maintenance of potency and proliferation in embryonic stem cells, neural progenitors, and mature postmitotic neurons. Histone methylation is critical for the maintenance of pluripotency in stem cells. Polycomb group proteins form complexes that add mono-, di-, or trimethyl groups to specific N-terminal histone tails and effectively silence genes involved in differentiation. The "histone code" is the specific combination of acetylated or methylated lysine residues on histone tails that together will determine whether chromatin will be "open" or "closed." ES cells are characterized by large amounts of euchromatin with bivalent domains containing H3K27me (Histone 3 methylated lysine residue 27) (Boyer *et al.*, 2006) to suppress genes involved in differentiation and H3K4, which is associated with open chromatin at the same site (Pray-Grant *et al.*, 2005). This suppression of differentiation was demonstrated to be required for the self-renewal and proliferation capacity of these cells. Bivalent portions of ES chromatin are said to be "poised" for differentiation. When the cell transitions from ES to progenitor, these signals become mutually exclusive in that promoter regions expressing H3K27me do not expression H3K4 and vice versa (Bernstein *et al.*, 2006). During differentiation there is also an increase in H3K9me and a dramatic decrease in H3Ac and H4Ac. These events have been demonstrated to be necessary for the appropriate transition of ES cell to multipotent progenitor.

Chromatin transition from neural progenitor to postmitotic neuron is accompanied by a switch in the components of the BAF complex, homologous to the Swi/Snf complexes in yeast. This complex is composed of 10 BAF proteins that specify distinct patterns of chromatin regulation germane to particular cell type. Neural progenitors express an assortment of core Baf proteins in addition to Baf45a and Baf53a. After differentiation, these subunits are downregulated and Baf45b and Baf53b are expressed (Lessard *et al.*, 2007). Expression of mi-RNA9 and 124 selectively repress the expression of Baf45a and Baf53a in postmitotic neurons (Yoo *et al.*, 2009). *In vivo* studies in mice expressing Baf53a or 45a under the nestin promoter or electroporation of constructs driving expression of these molecules in the mature chick spinal cord demonstrate that these subunits can enhance proliferation in progenitors and are incompatible with the differentiation postmitotic neurons as well as activity-dependent dendritic outgrowth (Yoo *et al.*, 2009). Brg1, an ATPase providing the energy for the BAF complex, collaborates with the different combinations of BAF complexes to maintain the self-renewal and proliferative state of neural progenitor cells. *Brg1lox/lox; Nestin-cre* animals showed enhanced mitosis of neural progenitors early and reduced neuronal number late in cortical development (Lessard *et al.*, 2007). Brg1-deficient neural stem cells show a reduced ability to form neurospheres. In contrast, in differentiated postmitotic neurons, Brg1 mediates activity-dependent neuronal gene

expression by recruitment of phosphoRB, and subsequent association with msin3a and HDAC at the c-fos promoter, and actively repressing transcription in resting neurons. Upon calcium entry, Rb is dephosphorylated and dissociates from the repressor complex. Creb and CrebBP are phosphorylated and associate via CREST, the Calcium RESponsive Transactivator protein, and activate transcription of c-fos (Qiu and Ghosh 2008). Therefore, chromatin remodeling complexes participate in tissue-specific and developmentally regulated modulation of chromatin accessibility and these processes may be directly or indirectly connected to the aforementioned changes in intracellular calcium.

Studies investigating chromatin remodeling complexes and their role in neuronal differentiation within the mammalian retina show similar changes in Baf subunit composition. In contrast to previous studies in other areas of the CNS, Baf53a is expressed in RPCs as well as a subset of differentiated early-born retinal neurons (Lamba *et al.*, 2008). Baf60c is expressed exclusively in RPCs. Conversely, Brm is expressed exclusively in differentiated inner nuclear layer neurons while Brg1 is expressed in both RPCs and differentiated neurons. The differential expression of the ATPase subunits for the Swi/Snf complex may play a critical role in the ability of these cells to form aggressive tumors. These data suggest that there are cell type-specific chromatin remodeling components that may facilitate or impede cell cycle reentry.

In the P107, single, horizontal cells reenter the cell cycle and successfully proliferate while maintaining neuronal specializations. The other cell types, Müller, rods, bipolars, etc., do not proliferate. A visual inspection of the chromatin of horizontal cells versus a highly specialized neuronal subtype, a rod, reveals a very striking dichotomy. Horizontal cells have larger nuclei and very "open" chromatin. In contrast, rods have very condensed chromatin with smaller nuclear volumes. In comparison, horizontal cells have chromatin more similar to a stem cell versus a rod whose chromatin is representative of a quintessential postmitotic neuron. These observations suggest that there may be differences in the histone code of a horizontal cell that render it more amenable to proliferation in the absence of the Rb family members. This genomic plasticity may confer metabolic and survival advantages by maintaining "open" conformations to areas of chromatin that are otherwise unavailable to other types of neurons. Another possible mechanism underlying the successful reentry of these cells into the cell cycle could be the expression of chromatin remodeling subunits that maintain the genome in a plastic "reprogrammable" state. BAF subunits are integral parts of the Swi/Snf chromatin remodeling complexes in mammals. In the nervous system, the switch from multipotent progenitor to postmitotic neurons is accompanied by a change in subunit composition. BAF45a and BAF53A, maintain neural progenitors in a proliferative state throughout the CNS and specifically BAF60c in RPC and dividing Müller glial

within the retina (Lamba *et al.*, 2008). Rb is known to collaborate with BAF complexes to shut down transcription at specific loci. Perhaps in the absence of Rb, horizontal cells may be able to upregulate BAF proteins involved in proliferation and together with a more open chromatin, be able to remodel that chromatin to sustain mitosis.

4.4. Neuronal morphology

Another possible barrier to the proliferation of differentiated neurons is its highly polarized morphology. Neuronal structure consists of the soma, or cell body, and the dendritic branches that receive synaptic input and the axon terminal that relays the outgoing signal. Within the retina, the cell bodies of the six neuronal cell types are segregated into well-defined layers with highly stereotyped projections of dendrites and neurons. The proliferation of differentiated neurons in the laminated structure of the retina seems implausible if the cytoarchitecture of the retina and the morphological specialization of the neuron are rigid. Studies in RD models clearly demonstrate morphological plasticity of individual neurons (Clapier and Cairns 2009; Jones and Marc 2005). As photoreceptors degenerate, the outer segments shorten, neurites sprout, and bypass appropriate targets. After the photoreceptors die, Müller glia proliferate and form a fibrotic seal between the outer nuclear layer and the retinal pigment epithelium. Deafferented bipolar and horizontal cells begin to sprout apical dendrites, migrate toward the ONL, and form ectopic synapses in the Dicer mutant (Damiani *et al.*, 2008). In contrast, the RD10 model of RD is characterized by the retraction of dendritic processes and the reduction in axonal terminal branching (Barhoum *et al.*, 2008). Each of these unrelated examples highlights the remarkable plasticity of retinal neurons raising the possibility of more far-reaching success in inducing other cell types to under cell cycle reentry.

5. Conclusions

Decades of research has led to a deeper understanding of RPCs and their ability to coordinate changes in proliferation with changes in competence. Until recently, it was widely assumed that once a RPC produced a postmitotic neuron, there was no possibility for cell cycle reentry. However, this dogma has now been overturned in the retina. Recent studies showing that mature differentiated horizontal neurons can reenter the cell cycle and proliferate while maintaining their differentiated features has led us to begin to explore the molecular and cellular mechanisms that help to keep differentiated neurons postmitotic. We propose that there are cell type-specific barriers to cell cycle reentry by differentiated neurons and these may include apoptosis, chromatin/epigenetics, cellular morphology,

and/or metabolic demands that are distinct across cell populations. These same cellular processes are important in RPCs and studies on postmitotic neurons may shed light on the coordination of changes in RPC competence during development with changes in the pattern of RPC proliferation. We propose that differentiated neurons span a continuum of cellular properties as it relates to their ability to reenter the cell cycle and undergo cytokinesis while maintaining their differentiated features. We believe it is important to further elucidate this continuum because it is possible that this may begin to explain cell type specificity of neuronal cell death and tumor susceptibility. Those neurons that have more barriers to cell cycle reentry may be less likely to form tumors but more likely to undergo degeneration. Conversely, those neurons that have fewer barriers to cell cycle reentry may be more likely to form tumors but less likely to undergo degeneration. Such a model would go a long way to begin to explain cellular underpinnings of human retinopathies and other disorders of the CNS.

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CHAPTER SEVEN

Planar Cell Polarity Signaling in the *Drosophila* Eye

Andreas Jenny

Contents

Abstract

Planar cell polarity (PCP) signaling regulates the establishment of polarity within the plane of an epithelium and allows cells to obtain directional information. Its results are as diverse as the determination of cell fates, the generation of asymmetric but highly aligned structures (e.g., stereocilia in the human ear or hairs on a fly wing), or the directional migration of cells during convergent extension during vertebrate gastrulation. Aberrant PCP establishment can lead to human birth defects or kidney disease. PCP signaling is governed by the noncanonical Wnt or Fz/PCP pathway. Traditionally, PCP establishment has been best studied in *Drosophila*, mainly due to the versatility of the fly as a genetic model system.

In *Drosophila*, PCP is essential for the orientation of wing and abdominal hairs, the orientation of the division axis of sensory organ precursors, and the polarization of ommatidia in the eye, the latter requiring a highly coordinated

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movement of groups of photoreceptor cells during the process of ommatidial rotation. Here, I review our current understanding of PCP signaling in the *Drosophila* eye and allude to parallels in vertebrates.

1. Introduction

Epithelia are polarized along the apical–basal axis in order to allow for directional transport of proteins within a cell or secretion of factors into lumina (Dow and Humbert, 2007; Wodarz and Nathke, 2007). In addition, most epithelia are also polarized within the epithelial plane. The latter polarization is called epithelial planar cell polarity (PCP) or tissue polarity. Planar polarization provides a cell not only with positional information but also directional (vectorial) information and correct PCP is a prerequisite for the formation of many organs.

Planar polarization can be very obvious: feathers of birds are nicely aligned; similarly, the fur of animals or hair on human skin is oriented in parallel. Less obvious examples of PCP are found in the organ of Corti in the mammalian inner ear, in which sensory neurons align their ciliary structures in a stereotypic fashion from cell to cell, a prerequisite for a proper response to sound (reviewed in Dabdoub *et al.*, 2005; Jenny and Mlodzik, 2006; Simons and Mlodzik, 2008; Vladar *et al.*, 2009). However, the consequences of PCP not only manifest in aligned cytoskeleton-derived structures but can also include cell fate decisions (as in the *Drosophila* eye) or the directed migration of single or groups of cells. To date, the most clinically relevant process shown to be affected by PCP signaling is convergent extension during vertebrate gastrulation and neurulation, during which mesenchymal cells migrate with respect to one another and toward the embryonic midline, where they intercalate, leading to a narrowing and elongation of the body axis (Keller *et al.*, 2000; Yin *et al.*, 2009). Aberrant convergent-extension results in neural folds that are spaced too far apart to correctly close and thus neural tube closure defects, one of the most frequent human birth defects (1–2 infants per 1000; Copp *et al.*, 2003; Doudney and Stanier, 2005). Indeed, mutations in the *vangl1* gene, one of the key players of PCP signaling discussed below, have been identified in spina bifida patients (Kibar *et al.*, 2007, 2009; Reynolds *et al.*, 2010). In addition, defects related to PCP signaling were shown to be responsible for certain cystic kidney diseases in humans and mice due to cilia-related malfunctions (Nephronophthysis and Bardet–Biedl syndrome; Ross *et al.*, 2005; Simons and Mlodzik, 2008; Simons *et al.*, 2005) or aberrant cell division axis orientation and cell migration (Karner *et al.*, 2009; McNeill, 2009; Saburi *et al.*, 2008).

PCP, however, is best studied in *Drosophila melanogaster*, mainly due to the ease of the fly as a model system. In *Drosophila*, PCP is externally visible in the alignment of sensory bristles and hairs (trichomes) on the thorax and abdomen, as well as the wing, where each cell produces a single hair pointing toward the distal tip (Adler, 2005; Lawrence *et al.*, 2007). Arguably the most beautifully polarized structure of *Drosophila* is the eye, with its roughly 800 precisely aligned facets. Facets or ommatidia are the building blocks of insect eyes and are meticulously oriented with respect to each other and to the general axes of the eye (Fig. 7.1). In this review, I will discuss the establishment of PCP in the *Drosophila* eye, which not only involves cytoskeletal rearrangements but also transcriptional responses, cell fate decisions, and directional movement (rotation) of groups of cells.

Most PCP phenomena studied to date, in *Drosophila* and vertebrates, are dependent on the noncanonical Wnt, a.k.a. Frizzled (Fz)–PCP pathway discussed in detail below. However, it is important to note that other mechanisms of planar polarization exist. For instance, Myosin II and Par3 (*bazooka* in flies) are subcellularly polarized during cell intercalation in *Drosophila* gastrulation independent of the Fz–PCP pathway and the interested reader is referred to reviews for further information (Lecuit, 2005; Lecuit and Lenne, 2007; Wirtz-Peitz and Zallen, 2009; Zallen and Blankenship, 2008).

2. PCP in the *Drosophila* Eye

2.1. *Drosophila* eye development

The adult *Drosophila* eye consists of some 800 ommatidia that develop during larval and pupal stages (reviewed in Singh *et al.*, 2005; Wolff and Ready, 1993). Each ommatidium is made up of 20 cells, including eight photoreceptors (PR or R-cells; Fig. 7.1), four lens-secreting cone cells, and bristle and pigment cells. The photoreceptors of each ommatidium—most easily identified by their rhabdomeres, the light-sensitive organelles (inset in Fig. 7.1B)—are organized in a trapezoid made up of the six "outer" (R1–6) and two "inner" photoreceptors (R7/8; R8 is underneath R7 and is not seen in the same plane as R7 in adult eye sections). The trapezoid of each facet is precisely aligned with those of its neighbors and the overall anteroposterior and dorsoventral axes of the eye. Furthermore, the ommatidia of the dorsal and ventral (black and red arrows, respectively, in Fig. 7.1) eye hemispheres are mirror images of each other and correspond to two chiral forms. This elaborate arrangement is the result of PCP signaling during the 3rd instar larval stage and is essential for proper image formation after wiring of the photoreceptor axons to the underlying lamina, medulla, and brain lobes (see also Chapter 8). Due to the curvature of the eye, individual

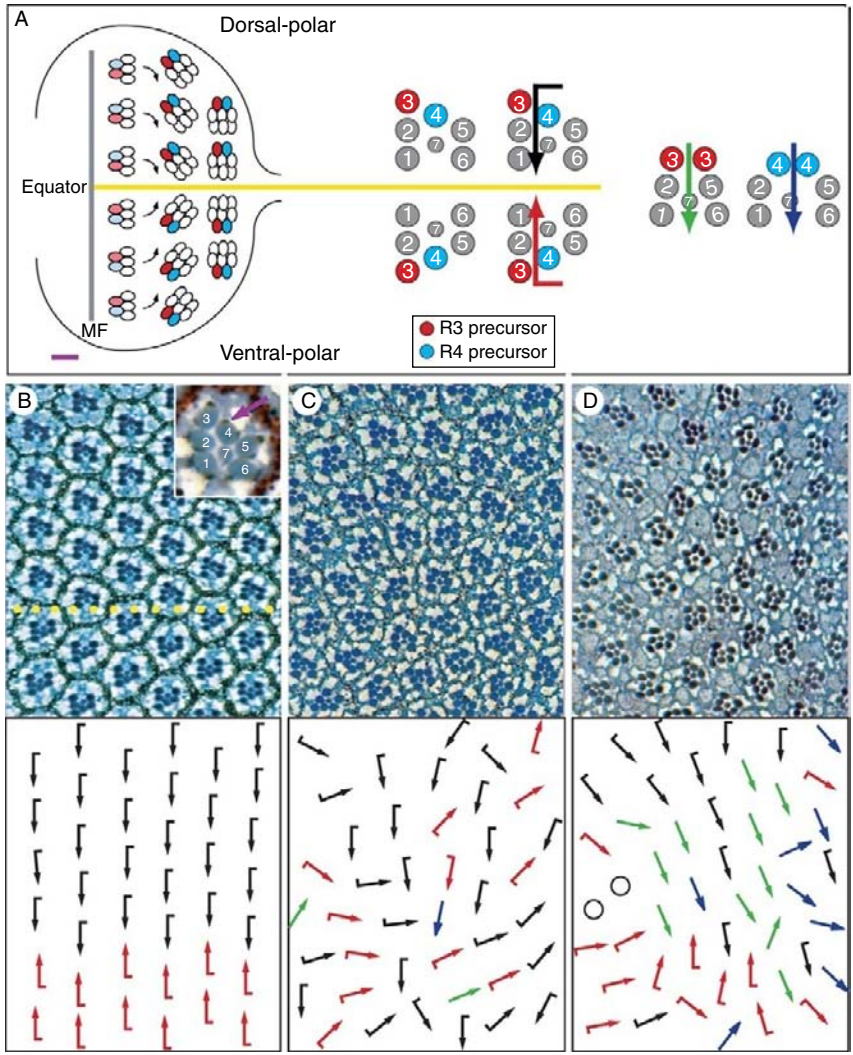

Figure 7.1 Establishment of PCP in the *Drosophila* eye. Anterior is to the left, dorsal is up in all panels. (A) Schematic of a 3rd instar eye imaginal disk with the dorsoventral midline (equator) in yellow and the morphogenetic furrow (MF) in gray. Purple bar outlines the approximate region of nonautonomous/*dachsous* signaling phase. Initially, ommatidial preclusters are symmetrical with the precursors for R3 (pale red) and R4 (light blue) next to each other. The cell of the R3/4 pair closer to the equator is specified as R3 (red) upon Fz–PCP signaling. The neighbor becomes R4 (blue). Ommatidia rotate 90° in opposing directions on either half of the eye. The rhabdomeres of adult ommatidia are thus mirror symmetric (chiral) with the rhabdomere of R3 at the polar-anterior tip and the R4 more equatorial and posterior (schematic on the right). Far right: schematic representing symmetric ommatidia of the R3/3 and R4/4 type

photoreceptors of an ommatidium point to different areas in space. In order to assemble a correct picture, corresponding photoreceptors of neighboring ommatidia that "see" the same point in space are wired together to represent a single point on the retinotopic map in the brain (Borst, 2009; Clandinin and Zipursky, 2002). In PCP mutants, ommatidia mostly form properly but their chirality and orientation are randomized and visual information thus cannot be properly processed (Fig. 7.1C, D).

The *Drosophila* eye develops from the eye imaginal disk which is initially specified by the *eyeless/Pax6* gene and its associated gene regulatory network (e.g., *eyes absent*, *dachsous*, *sine-oculis*, etc.; Desplan, 1997; Pappu and Mardon, 2004; Treisman and Heberlein, 1998; recently reviewed in this series: Cagan, 2009; see also Chapter 1). During early larval development, *wingless* (*wg*) expression is induced at the dorsal tip of the eye disk by the GATA factor *pannier* (Cavodeassi *et al.*, 1999; Heberlein *et al.*, 1998). Canonical Wg signaling (see below) then induces the homeobox genes of the Iroquois complex thereby specifying dorsal cell fates in the eye disk. Ultimately, this leads to the activation of Notch (N) along the midline (equator) of the eye separating dorsal from ventral and controlling overall growth of the eye disk.

During the early 3rd larval instar, *wg* is expressed at both poles of the eye disk and forms an activity gradient that is lowest at the equator (Singh *et al.*, 2005; Wehrli and Tomlinson, 1998). At this stage, canonical Wg signaling causes long range, mirror image polarization of the dorsal and ventral eye hemispheres. Clones, patches of mutant cells surrounded by wild-type tissue, of Wg pathway components such as the coreceptor Lrp6/*arrow* lead to the induction of ectopic equators and thus polarity reversals of whole fields of ommatidia (Singh *et al.*, 2005; Wehrli and Tomlinson, 1998). In addition, Wg emanating from the poles is required for proper separation of the eye field from head cuticle structures by maintaining *ey* expression anterior to the morphogenetic furrow (MF; Baonza and Freeman, 2002; Lee and Treisman, 2001; Royet and Finkelstein, 1997).

occurring in certain mutant situations. Colors of the flagged arrows correspond to the ones shown in the sections in B–D. (B–D) Tangential sections through wild-type (B), *fz* (C), and *dsh* (D) mutant adult *Drosophila* eyes. Note the randomized chirality and degree of rotation in the mutants. Schematic below the sections indicates the polarity of ommatidia (see (A) for arrows). Circles represent ommatidia with defects in the photoreceptor complement. Yellow dots represent the equator. Inset in (B): high magnification of a single ommatidium with numbered photoreceptors. Note that R8 is below R7 and thus cannot be seen. Purple arrow points to pigment granules associated with the rhabdomere of photoreceptors. The presence of these granules is used as a marker during genetic mosaic analysis. See text for details.

2.2. Establishment of ommatidial polarity

The MF is an indentation in the eye disk epithelium that functionally corresponds to the site of a switch from cell proliferation to differentiation. Like a wave of differentiation, it sweeps from posterior to anterior across the eye imaginal disk, leaving in its wake rows of differentiating photoreceptor clusters. Roughly every 2 h, a new row of PR clusters is specified (Campos-Ortega and Hofbauer, 1977; Tomlinson and Ready, 1987b). The eye imaginal disk thus represents at any moment a "natural time-course" of differentiation.

R-cell recruitment from the pool of undifferentiated cells occurs in a highly stereotypic fashion (reviewed in Baker, 2001; Roignant and Treisman, 2009). The first photoreceptor specified is R8 in a process strongly dependent on Notch signaling, followed by EGFR-dependent, pairwise, recruitment of R2/5 and R3/4. Together with R8, the R2/5 and R3/4 equivalence pairs form the five-cell precluster (Figs. 7.1 and 7.2A; note that preclusters can transiently contain one or two "mystery cells"; Tomlinson and Ready, 1987b). The precluster emerging from the MF is initially arc shaped with R3/4 at the outermost positions (Wolff and Ready, 1991). Upon maturation, the precluster tightens up and R3/4 come into contact (Fig. 7.2A). The precluster is subsequently joined by the R1/6 pair and R7. Accessory cells are recruited last (for more information about cell fate decisions see Chapter 6). Thus, ommatidia are generated by recruitment of cells from pools of undifferentiated, clonally (almost) unrelated cells, a fact that has proven enormously helpful to analyze the mechanism of PCP signaling.

Around the stage of PCP signaling, the five-cell precluster is symmetric with respect to the R3/4 cells, with the R3 precursor closer to the equator and the R4 precursor abutting R3 on the polar side (red and blue in Fig. 7.1A, respectively). The immature five-cell preclusters start to rotate in opposing directions on the dorsal (clockwise) and ventral (counterclockwise) sides of the equator once they are about four to five rows away from the MF (Tomlinson and Ready, 1987a; Wolff and Ready, 1991; reviewed in Jenny and Mlodzik, 2006; Mlodzik, 2005; Seifert and Mlodzik, 2007). After a first fast phase of rotation by 45°, ommatidial rotation slows down. Around rows 8/9 after the MF, undifferentiated cells divide once more ("second mitotic wave") and R1/6/7 are recruited from these newly divided cells (Ready *et al.*, 1976; Wolff and Ready, 1991). The ommatidia then rotate more slowly until they complete a full 90° turn with respect to their initial orientation when they were near the MF (Figs. 7.1 and 7.2). The precise organization, as well as timing and extent of ommatidial rotation has been followed by histochemical staining and more recently by examining apical markers (such as E-Cadherin; Figs. 7.2 and 7.5) or markers that reveal specific R-cell fates.

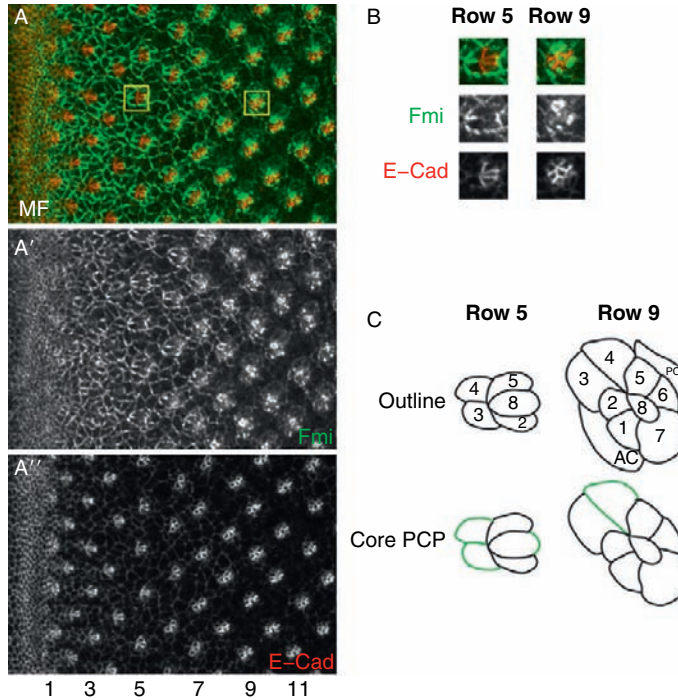

Figure 7.2 Subcellular localization of core PCP genes during PCP signaling. (A) Dorsal area at the apical level of a 3rd instar eye disk stained for Fmi in green (single channel image in A′) and E-Cad in red (single channel image in A″). The morphogenetic furrow (MF) is on the anterior (left). Note the progressive rotation of anterior (young) to posterior (more mature) ommatidia. Ommatidial row numbers are indicated at the bottom. (B, C): (B) Higher magnification of the ommatidial clusters indicated with yellow squares in A. In row 5, Fmi, a representative core PCP protein, is enriched in apical membranes in a "double horseshoe" pattern in R3 and R4, but reduced where they are in contact with R2/5. After the initial phase of rotation (about row 9), Fmi enrichment is visible as a single "horseshoe" in R4. Colors as in (A). (C) Schematic drawing outlining the cluster cells and localization of typical core PCP proteins (as described in B). In the upper panel, PRs are numbered. AC, PC: the anterior and posterior cone cells. Note that based on localization at the cell membrane it is not possible to determine of which cells touching each other actually expresses the protein (see also Fig. 7.5). Images courtesy of K. Gängel and M. Mlodzik.

A central step in PCP signaling is the cell fate specification of the R3 versus the R4 cells, the precursors of which initially are equivalent. This cell fate choice sets the basis for the opposite direction of rotation of photoreceptor clusters on the dorsal and ventral sides of the equator, and for the two chiral forms of ommatidia in the adult eye with their asymmetric position of R3 at the polar-anterior, and R4 posterior-equatorial position (Fig. 7.1). Indeed, this asymmetry can already be detected by molecular markers such

as pipsqueak-GFP (enriched in R3) or mδ–lacZ (expressed in R4) earlier during the rotation process (Cooper and Bray, 1999; Weber *et al.*, 2008). Even though mutations in PCP genes can uncouple the rotation direction from the chirality choice, the R3/R4 decision probably determines the direction of rotation. Evidence for this comes from the finding that in $pk^{spin\gamma\text{-}leg1}$ mutants, an allele of the core PCP gene *pk* (see below), the chirality is fully randomized, but ommatidia rotate in the direction appropriate for their acquired chirality (Gubb *et al.*, 1999; Jenny *et al.*, 2003).

3. The Core PCP Pathway and Its Effectors

Most components known to be involved in PCP establishment were initially identified in genetic screens in *Drosophila* and thus were genetically characterized before their molecular interplay was analyzed. Generally, PCP relevant factors fall into three categories (Fig. 7.3; Sopko and McNeill, 2009; Strutt and Strutt, 2009; Vladar *et al.*, 2009). First, the so-called Fat and Dachsous module appears responsible for a more global polarization of

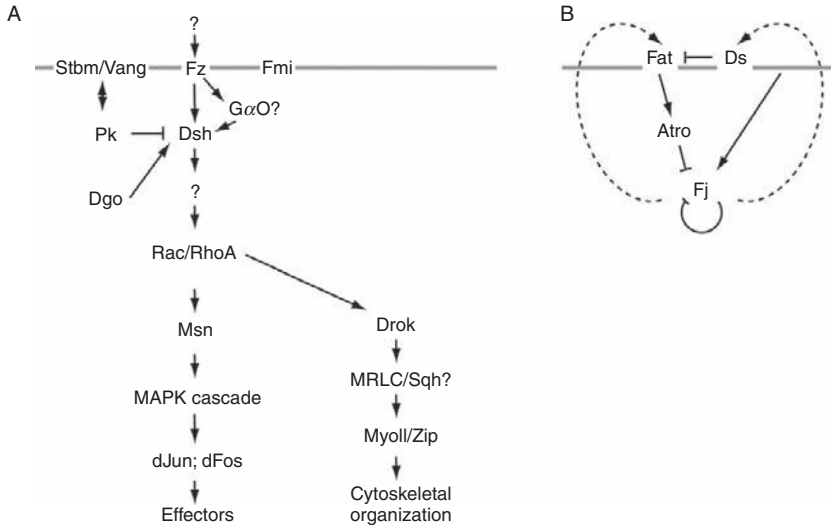

Figure 7.3 Schematic of the genes involved in PCP signaling. (A) Core PCP genes, JNK cascade, and cytoskeletal branch. In flies, the link between Dsh and the Rho family GTPases is currently unknown. The involvement of MRLC is inferred from wing data. (B) Ft/Ds module. Note that the hierarchical relationship between Ft/Ds and the core PCP pathway is unclear.

eye, wing, and abdominal tissues. Second, the core PCP genes such as *frizzled* (*fz*) comprise another module required for the establishment of polarity in all tissues. They regulate the third category of genes, the tissue-specific effectors. These "secondary polarity genes" allow for the appropriate tissue-specific responses such as the growth of an actin hair at the distal end of a wing cell (e.g., *inturned* or *multiple wing hairs*) or correct ommatidial rotation (e.g., the *nemo* gene coding for a kinase that is specifically required only for rotation; see also Table 7.1).

3.1. The core PCP module

PCP signaling is controlled by the noncanonical Wnt pathway, which shares some of its core components with the canonical Wnt/Wg pathway. During canonical signaling, a Wnt ligand activates a Fz/Lrp receptor/coreceptor complex leading, via the adapter protein Dishevelled (Dsh), to a stabilization and nuclear translocation of the transcription cofactor β-Catenin. Generally, canonical Wnt signaling controls cell proliferation, differentiation, and embryonic axis formation (reviewed in Clevers, 2006; Sokol, 1999). Both branches of Wnt signaling share Wnt ligands (in vertebrates), Fz, and Dsh, but are otherwise distinct (Jenny and Mlodzik, 2006; Mlodzik, 2002; Veeman *et al.*, 2003).

In addition to *fz* and *dsh*, *strabismus* (*stbm*, a.k.a. *van-gogh*, *vang*), *flamingo* (*fmi*, a.k.a. *starry-night*, *stan*), *prickle* (*pk*, also known as *prickle-spiny legs*), and *diego* (*dgo*) are members of the core PCP genes (Chae *et al.*, 1999; Feiguin *et al.*, 2001; Gubb and García-Bellido, 1982; Gubb *et al.*, 1999; Krasnow and Adler, 1994; Krasnow *et al.*, 1995; Taylor *et al.*, 1998; Usui *et al.*, 1999; Vinson *et al.*, 1989; Wolff and Rubin, 1998; Zheng *et al.*, 1995). All of the core genes affect R3/4 cell fate specification and ommatidial rotation (as well as PCP in other tissues) and have orthologs in vertebrate species that affect convergent extension or inner ear development (Table 7.1; reviewed in Jenny and Mlodzik, 2006; Simons and Mlodzik, 2008; Vladar *et al.*, 2009).

Key to PCP signaling is the G-protein coupled receptor-related, seven-pass transmembrane receptor Fz, which was among the first PCP factors identified (Fig. 7.3; Krasnow and Adler, 1994; Vinson *et al.*, 1989; Wang *et al.*, 1994). Work in the wing showed that Fz is required cell autonomously and can also have nonautonomous instructive roles in PCP signaling. Initial data was consistent with the wing hairs pointing along a decreasing gradient of *fz* activity due to either a diffusible signal or a relay-like signal propagation (Adler, 2005; Axelrod, 2009b; Wu and Mlodzik, 2009). Analogously, Zheng and Carthew showed that *fz* not only affects chirality and ommatidial rotation within mutant tissue but also can influence PCP in about one row of neighboring wild-type ommatidia located on the polar (but not equatorial) side of *fz* mutant clones (Zheng *et al.*, 1995).

Table 7.1 PCP genes involved in eye development

PCP gene	Tissues affected in *Drosophila*	Function in vertebrate PCP	PR requirement	Molecular features
Core genes				
frizzled (fz)	All adult tissues	M, X, Z	R3	Seven-pass transmembrane receptor, binds Wnt ligands, Dsh; recruits Dsh and Dgo to membrane
dishevelled (dsh)	All adult tissues	M, X, Z	R3	Cytoplasmic protein containing DIX, PDZ, DEP domains, recruited to membrane by Fz, binds Fz, Pk, Stbm, and Dgo
flamingo (fmi)/ starry-night (stan)	All adult tissues	Z, M	R3, R4	Cadherin with seven-pass transmembrane receptor features, homophilic cell adhesions
diego (dgo)	Eye, wing, notum in GOF[a]	X, Z	R3	Cytoplasmic Ankyrin repeat protein, recruited to membrane by Fz, binds Dsh, Stbm, and Dgo. Diversin/Inversin in vertebrates.
strabismus (stbm)/ van-gogh (Vang)	All adult tissues	H, M, X, Z	R4	Novel four-pass transmembrane protein, binds Pk, Dsh, and Dgo, recruits Pk to membrane
prickle (pk)	All adult tissues	M, X, Z	R4	Cytoplasmic protein with 3 LIM domains and PET domain, recruited to membrane by Stbm, physically interacts with Dsh, Stbm, and Dgo
Ft/Ds module				
ft	All adult tissues	M	R3 (?)	Atypical cadherin
ds	All adult tissues		R4 (?)	Atypical cadherin
fj	All adult tissues		R3 (?)	Golgi resident luminal kinase

Secondary genes				
RhoA/Rac	Eye, wing[a]	X		Small GTPase, acts downstream of Dsh
rho kinase (drok)	Eye, wing	M, X, Z		Ser-Thr kinase
daam	?[b]	X		Formin, actin polymerizing
misshapen (msn)	Eye (wing)		R3	Ste20 like kinase
jun	Eye	X (JNK)	R3	AP1 transcription factor component
fos (kajak)	Eye		R3	AP1 transcription factor component
pointed (pnt)	Eye		R4	Transcription factor
yan	Eye		R3	Transcriptional repressor
Notch (N)	Eye		R3	Transmembrane protein
Delta (Dl)	Eye		R4	Transmembrane protein
neuralized (neur)	Eye		R3	E3 ubiquitin ligase
nemo (nmo)	Eye			Kinase distantly related to MAPKs
argos (aos)	Eye			EGF (Spitz) binding inhibitor
Cell adhesion				
E-cadherin (shotgun)	Eye	X, Z		Cadherin, cell adhesion
N-cadherin	Eye			Cadherin, cell adhesion
echinoid (ed)	Eye		R1, R6, R7, CC	Ig CAM
fred	Eye		R1, R6, R7, CC	Ig CAM

H: human; M: mouse; X: *Xenopus*; Z: Zebrafish. Functional homolog unknown. CC: cone cells. See text for references.

[a] Other tissues were not tested.

[b] *Drosophila* Daam has no PCP LOF phenotype.

Fully mutant *fz* eyes show a random chirality over the whole eye (see also Fig. 7.1C). However, the phenotype of *fz* clones is stronger on the polar side than on the equatorial side, consistent with an activity gradient that declines toward the poles of the eye (Zheng *et al.*, 1995) though no ligand (see below) or other mechanistic cause of a graded activity is known to date. In addition, overexpression of Fz is sufficient to induce PCP phenotypes throughout the eye (Strutt *et al.*, 1997). The results of the *fz* clonal analysis are therefore consistent with a model in which Fz is constitutively active, with its activity dampened by a repressive activity declining from the poles toward the equator. Again, although antagonists are known, to date, neither such activity nor evidence for a relay mechanism in the eye has been described.

Downstream of Fz, the signal is transduced to Dishevelled (Dsh), an adapter protein consisting of a DIX, a PDZ, and a DEP domain, followed by a less well-conserved C-terminal region. Dsh is able to interact with a variety of proteins (Wallingford and Habas, 2005) including several components of the core PCP pathway. For example, its PDZ domain binds to Fz and there is evidence for an interaction of the DEP/C-terminal region with the intracellular loops of Fz. These interactions mediate recruitment of Dsh by Fz to the plasma membrane (Axelrod *et al.*, 1998; Wong *et al.*, 2003; Wu *et al.*, 2008).

Genetically downstream of *dsh* are Rho family GTPases such as Rho and Rac (Fanto *et al.*, 2000; Strutt *et al.*, 1997), with the caveat that due to genetic redundancies only *rhoA* mutations were shown to have PCP defects such as chirality inversions and rotation defects (Hakeda-Suzuki *et al.*, 2002; Munoz-Descalzo *et al.*, 2007; Strutt *et al.*, 1997). Reduction of Rho suppressed the overexpression phenotype of Fz or Dsh at the time of PCP signaling (Boutros *et al.*, 1998; Strutt *et al.*, 1997). Furthermore, constitutively active forms of RhoA and Rac cause ommatidial rotation defects and overexpression of Rac can partially suppress the PCP-specific allele dsh^1 (Fanto *et al.*, 2000). A direct molecular link between Dsh and Rho GTPases in flies remains elusive.

Fz has characteristics of a G-protein coupled receptor (Gilman, 1987) and the G-protein GαO has been reported to act just downstream of Fz in canonical Wnt signaling (Katanaev *et al.*, 2005). Although its effects on PCP signaling in the eye have not been analyzed, GαO loss causes PCP defects in the wing. However, the analysis of GαO function in PCP signaling showed that it is not simply downstream of Fz as in canonical Wnt signaling, but its relationship to the core PCP factors is more complex. For example, the nonautonomous effects of GαO are opposite of those due to loss of *fz* (Katanaev *et al.*, 2005).

As mentioned, a key step in PCP signaling is the determination of the R3/4 cell fates in the eye. The requirement of a gene in a particular cell can be determined by correlating mutant phenotypes of ommatidia with the

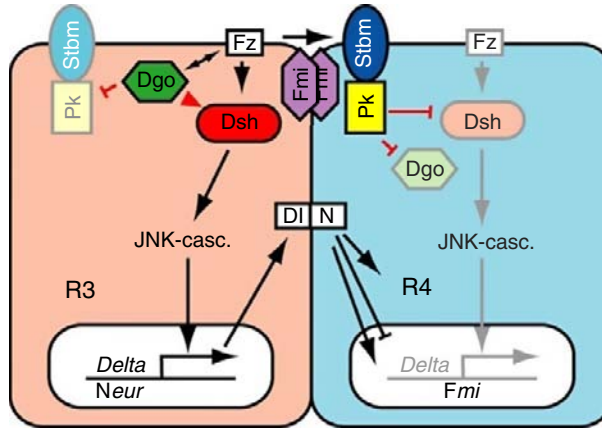

Figure 7.4 Schematic summarizing PCP signaling during R/3/4 cell fate specification. Factors in bright colors are genetically required in the respective cell.

genotype of single cells in ommatidia consisting of homozygous mutant and wild-type cells. Such a genetic mosaic analysis showed that *fz* is required in the R3 precursor: ommatidia with a wild-type R4 precursor and a genotypically mutant *fz* R3 precursor adopted the wrong chirality in >95% of all cases examined (Zheng *et al.*, 1995). Therefore, a Fz-signaling difference between R3/4 is instructive for cell fate (Fig. 7.4). Similar to Fz, Dsh and Diego (Dgo)—an Ankyrin containing protein—act in R3 (Feiguin *et al.*, 2001; Jenny *et al.*, 2005). On the other hand, the four-pass transmembrane protein Strabismus (Figs. 7.3 and 7.4) and the cytoplasmic protein Prickle were demonstrated to be required in R4 for the R3/4 cell fate decision (Jenny *et al.*, 2003; Wolff and Rubin, 1998; Zheng *et al.*, 1995). In general, factors that have positive effects on Fz–PCP signaling are required in R3, while "antagonists" are required in R4. Interestingly and in contrast to *fz* in the eye and wing and *stbm* in the wing, clones of cells mutant for *stbm* in the eye do not affect wild-type ommatidia and thus *stbm* does not act non-autonomously in the eye. Nevertheless, mosaic ommatidia with a wild-type R3 precursor but a *stbm* mutant R4 precursor will adopt the wrong chirality, indicating that Stbm can antagonize and override a Fz signal (Wolff and Rubin, 1998).

Flamingo (Fmi, also known as Starry-night or Stan) is another component of the core PCP machinery. *fmi* encodes an atypical cadherin with seven transmembrane domains capable of promoting homotypic cell adhesion (Casal *et al.*, 2006; Chae *et al.*, 1999; Usui *et al.*, 1999). Fmi's function in cell adhesion has not been explored, but its loss causes typical PCP defects in the eye, wing, and on the abdomen and thorax (Chae *et al.*, 1999; Das *et al.*, 2002; Usui *et al.*, 1999). In the wing, Fmi antagonizes Fz–PCP signaling

(Usui *et al.*, 1999) but its role in the eye is more complicated. Fmi is required in R3 and in R4, consistent with its ability to form homotypic interactions (Fig. 7.4; Das *et al.*, 2002). Initially, Fmi is required for proper membrane localization of Dsh, which could also explain its requirement in R3 (see also below).

Recently, protein–protein interactions between core PCP components were analyzed in more detail in both vertebrates and *Drosophila*. Interestingly, the extracellular domain of Fz can bind to the extracellular surface of Stbm (Wu and Mlodzik, 2008) and Fz coimmunoprecipitated with Fmi (Chen *et al.*, 2008; Montcouquiol *et al.*, 2006). Similarly, mouse orthologs of Stbm and Fmi interacted in coimmunoprecipitation experiments (Devenport and Fuchs, 2008) and these interactions can explain some of the nonautonomous effects in PCP signaling in the wing and abdomen (see Axelrod, 2009a,b; Lawrence *et al.*, 2008; Strutt and Strutt, 2009; Wu and Mlodzik, 2009). In the eye, interactions between Fz, Stbm, and Fmi may allow communication between the R3/4 precursors of an ommatidium, explaining why R3 precursors lacking *stbm* or R4 precursors lacking *fz* always become R3 and R4 cells, respectively, in R3/4 mosaics.

Physical interactions between Dsh and Pk (Tree *et al.*, 2002), Dgo (Jenny *et al.*, 2005), and Stbm (Park and Moon, 2002) were also described, as well as interactions of Pk, Dgo, and Stbm (Bastock *et al.*, 2003; Das *et al.*, 2004; Jenny *et al.*, 2003). The interaction of Pk with Stbm is required for Pk's localization at the plasma membrane, which is lost in *stbm* mutant clones (Bastock *et al.*, 2003; Jenny *et al.*, 2003). Supporting the importance of a Pk/Stbm interaction are experiments in *Xenopus* animal cap assays showing that ectopic expression of Stbm can recruit Pk to the membrane. Furthermore, such membrane recruitment during gastrulation and neurulation in zebrafish requires the *stbm* orthologs *vangl1* and *vangl2* (Ciruna *et al.*, 2006; Jenny *et al.*, 2003). The functional consequences of a direct interaction between Dsh and Stbm are currently unknown.

It was shown that Pk can prevent Dsh recruitment to the cell membrane in U2OS cells in culture, suggesting that sequestration of Dsh from the Fz–Dsh axis could explain Pk's ability to antagonize PCP signaling (Tree *et al.*, 2002). Interestingly, along with Pk and Stbm, Dgo (and its vertebrate orthologs) also interacts with Dsh (Jenny *et al.*, 2005; Moeller *et al.*, 2006; Simons *et al.*, 2005). In *Drosophila*, the basic-PDZ region of Dsh can bind both the C-terminal region of Dgo and Pk (Jenny *et al.*, 2005). Furthermore, an interaction between the Dsh DEP domain and the central PET/Lim region of Pk was also reported (Tree *et al.*, 2002). Importantly, Dgo and Pk compete for Dsh binding *in vitro*. The *in vitro* competition together with genetic interaction assays, as well as co-overexpression experiments in the wing, suggest a model in which the antagonistic effects of Pk and Dgo on Fz–PCP signaling are explained by direct competition for Dsh binding (Jenny *et al.*, 2005). It is, however, unknown mechanistically why Pk

binding to Dsh has repressive effects or why Dgo has enhancing effects. Potentially, either protein could recruit (or prevent the recruitment of) additional factors such as kinases or phosphatases, or could directly alter the stability of higher order PCP signaling complexes. Except for the membrane recruitment of Pk by Stbm (above), the function of the mutual interactions between Stbm, Dgo, and Pk is not well understood and it is unknown whether these interactions are mutually exclusive. *In vivo* analysis of the functional relevance of such interactions is complicated by the lack of a robust functional assay, as apical membrane recruitment during PCP signaling in the eye appears to be partially redundant (Das *et al.*, 2004).

3.1.1. Asymmetric core protein localization

PCP signaling can initiate the formation of asymmetric structures or be required for directional migration of cells (e.g., during vertebrate gastrulation). The core PCP proteins themselves become asymmetrically localized in a variety of situations investigated. For example, in wing cells, after initially localizing uniformly around the apical circumference, proteins required positively for PCP signaling such as Fz, Dsh, and Dgo become (transiently) enriched at the distal edge of the cells (Axelrod, 2001; Das *et al.*, 2004; Strutt, 2001). In contrast, Stbm and Pk become concentrated on the proximal side of the cells, where they abut Fz on the distal side of the more proximal cell. Similarly, in the eye, core PCP proteins are enriched in a double horseshoe-like pattern at the level of apical junctions in R3/4 shortly prior to and during early rotation (e.g., row 5). They are mostly excluded from contact between R3/4 and R2/5 (Fig. 7.2B and schematic in Fig. 7.2C; Das *et al.*, 2002, 2004; Jenny *et al.*, 2003; Rawls and Wolff, 2003; Strutt *et al.*, 2002). Around row 9, they concentrate in a single horseshoe around R4 (Fig. 7.2B, C). Clonal expression of functional GFP fusion proteins showed that Stbm, Dgo, and Fz are asymmetrically localized with respect to the cell border between R3/4 at the later stage: Fz and Dgo are concentrated on the R3 side of the R3/4 border, while Stbm localizes to the equatorial border of R4 (schematics in Fig. 7.5A; Das *et al.*, 2004; Strutt *et al.*, 2002). Thus in the eye and wing, PCP proteins sort to opposite sides within a cell and each group abuts the other across a cell membrane. It is unknown, however, if the asymmetric localization of the core PCP proteins is functionally significant, instructive, indicative of active signaling, or just a "readout" of acquired polarity, because mutant scenarios have been described in which cells can be polarized without apparent asymmetric PCP proteins (e.g., Lawrence *et al.*, 2004, reviewed in Strutt and Strutt, 2009).

Nevertheless, asymmetric localization of PCP proteins is conserved in a variety of PCP signaling scenarios in different organisms. For example, in the organ of Corti of the mammalian inner ear, Vangl2, a Stbm paralog, and Dsh–GFP localize to opposite sides of the sensory neurons (although Fz3 in this case colocalizes with Vangl2; Montcouquiol *et al.*, 2006; Wang *et al.*, 2005).

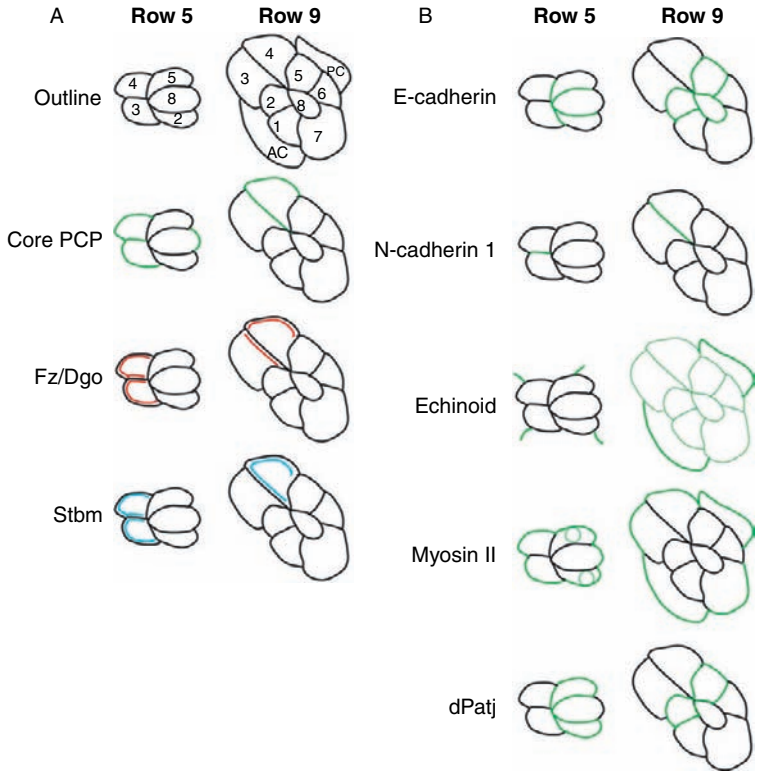

Figure 7.5 Schematic of protein expression patterns during PCP signaling in ommatidial clusters. (A) Subcellular core PCP protein expression. Use of clonal expression of GFP fusion proteins of Fz, Dgo, and Stbm showed that the overall similar single horseshoe pattern (green) is actually due to asymmetric protein localization that is distinct for Fz/Dgo (red) and Stbm (blue). While Fz and Dgo localize to the polar border of R3 (and R4), but are excluded from the equatorial side of R4, Stbm localizes to the equatorial side of R4 (and its polar side). The situation at the R3/4 cell interface is thus equivalent to the proximal/distal border of wing cells, where Stbm/Pk on the proximal side of distal cells abut Fz/Dgo/Dsh on the distal side of the more proximal cell. (B) Schematic summarizing simplified expression patterns of E- and N-cadherin 1, Echinoid, Myosin II (Zip), and dPatj. See text for a more detailed description and references. Note that these proteins are expressed at lower levels in most cells and green color indicates a strong enrichment. In particular, Ed and Myosin II expression is highly simplified. Ed is absent from early preclusters and later enriched at the interface between the ommatidia and IOCs. Similarly, Myo II is also expressed within the cytoplasm of cluster cells (as indicated by pale green circles).

Analogous opposite localizations for Pk2 and Fz6 have been described for the vestibular system (Deans *et al.*, 2007) and for Dsh and Pk in migrating cells during convergent extension in zebrafish (Ciruna *et al.*, 2006; Yin *et al.*, 2008). Although there are variations between the different cell types,

the strong conservation of asymmetric PCP protein localization in the different organisms suggests some function, whether causative or purely as a feedback reinforcement of a distinct initial asymmetric cue. Furthermore, a lack of visible, macroscopic protein asymmetry does not exclude asymmetric protein activities and could explain the cell polarizations observed without apparent asymmetric PCP protein localization (Lawrence *et al.*, 2004).

3.2. Effectors of PCP signaling in the eye

Effectors of the core PCP genes, also called secondary PCP genes, are generally tissue specific. For example, genes such as *multiple wing hairs* or *inturned* only affect PCP signaling in the wing (Adler, 2005). In the eye, several groups of genes were placed downstream of the core genes. For example, a JNK-MAPKinase module signals to the transcription factors Jun and Fos, and their target genes contribute to R3 specification (Fig. 7.3; Table 7.1; Weber *et al.*, 2000, 2008). In addition, Fz signaling regulates a Delta (Dl)–Notch (N) signal that reinforces the R3/4 cell fate decision (Fig. 7.4, Table 7.1; Cooper and Bray, 1999; Fanto and Mlodzik, 1999; Tomlinson and Struhl, 1999). Other genes, such as Nemo (Nmo; Choi and Benzer, 1994), the founding member of the Nemo-like kinase (NLK) family distantly related to MAP Kinases, only affect ommatidial rotation and are not required for PCP signaling in other tissues. Yet other genes such as *misshapen* (msn) or *rho kinase* (*drok*) have functions in the eye and wing (Paricio *et al.*, 1999; Winter *et al.*, 2001) and are considered effector genes here, because it is unclear whether their mode of action differs between the eye and wing.

3.2.1. Misshapen and a JNK module act downstream of Fz/Dsh

MAPKinase modules are usually activated by small GTPases (e.g., Ras during EGF signaling) leading to consecutive phosphorylation of a MAP Kinase Kinase Kinase (MAP3K), a MAP Kinase Kinase (MAPKK), and a MAP Kinase (MAPK), ultimately phosphorylating and activating a transcription factor in the nucleus (reviewed in Pearson *et al.*, 2001). Dominant gain of function interaction tests identified a JNK MAPK cassette acting downstream of the Rho GTPases during PCP signaling in the eye (Fig. 7.3). The PCP phenotype due to overexpression of Fz or Dsh is suppressed by removing one gene dosage of the MAPK *basket* (*bsk*; Strutt *et al.*, 1997) or the MAPKK *hemipterous* (*hep*) or by deficiencies removing those genes (Boutros *et al.*, 1998; Paricio *et al.*, 1999). Furthermore, the PCP phenotype resulting from overexpression of the GTP-bound form of Rac (Rac^{V14}) is dominantly suppressed by removing a copy of *hep* and *bsk*, as well as the transcription factor *jun* (Fanto *et al.*, 2000). Similarly, the

PCP-specific allele *dsh*^{1} can be rescued by overexpressing Hep, Bsk, or Jun at the time of PCP signaling (Boutros *et al.*, 1998). Thus, dominant gain of function genetic interaction experiments indicate that a JNK module acts downstream of RhoA or Rac in PCP signaling in the eye. This is further supported by experiments showing that dominant negative Bsk induces strong PCP defects and that Dsh overexpression can induce Jun phosphorylation in cell culture (Boutros *et al.*, 1998; Weber *et al.*, 2000). Furthermore, clones mutant for the STE20 like kinase Misshapen (Msn; a.k.a. MAP4K) show rotation defects and, more rarely, chirality inversions. Epistasis experiments place *msn* downstream of *rhoA* or *rac*, but upstream of *hep* and *bsk* (Paricio *et al.*, 1999). Analogous genetic interaction tests suggest that the MAP3K dTak can act upstream of Hep and Bsk, but downstream of Msn (Mihaly *et al.*, 2001).

However, the involvement of JNK signaling is controversial (Strutt *et al.*, 2002). To date, it has proven difficult to detect strong loss of function PCP phenotypes for JNK cascade components or their effector Jun. For instance, *hep* or *bsk* mutant tissue in 3rd instar eye imaginal disks show no or only weak PCP defects, which has been attributed to redundancy with other MAPKKs or MAPKs (such as p38). Indeed, deficiencies removing MKK4 or p38 also suppress the PCP phenotype of Dsh overexpression (Paricio *et al.*, 1999). In addition, *jun* clones show weak PCP defects and *jun* could function partially redundantly with *fos*. Jun and Fos are components of the AP1 transcription factor and can either hetero- or homodimerize (Wagner and Eferl, 2005). *fos* is required for tissue survival and progression through mitosis (Ciapponi *et al.*, 2001) and *jun/fos* double mutants are not viable and clones cannot be recovered. Hypomorphic alleles of *fos* (*kaj*) show mild PCP defects in adult eye sections and clonal analysis using molecular markers in eye disks show a requirement for *fos* in the R3 precursor, similar to *fz, msn,* and *jun* (Paricio *et al.*, 1999; Weber *et al.*, 2000, 2008; Zheng *et al.*, 1995), consistent with a role downstream of, or in parallel to, *fz* in R3/4 cell fate specification. Also consistent with a role for JNK in PCP signaling in the eye is that loss of *hindsight/pebbled*, which is required to downregulate JNK signaling, causes PCP phenotypes in the eye (Pickup *et al.*, 2002).

Interestingly, a conservation of JNK involvement in convergent extension further supports its involvement in PCP signaling. In *Xenopus*, knockdown of JNK using morpholinos or dominant negative MKK7 affects convergent extension, and JNK knockdown is also sufficient to suppress convergent-extension defects induced by the overexpression of Dsh (Yamanaka *et al.*, 2002). However, new data showed that JNK can also function downstream of Wnt5/Ror and thus can also work in parallel to Fz/Dsh during convergent extension (Schambony and Wedlich, 2007).

Additional evidence suggests an influence of EGFR signaling on R3/4 cell fate choice. In particular, EGFR signaling stimulates its downstream

transcription factor Pointed (Pnt) to promote R4 specification, while inhibiting Yan, a transcription repressor that can promote R3 fate (Weber *et al.*, 2008).

3.2.2. Delta and Notch reinforce the R3/4 fate decision

Typically, after loss of core PCP function, ommatidia adopt a chirality stochastically rather than in a directed manner, suggesting a mechanism that can reinforce a weak cellular bias induced by Fz–PCP signaling. Indeed, the Delta (Dl)/Notch (N)-signaling pathway has been shown to affect the specification of cell fates downstream of the core PCP module (Cooper and Bray, 1999; Fanto and Mlodzik, 1999; Tomlinson and Struhl, 1999). mδ–*lacZ*, a reporter line in which a short fragment of the N target gene mδ controls expression of β-Galactosidase, is expressed strongly in developing R4 cells in 3rd instar eye imaginal disks (Cooper and Bray, 1999). Importantly, mδ–*lacZ* expression is reduced in *fz* and *dsh* mutants. Overexpression of constitutively active N leads to expression of mδ–*lacZ* in both R3 and R4 precursors and gives rise to ommatidia that appear symmetric and contain two R4 cells (as judged by the morphology of adult ommatidia; Fig. 7.1A; Cooper and Bray, 1999). Conversely, temperature-sensitive alleles of *N* or *Dl* clones give rise to R3/3 symmetric ommatidia (Cooper and Bray, 1999; Fanto and Mlodzik, 1999; Tomlinson and Struhl, 1999). Clonal analysis showed that Dl is required in R3 to promote R4 fate in its neighbor (Table 7.1). Similarly, reduction of N activity in a mosaic fashion showed that N in R4 specifies R4 cell fate (Tomlinson and Struhl, 1999). Furthermore, *N* signaling is epistatic to *fz* (Cooper and Bray, 1999; Fanto and Mlodzik, 1999; Tomlinson and Struhl, 1999) and *N* can repress *Dl* in R4 (Cooper and Bray, 1999).

Several transcriptional targets of Fz–PCP signaling were identified (Fig. 7.4). First of all, *Dl* is expressed at higher levels in R3 at the time of PCP signaling and is upregulated upon overactivation of Fz, initiating the Dl/N bias between R3 and R4 (Cooper and Bray, 1999; Fanto and Mlodzik, 1999). The RING-finger E3-ubiquitin ligase *neuralized* (*neur*) is a transcriptional target of the Fz pathway as well. *neur* is required in R3 to promote Dl activity (Del Alamo and Mlodzik, 2008), either via the stimulation of Dl endocytosis leading to higher N activation (Lai *et al.*, 2001) or by modification of the Dl/N binding interaction (reviewed in Le Borgne *et al.*, 2005). Conversely, N represses *neur* in R4 to reiterate the bias (Del Alamo and Mlodzik, 2008). Furthermore, a *Dl* transcriptional reporter is upregulated in *fmi* clones while Fmi overexpression in R3/4 leads to a reduction of *Dl* and thus the formation of R3/R3 symmetric ommatidia due to a loss of N activity (Das *et al.*, 2002). In addition, Fmi itself is a target of N signaling: if only one of the R3/4 precursors of an ommatidium overexpresses Fmi, that cell will become R4 because Dl levels in that cell are lower than its partner, resulting in higher *cis*-N activity (thus reinforcing

the bias). It is currently unclear how Fmi represses Dl. However, a repressive effect directly on Fz–PCP signaling would be consistent with data from the wing, where it has been shown that overexpression of Fmi antagonizes Fz (tissue-specific expression of Fmi reorients wing hairs toward the expression domain, while upon Fz overexpression, hairs point away from the source of Fz; Usui *et al.*, 1999).

Taken together, experimental evidence suggests a model in which Fz controls Dl at the transcriptional level in R3, which then nonautonomously activates N in the neighboring R4 precursor to cement the *fz* activity bias between R3 and R4. However, an alternative model was proposed in which Dsh, previously shown to bind to N, directly inhibits N in R3 (Strutt *et al.*, 2002). However, this model is difficult to reconcile with the finding that the N-reporter mδ–*lacZ* is reduced, rather than upregulated, in a dsh^1 mutant background (Cooper and Bray, 1999).

3.2.3. Ommatidial rotation

As described above, dorsal and ventral ommatidia rotate 90° clockwise and counterclockwise, respectively (Figs. 7.1 and 7.2). The initial rotation of 45° is relatively fast and occurs over ommatidial rows 4–7/9, while a second, slower phase is completed around rows 15–18 (Gaengel and Mlodzik, 2003; Wolff and Ready, 1991). During rotation, groups of adhering photoreceptors move between stationary, interommatidial cells (IOCs; Fiehler and Wolff, 2007). To date, it is unknown whether this specialized type of cell migration is dependent on protrusive activities of cells such as lamellipodia and/or filopodia (as during convergent extension; Wallingford *et al.*, 2002; Yin *et al.*, 2009), or whether rotation is mainly achieved via restructuring of apical junctions (as in germband extension during *Drosophila* embryogenesis; Bertet *et al.*, 2004; Blankenship *et al.*, 2006). Unfortunately, no *in vivo* imaging of the rotation process has been reported to date, mainly because of the difficulties in culturing eye disks. It is, however, expected that rotation is controlled by cytoskeletal proteins, cell adhesion, and the extracellular matrix. In addition there are mechanisms regulating the start and stop of the process. Indeed, genes affecting rotation have been described for each of these classes and include *nemo, argos, drok, cadherins,* and *laminin A*, but their interplay with the core genes and each other is not well understood (Choi and Benzer, 1994; Henchcliffe *et al.*, 1993; Mirkovic and Mlodzik, 2006; Winter *et al.*, 2001).

Mutations in *nemo* (*nmo*), a distant MAPK relative and Wnt antagonist, arrest rotation at about 45° and *nmo* regulates the speed of rotation (note it is unclear whether available alleles are null mutations; Choi and Benzer, 1994; Fiehler and Wolff, 2008). Not much is known about the mechanism of action of Nmo during PCP signaling, but it could potentially phosphorylate members of the core PCP proteins or components of the cytoskeletal or

adhesion machineries to regulate their activities. Mutations in *argos* (*aos*), a diffusible inhibitor of EGFR signaling initially identified as relevant for PCP signaling due to the phenotype of the $aos^{roulette}$ (aos^{rlt}) allele, lead to strong over- and under-rotation of ommatidia (Brown and Freeman, 2003; Choi and Benzer, 1994; Gaengel and Mlodzik, 2003; Strutt and Strutt, 2003). Initially, since *nmo/aos* double mutants arrest rotation at 45°, it was assumed that Aos controlled the second phase of rotation. More recently, it appears that precise control of EGFR signaling is crucial for the correct extent of rotation. Not only the inhibitor Aos but also weak alleles of *egfr* (a.k.a. *torpedo, top*) show rotation defects. Multiple explanations, which are not necessarily mutually exclusive, were proposed to explain the observed phenotypes. First, a higher frequency of mystery cells, an R-like cell normally only briefly associated with ommatidial preclusters, was observed. These mystery cells also remain part of the photoreceptor precluster for longer than normal and affect Fz and Fmi subcellular localization and thus might perturb Fz–PCP activity (Strutt and Strutt, 2003). In addition, a genetic interaction between EGFR signaling components and *E-cadherin* was observed, suggesting a regulation of cell adhesion by EGF signaling (Brown and Freeman, 2003).

The EGF signal is usually mediated via the small GTPase Ras that activates different downstream branches such as Raf/Rolled MAPK, PI3 Kinase, Rgl, or Canoe (Cno)/AF-6 (Prober and Edgar, 2002). Using Ras effector loop mutants that are able to activate only subsets of the different Ras branches, it was shown that EGFR can affect rotation not only via the Raf/Rolled MAPK cascade but also via Canoe/AF-6 and potentially Rgl/Ral or PI3 Kinase (Gaengel and Mlodzik, 2003). Indeed, mutations in the adherens- and tight-junction associated protein Canoe show over- and underrotation defects similar to aos^{rlt}, even early during the larval 3rd instar stage. Although not assessed in PCP signaling, *cno* genetically interacts with *scabrous* (sca), an endosome-associated protein involved in N signaling (Li *et al.*, 2003; Miyamoto *et al.*, 1995). Ommatidia posterior to *sca* mutant clones overrotate and *sca* thus appears to be required non-cell autonomously for ommatidia to stop at the right position (Chou and Chien, 2002). It remains puzzling to explain the genetic interaction of *cno* and *sca* mechanistically. However, consistent with an involvement of EGFR signaling in rotation, mutations in the phospholipase Cγ, *small-wing*, which is involved in the ER retention of the processed EGFR ligand Spitz, show rotation defects (Schlesinger *et al.*, 2004).

3.2.4. Impact of cell adhesion and the cytoskeleton on ommatidial rotation

Ommatidial rotation can be considered a special type of cell migration, in which groups of tightly adhering cells change their position relative to cells in their circumference. Cells not part of maturing ommatidia (IOCs) are not

moving along with the clusters (Fiehler and Wolff, 2007; Wolff and Ready, 1991), implying that members of the PR clusters adhere more strongly to one another than to undifferentiated neighbors and that a force generator must exist to allow movement of cluster cells with respect to IOCs. Cytoskeletal as well as junctional components are thus expected to play key roles downstream of core PCP genes and indeed, members of each group have been identified that affect rotation.

Downstream of RhoA, Rho Kinase (Rock, Drok in flies) regulates acto-myosin contractility (Riento and Ridley, 2003). Rock activates Myosin regulatory light chain (Spaghetti squash, Sqh) and inhibits Myosin phosphatase, which itself inactivates Sqh, ultimately increasing Myosin II activity (Lee and Treisman, 2004; Riento and Ridley, 2003; Winter *et al.*, 2001). *drok* mutant clones show rotation defects (in addition to a severe loss of photoreceptors) and in the wing, *drok* genetically interacts with *fz* and *dsh* consistent with being downstream of the core PCP cassette (Winter *et al.*, 2001). Similarly, hypomorphic alleles of Myosin II heavy chain (*zipper*, *zip*), as well as overexpression of wild-type or dominant negative forms of Zip, cause over- and under-rotation of ommatidia (Fiehler and Wolff, 2007). Furthermore, *zip* and *drok* genetically interact in the eye (although most evident by the suppression of PR defects caused by overexpression of constitutively active Drok, rather than by effects on rotation; Fiehler and Wolff, 2007; Verdier *et al.*, 2006). Interestingly, MyoII is concentrated around (although not restricted to) the perimeter of the cells already recruited into the PR cluster (schematic in Fig. 7.5B), which led Fiehler *et al.* to suggest that it might be the driving force for rotation, similar to the zippering up of the *Drosophila* embryo during dorsal closure (Fiehler and Wolff, 2007; Franke *et al.*, 2005). Alternatively, such a localization pattern could also be indicative of a mechanism compacting the ommatidial cluster, or, since confocal imaging cannot resolve on which side of the cell–cell border Zip is localized, Zip could be enriched in non-PR cells around the rotating cluster. Importantly, the function of Rho kinase and MyoII downstream of the core PCP module is conserved during convergent extension in mouse, *Xenopus,* and zebrafish (Kim and Han, 2005; Marlow *et al.*, 2002; Skoglund *et al.*, 2008; Ybot-Gonzalez *et al.*, 2007).

During cellular movements, cell–cell contacts dynamically change. A major component of adherens junctions are cadherins (Halbleib and Nelson, 2006; Hulpiau and van Roy, 2009). Classical cadherins form homotypic interactions between adjacent cells, bind intracellularly to α/β Catenin complexes, and are responsible for cell adhesion. The *Drosophila* genome codes for an *E-cadherin* (*shotgun, shg*) and two *N-cadherin* genes, all of which are involved in the rotation process (in addition to E-Cadherin's requirement for the maintenance of epithelial integrity; Mirkovic and Mlodzik, 2006). Hypomorphic *shg* mutations show a clear underrotation

defect visible already in very young ommatidia close to the MF. In contrast, N-Cad double mutant ommatidia rotate too quickly, but stop at the correct angle of 90°, indicating not only that N-Cad slows down the rotation process but also that an N-Cad independent process exists that stops ommatidia (Mirkovic and Mlodzik, 2006). Analysis of *N-Cad/sca* double mutants could elucidate whether Sca function (see above) is part of such a stopping process.

Particularly striking are the complementary expression patterns of E-Cad and N-Cad1: E-Cad is relatively uniformly expressed at lower levels throughout the developing eye imaginal disk, but highly enriched at the borders where R2 touches R3 and R8, and where R5 touches R4 and R8 (Fig. 7.2 and schematic in Fig. 7.5B; Mirkovic and Mlodzik, 2006) but is not enriched at the contacts of R3/4 or where cluster cells touch IOCs. In contrast, N-Cad1 is mainly enriched at the shared border of R3/4 (Fig. 7.5B), where core PCP localization is asymmetric. With their complementary expression pattern and qualitatively opposing phenotype, it is tempting to speculate that the cadherins mediate precluster integrity/adhesion as well as balance the speed of ommatidial rotation. As mentioned above, E-Cad genetically interacts not only with EGFR signaling pathway components but also with a subset of the core PCP genes and RhoA (Mirkovic and Mlodzik, 2006). It is, however, unknown how these interactions function on a molecular basis; in particular, it is unknown whether Rho kinase can (differentially) phosphorylate components of adhesion complexes. Alternatively, genetic interactions could also be mediated by the Ras family GTPase Rap1 which also affects rotation, binds to Canoe, and modifies E-cadherin localization (O'Keefe *et al.*, 2009). Furthermore, due to the low level of uniform E-Cad expression, its ability to form homotypic interactions, and the existence of other adhesive proteins, it is expected that the effects of adhesion on rotation are more complex, as they are not fully "ommatidium autonomous" and could be affected by interactions with IOCs.

Indeed, two additional cell adhesion molecules, the IgCAM Echinoid (Ed), capable of homophilic and heterophilic interactions (Islam *et al.*, 2003), as well as its paralog Fred (Friend of Echinoid), have recently been described to be required for proper rotation (Fetting *et al.*, 2009; Ho *et al.*, 2010). While loss of their function causes a wide variation in the rotation angle of individual ommatidia, genetic interactions showed that *ed* and *fred* antagonistically interact with the EGFR signaling components *pointed* and *canoe* (Fetting *et al.*, 2009). Ed is a known inhibitor of EGFR signaling in other contexts (Bai *et al.*, 2001), but enhances rotation defects of *pnt* and *cno* (Fetting *et al.*, 2009). In contrast, *fred* mutations suppress loss of *cno* or *pnt*, again pointing toward a tight interplay between EGFR signaling, cell adhesion, and rotation. Interestingly, Ed appears to be enriched in cells newly recruited into the PR clusters and the neighboring IOCs, but

reduced in the rotating preclusters and their contact interfaces with IOCs (Fetting *et al.*, 2009; Ho *et al.*, 2010; Fig. 7.5B). Fred, on the other hand, shows an expression pattern more similar to the core PCP proteins with a double horseshoe around R3/4 at early stages of rotation and a higher level on membranes around R4 cells (with the exception of where R4 abuts R3; Fetting *et al.*, 2009). How the dynamic localization patterns of Ed and Fred mechanistically can be reconciled with their genetic requirements in R1,6,7 and cone cells (Table 7.1; Fetting *et al.*, 2009) remains to be addressed in greater detail. Recently, an indirect mechanism for Ed's effect on rotation has been proposed (Ho *et al.*, 2010). Ed is required for the endocytosis of Fmi in IOCs. In the absence of Ed, Fmi levels strongly increase in non-PR cells and such excessive ectopic Fmi levels can lead to PCP defects, similarly to prolonged Fmi presence in perduring mystery cells in *argos* mutants (see also above Strutt and Strutt, 2003).

4. Upstream PCP Components

The least understood and potentially most interesting, as well as controversial, questions in the PCP field currently are how the global polarization of the eye field, wing, and abdomen relate to the polarization of the individual structural units. Are there secreted factors such as Wnts involved? How do the atypical cadherins Fat and Dachsous act, and how and where are they connected to the PCP pathway?

4.1. Involvement of a Wnt in PCP signaling?

Wnts (Wingless, Wg in flies) are a family of secreted proteins functioning as classical ligands for Fz receptors during canonical Wnt signaling and could be responsible for a global activation of the Fz–PCP pathway (van Amerongen and Nusse, 2009). Considerable data is available that Wnts are involved in PCP signaling in vertebrates. Wnt7a induced PCP signaling regulates the number and expansion of satellite stem cells after muscle injuries in mice (Le Grand *et al.*, 2009). In *Xenopus* and zebrafish, Wnt5a and Wnt11 are both required for convergent-extension movements (Heisenberg *et al.*, 2000; Lele *et al.*, 2001; Moon *et al.*, 1993; Tada and Smith, 2000), and double mutants of Wnt5a and Wnt11 with concomitant morpholino-mediated knockdown of Wnt4 leads to neurulation defects in zebrafish similar to loss of *vangl1/2*, the paralogs of *stbm/vang* (Ciruna *et al.*, 2006). Similarly, Wnt5 knockout mice show convergent-extension defects in the organ of Corti in the inner ear, a typical vertebrate PCP-related effect (Qian *et al.*, 2007). Because injection of Wnt11 mRNA into the oocyte can rescue convergent-extension defects of zebrafish *wnt11* mutants, the

directionality of Wnt input in PCP signaling has been questioned. It has, however, not been assessed whether ubiquitous Wnt11 mRNA leads to ubiquitous Wnt11 protein or, even more importantly, activity. Support for directional input of Wnts on PCP signaling comes from the finding that Wnt11 provides a directional cue for the orientation and elongation of muscle fibers during early chicken development (Gros *et al.*, 2009). Muscle fiber orientation and elongation are also perturbed by manipulating core PCP components and the Wnt11 effects were thus attributed to noncanonical Wnt/Fz–PCP signaling.

In contrast to vertebrates, although not assessed in the eye, no data currently supports the involvement of *wg* or any of the other six *Drosophila* Wnts in PCP signaling (Chen *et al.*, 2008; Lawrence *et al.*, 2002). In particular, compound mutant clones of the five *wnts* expressed in the wing during PCP signaling cause no PCP defects (Chen *et al.*, 2008). These results thus show that simple redundancy does not account for the failure to detect Wnt involvement in PCP signaling in *Drosophila*. To date, the existence of a Fz ligand required for PCP signaling remains elusive, and the absence of a Wnt requirement is the biggest difference between PCP establishment in flies and vertebrates. However, Wg can affect ommatidial orientation more globally, due to its involvement in the establishment of the equator of the eye (see above). Loss of canonical Wnt signaling, as occurs in clones of the obligate canonical coreceptor *arrow* (Lrp5/6 ortholog) or *dsh*, leads to the formation of ectopic equators on the equatorial side of such clones relative to the endogenous equator (Strutt and Strutt, 2002; Wehrli and Tomlinson, 1998), as characterized by the induction of an equatorial marker (i.e., the enhancer trap line *eq1*; Wehrli and Tomlinson, 1998), arguing that this mechanism is distinct form PCP signaling.

4.2. The Fat/Ds system

fz clones in the eye, wing, and abdomen not only have cell autonomous effects but also affect neighboring wild-type tissue (Lawrence *et al.*, 2004; Vinson and Adler, 1987; Zheng *et al.*, 1995). Multiple molecular mechanisms have been invoked in order to explain noncell autonomy involving either diffusible factors ("factor X"; Lawrence *et al.*, 2002; Struhl *et al.*, 1997; Wehrli and Tomlinson, 1998) or relay systems and direct intercellular protein–protein interactions (Amonlirdviman *et al.*, 2005; Chen *et al.*, 2008; Tree *et al.*, 2002; Wu and Mlodzik, 2008). Most experiments addressing cell nonautonomous functions of PCP components were performed in the wing and abdomen, and models have been reviewed recently (Axelrod, 2009a; Lawrence *et al.*, 2007, 2008; Strutt and Strutt, 2009; Wu and Mlodzik, 2009). This review concentrates on experimental data obtained from the eye. Basically, analogous mechanisms involving intermediary,

diffusible factors, or locally relayed activities can be envisaged to explain nonautonomous effects in the eye. To date, nonautonomy in the eye has been neglected and no data exists that favors a particular model.

In addition to Fz, the atypical cadherins Fat (Ft) and Dachsous (Ds), as well as the Golgi resident, type II transmembrane protein Four-jointed (Fj) cause nonautonomous PCP defects in the eye (Fanto *et al.*, 2003; Rawls *et al.*, 2002; Strutt and Strutt, 2002; Yang *et al.*, 2002; Zeidler *et al.*, 1999b) and are implicated in setting up a global orientation gradient in the eye and wing (Ma *et al.*, 2003; Matakatsu and Blair, 2004; Strutt and Strutt, 2002). While fully mutant eyes for *ds* and *ft* show random chirality inversions, *fj*$^{-}$ mutants show only very rare PCP defects. Surprisingly, mutant clones of all three genes induce very strong PCP defects with strikingly distinct nonautonomous defects (Rawls *et al.*, 2002; Yang *et al.*, 2002; Zeidler *et al.*, 1999b). *fj* and *ft* clones show chirality inversions on the polar side of the mutant tissue, while *ds* clones are affected only on the equatorial side. Thus, wild-type tissue next to mutant tissue can correct PCP defects on the equatorial side of *ft* and *fj* clones and on the polar side of *ds* clones (Fanto *et al.*, 2003; Rawls *et al.*, 2002; Strutt and Strutt, 2002; Yang *et al.*, 2002; Zeidler *et al.*, 1999b). In addition, wild-type tissue on the equatorial side of *ds* and the polar side of *ft* and *fj* clones can revert the chirality, demonstrating an effect of mutant tissue on wild-type tissue (Fanto *et al.*, 2003; Strutt and Strutt, 2002; Yang *et al.*, 2002; Zeidler *et al.*, 1999b), although one report explicitly did not find the latter type of defects (Rawls *et al.*, 2002).

Initially, it was shown that *ft* and *fj* are required in the R3 cell, while *ds* was required in R4 (Fanto *et al.*, 2003; Yang *et al.*, 2002), and it was proposed that these factors directly act on the R3/4 cell fate specification, because Ft and Fj can promote R3, and Ds can promote R4 fates in R3/4 mosaic analysis (Table 7.1; Fanto *et al.*, 2003; Yang *et al.*, 2002). However, the validity of this clonal analysis was questioned because of a nonrandom incorporation of photoreceptor precursors into the ommatidial preclusters (Rawls *et al.*, 2002). The main reason for concern over the strict requirement of Ft, Fj, and Ds, however, comes from the fact that the induced clonal phenotypes can induce mirror symmetry lines similar (although mechanistically not necessarily equivalent) to ectopic equators, and the chirality choice of a single ommatidium is thus not "ommatidia autonomous," but can be influenced by its neighbors (Fanto *et al.*, 2003; Rawls *et al.*, 2002; Strutt and Strutt, 2002; Zeidler *et al.*, 1999b). Nevertheless, since the effects of *ft* and *ds* in R3/4 mosaic ommatidia for promoting R3 and R4 fates, respectively, are >80–90% penetrant, *ft* and *ds* probably can have an instructive role for the R3/4 cell fate (Fanto *et al.*, 2003; Yang *et al.*, 2002). This interpretation is also supported by the finding that the instructive role of *ft* requires *fz*: *ft* loses its ability to promote R3 in R3/4 mosaics in a homozygous *fz* mutant background (Yang *et al.*, 2002), indicating that the

initial bias is not simply due to the geometry of the eye disk and the manner cells are recruited into the ommatidial preclusters.

Ft protein is expressed uniformly in the eye disk but elevated around the MF. Ds, in contrast is expressed more strongly at the poles than at the equator close to the MF, while Fj (as assessed by a *fj–lacZ* transcriptional reporter) is expressed in an equatorial to polar gradient (Yang *et al.*, 2002). It is currently thought that Fj antagonizes Ds, which in turn can antagonize Ft activity (Fig. 7.3B) resulting in a Ft activity gradient (Sopko and McNeill, 2009; Strutt, 2009). In addition, *fj* transcription is repressed by Ft signaling and by Fj itself, while Ds can activate *fj* transcription (Fig. 7.3B; Yang *et al.*, 2002). Ft and Ds form heterotypic interactions (Ma *et al.*, 2003), which could explain their antagonism in the R3/4 fate decision. Furthermore, Fj is an extracellular kinase that can phosphorylate Ft as well as Ds on their transit through the Golgi (Ishikawa *et al.*, 2008). How such a phosphorylation could modify Ft and Ds activity and/or interaction, and whether their extracellular phosphorylation is essential, remains to be demonstrated.

A direct effect on R3/4 cell fate choice is not sufficient to explain the nonautonomous phenotypes of loss of *fj*, *ft*, and *ds* in clones and it has thus been proposed that the Ft/Ds system additionally modulates the activity of a factor X (Fanto *et al.*, 2003). Consistently, Atrophin (Atro), a transcription repressor that binds to the intracellular C-tail of and acts downstream of Ft shows PCP phenotypes similar to Ft. Importantly, these phenotypes include autonomous and nonautonomous effects. This supports the existence of a relay molecule regulated by the Ft/Atro axis (Fanto *et al.*, 2003). Again, such a factor X would need to be identified and does not necessarily have to be secreted, but could also be a factor that alters a cell-relay mechanism.

One of the most intriguing, complicated, and controversial questions is how Ft/Ds signaling is mechanistically related to the core PCP genes (Fig. 7.3B). Based on experiments in the abdomen, in which a fz^{-} cell can be repolarized by the Ft system, it was proposed that the two systems act in parallel (Casal *et al.*, 2006), although it is not clear where the two systems would converge (recently reviewed in Axelrod, 2009a; Lawrence *et al.*, 2007, 2008; Strutt and Strutt, 2009; Wu and Mlodzik, 2009). Similarly, *ds* and *fz* can function additively under certain conditions in the eye (Strutt and Strutt, 2002). Based on experiments in the wing, it was proposed that Ft/Ds provide a global signal inducing local refinement by the core Fz system (Ma *et al.*, 2003). The dependence on *fz* of the Ft instructive effect in R3/4 mosaics discussed above (Yang *et al.*, 2002) suggests that, at least to a certain extent, the Ft module might have upstream functions. Consistent with this, the asymmetric localization of core PCP proteins in the wing follows the "induced global polarity" upon Ft/Ds manipulation in the wing (Ma *et al.*, 2003; Strutt and Strutt, 2002). Importantly, forced asymmetric expression of Ft and, in particular, reverse asymmetric expression of Ds in an equatorial to

polar gradient in a *fj* mutant background can almost fully invert the chirality in the whole eye, demonstrating that graded *ds* expression can polarize the eye field (Simon, 2004). Surprisingly, *ds* and *fj* gradients in the wing are dispensable (Matakatsu and Blair, 2006; Simon, 2004). Furthermore, ubiquitous expression of Fat lacking the extracellular domain is sufficient to rescue the PCP defects of Fat mutants in the wing, suggesting that if a graded Ft activity is required, an intracellular system must exist to control it (Matakatsu and Blair, 2006).

Puzzling questions remain. First, comparison of a potential Fz activity gradient in the eye (equatorial to polar) and in the wing (proximal to distal) to a hypothetical Ft activity gradient shows that the latter would be reverted with respect to Fz in the wing (distal to proximal vs. equatorial to polar in the eye; Matakatsu and Blair, 2004, 2006; Simon, 2004). The situation is even more complicated on the abdomen, where the relative gradient orientation inverts within each segment (Casal *et al.*, 2006; reviewed in Strutt, 2009). In spite of these apparently contradictory findings, it is worth noting that Ft plays a role in PCP signaling not only in the fly, but knockouts of Fat4, the mouse *ft* paralog, also show PCP-related phenotypes in the inner ear and kidney, clearly revealing a conserved role in PCP signaling (Saburi *et al.*, 2008).

Second, in elegant experiments using heat-shock time-course rescue, it was shown that the nonautonomous PCP signaling phase and the autonomous requirement of Fz can be temporally separated with the nonautonomous phase preceding the autonomous one in the wing as well as in the eye (Strutt and Strutt, 2002). Intriguingly, it was concluded that the nonautonomous, *ds*-like phase of PCP signaling in the eye occurs ahead of the MF prior to the recruitment of photoreceptors into ommatidial clusters (Fig. 7.1). R3/4 mosaic analysis demonstrated that *fz* and *stbm* mutant cells push the wild-type partner cell into R3 and R4 fate, respectively, with $>95\%$ efficiency, which clearly requires communication between the future R3/4 cells of the same cluster. If R3/4 cells are globally prespecified even before they are photoreceptors, and even longer before they are in contact with each other (ommatidial row 3; Fig. 7.2; Wolff and Ready, 1991), a mechanism would have to be predicted that is able to revert the previously imposed R3/4 fates later.

Third, in the wing and abdomen, not only *fz* but also *stbm*/vang clones show domineering nonautonomy (Lee and Adler, 2002; Taylor *et al.*, 1998) and feedback models explaining nonautonomy consider both of these transmembrane molecules crucial (e.g., Amonlirdviman *et al.*, 2005; Chen *et al.*, 2008; Lawrence *et al.*, 2002; Wu and Mlodzik, 2008). However, in the eye, no nonautonomy has been observed for *stbm* clones (Wolff and Rubin, 1998) and a more comprehensive analysis of the effects and mechanistic causes of nonautonomy in the eye is thus required in the future.

4.3. JAK/Stat signaling

The JAK (Janus kinase)/STAT (signal transducer and activator of transcription) signaling pathway was initially discovered in mammals for its role mediating cytokine signaling (Hou *et al.*, 2002; Rawlings *et al.*, 2004; Shuai and Liu, 2003). In *Drosophila*, Unpaired (Upd) signals to the receptor Domeless, which activates the JAK Hopscotch (Hop), leading to phosphorylation and nuclear translocation of the transcription factor Stat. Interestingly, loss of *upd*, *hop,* and to a lesser extent *stat92e* leads to polarity inversions similar to loss of *ft* (Luo *et al.*, 1999; Zeidler *et al.*, 1999a). In particular, mutant clones of *hop* and *stat92e* lead to inversion of ommatidial chirality on the polar side of clones and show a nonautonomous behavior similar to *ft* clones (Zeidler *et al.*, 1999a). However, in contrast to loss of canonical Wnt signaling which causes induction of true ectopic equators based on the induction of the equatorial marker *eq1* (see above; Wehrli and Tomlinson, 1998), *hop* clones do not change the expression of equatorial markers (Zeidler *et al.*, 1999a). The phenotype reminiscent of ectopic equators seen in *hop* or *stat92e* clones is instead due to effects on PCP signaling. These findings have led to the suggestion that JAK/STAT signaling gradients are another means to regulate the elusive "factor X" controlling ommatidial orientation (Zeidler *et al.*, 1999a; reviewed in Strutt, 2009). Interestingly, however, the ligand Upd can also act independent of JAK/STAT to influence the equator by changing the expression of *mirror*, a key gene required for the establishment of the endogenous equator (McNeill *et al.*, 1997; Zeidler *et al.*, 1999a). Similar to the Ft system, future studies are needed to decipher the interplay between the JAK/STAT and the core Fz–PCP module during PCP establishment in the eye.

5. Additional Components Affecting PCP Signaling in the Eye

Several additional genes have been identified that also affect PCP signaling, but do not belong the groups of genes described so far. For example, genes required for apical/basal cell polarity can affect the establishment of PCP in the eye. In particular, the apical dPatj (PALS-1 associated tight-junction) protein recruits aPKC to Fz, which leads to a reduction of Fz activity in non-R3/4 cells of the ommatidial precluster (Djiane *et al.*, 2005). Consistent with this function, dPatj is strongly expressed around the apical circumference of R2 and R5 (Fig. 7.5B) and reduced around R3/4. In contrast, Par3/Bazooka is enriched more like a typical core PCP protein and can prevent aPKC from phosphorylating Fz in R3/4 and thus prevents its inhibition in the R3/4 precursor cells (Djiane

et al., 2005). Similarly, Scribble, a member of the Scribble/Lethal giant larva (Lgl)/Discs large (Dlg) complex localized to septate junctions (Bilder, 2004), has been shown to interact with Stbm and *scrib* mutants to cause PCP defects (Courbard *et al.*, 2009). Importantly, mouse Scrb1 (*spin-cycle*) genetically interacts with *vangl2* mutants during PCP establishment in the inner ear (Montcouquiol *et al.*, 2003).

Furthermore, Nhe2, a Na^+/H^+ exchanger has been shown to be required for Dsh membrane recruitment, a process considered critical for canonical as well as noncanonical Wnt signaling (Axelrod, 2001; Bilic *et al.*, 2007). *Nhe2* genetically interacts with *fz* and its overexpression can induce PCP defects. It has thus been suggested that a local more-alkaline pH favors an additional, direct membrane binding of Dsh upon recruitment by Fz (Simons *et al.*, 2009). Interestingly, *bedraggled* (*bdg*), another putative membrane transporter also genetically interacts with PCP genes and Bdg overexpression can induce PCP defects in the eye (Rawls *et al.*, 2007), suggesting that the membrane microenvironment is critical for PCP signaling.

In addition, mutations in *rasputin* (*rin*) cause photoreceptor recruitment defects as well as typical PCP defects in the eye (Pazman *et al.*, 2000). Rin is the *Drosophila* ortholog of the Ras-*G*TPase-activating SH*3* domain-*b*inding *p*rotein (G3BP). G3BPs contain, amongst others, several SH3 domain-binding sites and RNA-binding motifs and interact with RasGAP. G3BPs are involved in a variety of functions from cell signaling to RNA metabolism and transcription, but how these functions relate to each other is unknown (reviewed in Irvine *et al.*, 2004). *rin* genetically interacts with Ras and Rho signaling, potentially via the regulation of a RasGAP and RhoGAP. Further experiments are required to more precisely determine the mechanism of *rin* function in PCP signaling. It would be of particular interest to know if the RNA-binding activities of Rin are required for PCP signaling.

6. Conclusions

Much progress has been made in the past years in the understanding of PCP establishment in the *Drosophila* eye. Most importantly, genetics and molecular and genetic interactions have defined requirements for genes in PCP signaling that are conserved from invertebrates to vertebrates. Nevertheless, several key questions remain to be answered in order to understand PCP signaling in the eye and ultimately in human development and disease.

The most obvious problem to solve is defining the precise molecular linkage between the Ft/Ds system and the core Fz PCP module. If these systems act in parallel, where do they converge? If the Ft/Ds system feeds into the Fz system, at what position? Is the intersection or lack thereof the same in every tissue in *Drosophila* and in vertebrates?

Probably intertwined with the last problem is the need to resolve the mechanistic aspects of the directional nonautonomy in more detail. In particular, are *fz* nonautonomous effects in the wing truly equivalent to those in the eye? As discussed, in contrast to the wing, *stbm/vang* acts cell autonomously in the eye. Furthermore, in the wing, asymmetric PCP protein localization reiterates from cell to cell. In the eye, PCP protein localization has only been studied in PR cells and no asymmetries have been reported that would support a relay system passing across IOCs. Along similar lines, the function of the cytoskeleton and cell adhesion components in PRs as well as IOCs need to be better analyzed in order to understand their respective contribution the rotation process, which in turn will help us to better understand this type of collective cell migration.

The molecular link between Dsh and the genetically downstream Rho family GTPases remains enigmatic. In *Xenopus*, the formin xDAAM was shown to bridge signaling between Dsh and Rho. Furthermore, xDAAM is activated by xDsh, which in turn leads to an activation of Rho by a poorly understood process (Habas *et al.*, 2001; Liu *et al.*, 2008). Genetic analysis of *Drosophila* did not implicate *daam* in PCP signaling in the fly; thus, the molecular link between RhoA/Rac and Dsh remains obscure (Matusek *et al.*, 2006).

Historically, the *Drosophila* eye has provided an excellent model system for studies of PCP establishment and recent genetic evidence demonstrating requirements for *stbm*, *fmi*, *vangl2,* and *celsr1* in mammalian eye development corroborate the relevance of the fly system and are thus very gratifying (Sugiyama *et al.*, 2010).

NOTE ADDED IN PROOF

The effect of PCP signaling on cilia has very recently been shown to crucial for the establishment of left-right asymmetry in mice and zebrafish (Borovina *et al.* 2010; Song *et al.* 2010). Furthermore, the Golgi resident kinase Fj was shown to phosphorylate the extracellular domains of Ft and Ds, thereby changing the affinity for each other (Brittle *et al.*. 2010; Simon *et al.*, 2010).

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CHAPTER EIGHT

Milestones and Mechanisms for Generating Specific Synaptic Connections between the Eyes and the Brain

Nicko J. Josten *and* Andrew D. Huberman‡

Contents

Abstract

All information about the visual world is conveyed to the brain by a single type of neurons at the back of the eye called retinal ganglion cells (RGCs). Understanding how RGC axons locate and wire up with their targets is therefore critical to understanding visual development. In recent years, several important technological and conceptual advances have been made in this area, and yet, many fundamental questions remain unanswered. Indeed, while much is now known about how RGC axons pathfind at the optic chiasm and form retinotopic

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maps within their targets, how RGCs select their overall targets in the first place is poorly understood. Moreover, the signals that direct mammalian RGC axons to their appropriate layer within those targets remain unknown. The recent advent of genetic tools to selectively label and manipulate defined groups of RGCs is starting to provide a way to resolve these and other important questions about RGC wiring specificity. This field is therefore positioned to reveal new principles of visual circuit development that no doubt will extend to other regions of the CNS.

1. Introduction

Retinal ganglion cells (RGCs) encode different features of the visual environment and send that information to the brain where it is processed into perceptions and behaviors. RGC connections are exquisitely precise to ensure accurate visual processing. For example, neighboring RGCs project to neighboring portions of their targets and thereby convey information about the spatial position of objects in the environment. Moreover, functionally distinct RGCs project to different depths or "layers" within their targets and thereby establish parallel circuits for analyzing different features of the visual scene such as motion, color, or brightness. From a developmental perspective, each component of eye-to-brain connectivity translates into a different requirement for axon growth, pathfinding, and target recognition during development. Thus, understanding the complete sequence of events that enable RGCs to wire up with their targets is not only critical for understanding the genesis of vision but it also provides a comprehensive model for exploring how complex neural circuits are built. Here, we review studies focused on how mammalian RGCs establish precise synaptic connections in the brain. In doing so, we often mention experiments that were carried out on lower vertebrates and Drosophila, because they provide a conceptual framework for thinking about the cellular and molecular mechanisms that generate circuit specificity. Throughout this review, we also emphasize important aspects of mammalian eye-to-brain development that remain poorly understood, in hopes that our readers will be inspired to design and implement experiments to elucidate them.

2. Connecting the Eyes to the Brain

2.1. Preliminary steps for connecting the eyes to the brain

Connecting the eyes to the brain is a multistep process that begins as RGCs migrate to their correct layer of the retina and extend their axons. At this stage, RGCs must achieve three important milestones before they can begin

to search for their targets: First, they have to grow their axons toward the exit point of the eye, the optic disk. Second, RGC axons have to pass through the optic disk and into the optic nerve. Third, when RGC axons reach the ventral chiasm midline, they have to decide whether to cross to the opposite (contralateral) side of the brain or, alternatively, to remain in the same (ipsilateral) hemisphere (Fig. 8.1).

Some of the cellular and molecular mechanisms that regulate these early milestones are known. For example, RGCs extend their axons toward the optic disk in response to axon repellants expressed at the retinal periphery or over the retinal surface (Birgbauer *et al.*, 2000; Brittis *et al.*, 1992). Once RGC axon growth cones arrive at the optic disk, local expression of the chemoattractant, netrin-1, causes them to pass through the disk, leave the eye, and form the optic nerve (Deiner *et al.*, 1997). The extension of RGC axons down the optic nerve likely reflects their robust capacity for growth at embryonic ages (Goldberg *et al.*, 2002a). How do RGCs decide which hemisphere of the brain to project to? Work from Mason and coworkers revealed a molecular pathway that endows a subset of RGCs with strong sensitivity to chemorepulsion at the optic chiasm. Consequently, this subset of RGCs steers away from the midline to remain on the ipsilateral side of the brain (Herrera *et al.*, 2003; Williams *et al.*, 2003). There also appears to be a molecular genetic program related to chiasm crossing (e.g., Pak *et al.*, 2004; Williams *et al.*, 2006). Given that several excellent and thorough reviews were recently published on the topics of pathfinding out the eye, into the nerve, and at the chiasm (see Oster *et al.*, 2004;

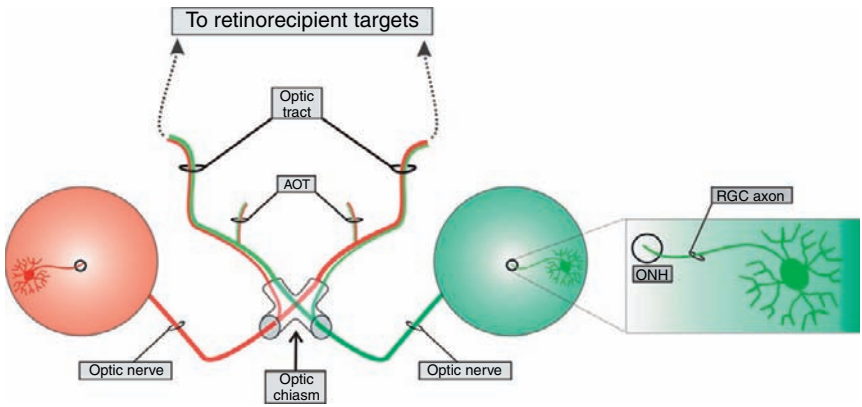

Figure 8.1 Critical early choice points for retinal ganglion cell (RGC) axons to reach the brain. In order for retinal ganglion cells to connect to the brain, they must first extend their axons toward the optic nerve head (ONH), down the optic nerve, and through the optic chiasm. Posterior to the chiasm, RGC axons travel to their retinorecipient targets via the optic tracts. A small subset of RGC axons travel to their targets via the accessory optic tracts (AOT).

Petros *et al.*, 2008), we do not discuss them in further detail here. We mention them because (i) they are necessary for RGCs to eventually reach their correct targets in the brain and (ii) RGC axon–axon interactions within the eye, nerve, or chiasm could impact the targeting of those axons at more distal locations along the visual pathway. Indeed, the degree to which the total RGC population is divided into decussating or nondecussating fractions impacts the targeting of those axons in downstream targets (see below). So with the importance of these early pathfinding events in mind, we now consider the choices RGC axons face as they enter the brain and grow toward their targets.

2.2. Choosing the correct retinorecipient target/s

What we refer to as "the connections between the eyes and the brain" actually consists of axons arising from ~20 functionally distinct subtypes of RGCs (Dacey *et al.*, 2003; reviewed in Berson, 2008). Each subtype carries information about a different feature of the visual environment such as edges, motion, or color (Dacey *et al.*, 2003; Roska and Werblin, 2001) and sends that information to a restricted number of retinorecipient targets, where it is processed into perceptions or behaviors (Callaway, 2005; Nassi and Callaway, 2009). For example, the RGCs that encode luminance project to the intergeniculate leaflet (IGL) and ventral lateral geniculate nucleus (vLGN), thalamic structures that mediate nonphotic entrainment of circadian rhythms (Hattar *et al.*, 2002; Muscat and Morin, 2006; reviewed in Harrington, 1997), whereas the RGCs that encode directional object motion project to the dorsal lateral geniculate nucleus (dLGN), a thalamic structure that relays visual information to the cortex for conscious image perception (Huberman *et al.*, 2009; Stewart *et al.*, 1971; for a comprehensive review of mammalian RGC subtypes and their central projections, see Berson, 2008). In order for the visual system to function properly, it is critical that each RGC subtype innervate the correct retinorecipient targets. How RGCs accomplish that task is not well understood, but the organization of the mature visual system displays several features that constrain how this could occur. The dominant constraint on RGC target selection is the existence of ~24 retinorecipient targets but only two ways for RGC axons to reach them (Figs. 8.1 and 8.2). All RGC axons travel to their targets via the main or accessory optic tracts—dense bundles that course next to (and past) each retinorecipient target (Fig. 8.2). The decision made by a RGC to innervate a particular retinorecipient target therefore reflects the decision to defasciculate from and exit the optic tract. It is also worth noting that, at maturity, many RGCs project to multiple retinorecipient targets (Bowling and Michael, 1980; Crook *et al.*, 2008; Huberman *et al.*, 2008a; Tamamaki *et al.*, 1995). Moreover, individual RGC axons often select one of several targets that reside adjacent to one another (e.g., Huberman *et al.*, 2008a,

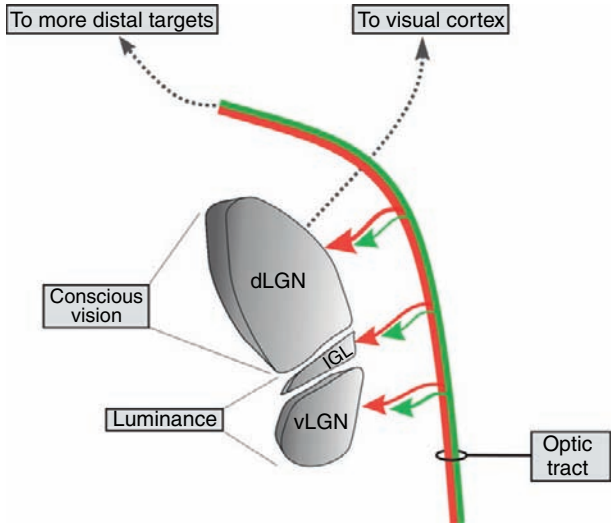

Figure 8.2 RGC axons enter their targets by defasciculating from the optic tract. RGC axons from the two eyes (red and green) travel together in the optic tract. The axons of functionally distinct RGC subtypes innervate different retinorecipient targets, such as the vLGN (ventral lateral geniculate nucleus), IGL (intergeniculate leaflet), or dLGN (dorsal lateral geniculate nucleus), by leaving the tract. The neurons in those targets serve different functional roles in visual perception and behavior and have outputs to different brain areas (e.g., dLGN neurons project to visual cortex).

2009; Fig. 8.2). Thus, stringent axon–target recognition systems must exist to allow RGCs to distinguish among closely positioned nuclei along the visual pathway.

Very little is known about the cellular and molecular mechanisms that control overall target recognition in the mammalian visual system. Lesion studies have shown if their visual targets are ablated, RGCs can innervate nonvisual targets such as the auditory or somatosensory thalamus (Frost and Metin, 1985; Sur *et al.*, 1988), but how different subtypes of RGCs distinguish among the various retinorecipient targets during normal development is not known. In large part, this gap in knowledge arose because of a lack of tools to label specific and defined subtypes of RGCs across development. In other words, without a means to visualize and unequivocally identify RGCs that are destined to innervate certain targets and not others, it is virtually impossible to study how target selection develops or changes in response to experimental manipulation.

A small number of studies examined how RGCs pick their correct targets by combining retrograde labeling and morphology-based classification of RGC subtype. Notably, Shatz and coworkers carried out a study in

which they retrogradely labeled RGCs from different retinorecipient targets across development. Their analysis of the back-filled RGCs showed that one particular RGC type—the so-called "X" RGCs—initially project to the dLGN and to the midbrain superior colliculus (SC), but then withdraw their connections to the SC (Ramoa *et al.*, 1989). Similar results were later obtained in ferrets (Wingate and Thompson, 1995). Those findings indicate that specificity of RGC axon–target connections can arise through removal of inappropriate connections, but whether this is a general rule for all RGC subtypes is not known. The recent discovery of transgenic mice that selectively express fluorescent proteins in defined subtypes of RGCs (Hattar *et al.*, 2002; Huberman *et al.*, 2008a, 2009; Kim *et al.*, 2008; Siegert *et al.*, 2009; Yonehara *et al.*, 2009) now make it possible to systematically study RGC target selection.

Studies of cold-blooded vertebrates reveal that RGC axons often follow one another to their targets. Chien and coworkers found that when early born RGCs are deleted from zebra fish, the axons of later born RGCs make large-scale targeting errors when they eventually reach the brain (Pittman *et al.*, 2008). This suggests that some RGC axons act as "pioneers" to guide later growing axons. The concept of pioneer pathfinding has longstanding support from studies of insects (e.g., Taghert *et al.*, 1982), but has received less experimental attention in mammals. It will therefore be exciting to see whether RGCs with similar functional characteristics (e.g., "luminance detecting" RGCs) are led to their targets by pioneers of the same or similar subtype. Alternatively, the axons of early born RGCs may pioneer the way for later born RGCs, irrespective of their functional or subtype identity. Again, these questions can now finally be addressed using the abovementioned transgenic mice that distinguish functionally unique RGC subtypes in the mature and developing brain (Hattar *et al.*, 2002; Huberman *et al.*, 2008a, 2009; Kim *et al.*, 2008; Siegert *et al.*, 2009; Yonehara *et al.*, 2009).

Studies in cold-blooded vertebrates revealed one of the molecular pathways that mediate target selection. Holt and coworkers discovered that in Xenopus, fibroblast growth factor (FGF) induces RGC axon extension (McFarlane *et al.*, 1995). Interestingly, levels of FGF are low within the target tectum (the structure homologous to the SC)—which could explain why RGCs slow down and enter this target. Indeed, if FGF is ectopically expressed at the tectal border, RGCs grow past the tectum (McFarlane *et al.*, 1996). These findings suggest such that growth factors may play a role in mammalian target recognition. As this field moves forward and the factors that promote mammalian target recognition are identified, it will be interesting to see if they generally operate by promoting axon–target adhesion or rather by promoting repulsion. Of course both mechanisms could collaborate to enforce target choice specificity. With many genetic tools now available for monitoring neurons in fixed tissue and *in vivo*, our understanding of how RGCs pick their targets is sure to expand rapidly in the next decade.

2.3. Finding the correct retinotopic termination zone

After RGC axons locate and enter their appropriate targets, they have to navigate to the correct retinotopic location. "Correct" in this context means that RGCs must conserve their spatial relationships by projecting to neighboring locations within the target and thereby establish an orderly representation of the visual field. Retinotopic mapping has been studied mainly by a technique called focal tracing in which the lipophilic dye, DiI, is injected into a restricted location on the retina and the precision of the labeled termination zone (TZ) is visualized in the brain. Classic work from O'Leary and coworkers used focal tracing to demonstrate that early in development, RGC axons overshoot their correct TZ by a large distance (Simon and O'Leary, 1992). In the rodent SC, this overshoot is particularly dramatic: RGCs axons from the temporal retina initially extend across the full anterior–posterior extent of the SC, bypassing their correct TZ by several millimeters. Once the overshoot is maximal, an interstitial branch forms at the correct retinotopic location and the mistargeted portion of the axon is pruned back through a process that may involve Wallerian-like degeneration (Hoopfer *et al.*, 2006; Simon and O'Leary, 1992; Fig. 8.3). The degree of axonal overshoot varies depending on the location in the retina from which the RGC axon arises, but all RGCs nonetheless appear to undergo these cellular changes before arriving at the appropriate TZ (see McLaughlin and O'Leary, 2005 for review).

It is worth mentioning that retinotopic maps are present in virtually all retinorecipient targets. Given that many RGC subtypes project to more

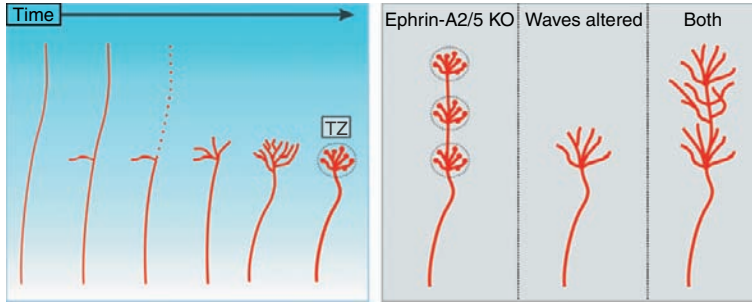

Figure 8.3 Retinotopic mapping in normal, ephrin-A, and retinal wave-deficient mice. The blue box includes the normal developmental sequence that RGC axons (red) undergo to find their correct retinotopic termination zone (TZ) in their targets. (See text for detailed description of these events.) The dashed red line indicates axonal degeneration. The blue gradient reflects a typical distribution of ephrin-A ligands across the target. The gray box encompasses the phenotypes seen after knockout (KO) of ephrin-A2/5, when early retinal waves are altered, or when ephrin-As *and* waves are both disrupted (see text for full description).

than one of these targets (Bowling and Michael, 1980; Crook *et al.*, 2008; Tamamaki *et al.*, 1995), RGC axons must form retinotopically correct TZs in multiple locations along the visual pathway. The two major forces by which RGC axons establish retinotopic maps are (i) spontaneous waves of neural activity and (ii) gradients of molecular guidance cues. We now consider how these forces work.

2.3.1. Correlated RGC firing drives retinotopic refinement

In mammals, retinotopic map formation occurs prior to vision but during the time when spontaneous "waves" propagate across the retina, causing neighboring RGCs to fire action potentials (reviewed in Huberman *et al.*, 2008b; Torborg and Feller, 2005a; Wong, 1999). This correlated RGC firing is hypothesized to drive retinotopic map refinement by engaging Hebbian-type plasticity at central synapses (Butts *et al.*, 2007; Katz and Shatz, 1996). The first direct test of this hypothesis was done by comparing the retinotopic maps in the brain of wild-type mice and mice lacking the beta2 subunit of the nicotinic acetylcholine receptor. Early experiments showed that beta2 mice lack retinal waves and instead exhibit noncorrelated RGC firing during the period of retinotopic refinement (McLaughlin *et al.*, 2003; Torborg and Feller, 2005a,b). More recent experiments suggest, however, that retinas from beta2 mice can support waves under certain conditions, but those waves are much larger and much faster than normal, and they lack a characteristic directional bias found in wild-type retinas (Stafford *et al.*, 2009; Sun *et al.*, 2008). Using focal DiI tracing to evaluate the precision of retinotopic mapping, McLaughlin *et al.* (2003) and Grubb *et al.* (2003) found that RGC axons fail to refine into a focal TZs in the dLGN or SC of beta2 mice (Fig. 8.3). Extracellular recordings (Cang *et al.*, 2008; Chandrasekaran *et al.*, 2005; Grubb *et al.*, 2003) and optical imaging studies (Mrsic-Flogel *et al.*, 2005) also showed that the spatial organization of dLGN and SC receptive fields are expanded in beta2 knockouts—essentially "smearing" the representation of the visual space. Collectively, these studies show that normally patterned retinal waves are essential for retinotopic mapping in the mammalian visual system.

After retinotopic maps are established, spontaneous retinal waves continue to drive the removal of excess RGC synapses onto target neurons. These refinements occur on a scale much too fine to detect with focal DiI tracing, but electrophysiological recordings have shown that the number of RGCs connecting to each dLGN neurons reduces from $\sim$12 to 1–3 during this period (Chen and Regehr, 2000; Jaubert-Miazza *et al.*, 2005; reviewed in Huberman, 2007). Waves are necessary for this pruning to occur because intraocular injection of the sodium channel tetrodotoxin (TTX) prevent fine-scale pruning (Hooks and Chen, 2006). Thus, even before vision, retinal waves drive refinement of RGC axons into progressively sharper and sharper TZs.

2.3.2. Guidance molecules and the polarity of retinotopic maps

In beta2 mutant mice, RGC terminations are abnormally diffuse (Chandrasekaran *et al.*, 2005; Grubb *et al.*, 2003; McLaughlin *et al.*, 2003) but they still project to roughly the correct area of the target (e.g., the temporal retina still maps to anterior SC, and the nasal retina to posterior SC). Thus, the basic polarity and global structure of retinotopic maps is likely to be controlled by activity-independent factors. In past two decades, studies in chicks and mice showed that the ephrins and their receptors (Ephs) are the molecular cues that set the basic structure of retinotopic maps—not just in the SC, but in multiple retinorecipient targets along the subcortical visual pathway (Feldheim *et al.*, 1998; and reviewed in McLaughlin and O'Leary, 2005). Here, we highlight the basic principles by which ephrins perform this role and we describe some recently published experiments that expand on those principles.

Ephrins establish retinotopic maps through "gradient matching." Several different ephrin-As (in mice, ephrins-A2/3/5) are expressed in high-posterior, low-anterior gradients across the SC. At the same time particular Eph-A receptors (EphA3 in chick, EphA5 in mouse) are expressed in high temporal, low nasal gradients across the ganglion cell layer of the retina (reviewed in Huberman *et al.*, 2008b; McLaughlin and O'Leary, 2005). Because ephrin-As generally act as repellants for axons expressing high levels of Eph-As, two logical predictions emerge from these expression patterns: first, RGCs in the temporal retina will avoid the ephrin-A-rich posterior SC and instead map to the anterior SC. Second, RGCs in the nasal retina will be able to map further "up" the ephrin-A5 gradient, into the posterior SC (Huberman *et al.*, 2008b; McLaughlin and O'Leary, 2005). Overexpression and genetic knockout data nicely support these predictions; in mice lacking ephrins-A2/5, RGC axons form ectopic terminations along the A–P axis of the target (Feldheim *et al.*, 2000; Frisen *et al.*, 1998; Fig. 8.3). Similar results are seen in mice lacking EphA5 receptors (Feldheim *et al.*, 2004). It should be noted, however, that multiple ephrin-As and Eph-As are expressed both in the retina and in the SC (Hornberger *et al.*, 1999). Indeed, removal of target-derived EphA7 alters RGC targeting (Rashid *et al.*, 2005). A simple model based on Eph-A receptors in the retina and ephrin-A ligands in the target therefore is not sufficient to explain the development of N–T retinotopic maps. Conditional, region-specific knockouts of ephrin-As and Eph-As are urgently needed to resolve precisely where and how these molecules influence visual map development. In the meantime, one can generally conclude that ephrin-A:Eph-A interactions are essential for delivering RGC axons to their correct retinotopic termination sites in the brain.

A hallmark principle of retinotopic mapping is that RGCs define their correct TZ according to the relative levels of ephrin-A:Eph-A signaling in neighboring RGC axons (McLaughlin and O'Leary, 2005; Reber *et al.*, 2004). This principle was elegantly demonstrated by Brown *et al.* (2000),

who made a knockin (ki/ki) mouse with EphA3 expressed in approximately every other RGC. The retinas of ki/ki mice thus have a gradient of EphA3 superimposed onto the normal endogenous gradient of EphA5. The striking consequence of this arrangement is that the EphA3/5 expressing RGCs establish a retinotopic map that is distinct from the retinotopic map formed by the EphA5-only expressing RGCs. Indeed, in ki/ki mice there are two complete, orderly retinotopic maps in the SC, each arising from RGCs *in the same eye*. Those results provide strong evidence that RGC axons map not according to the absolute amount of ephrin-A they encounter in their targets but rather according to the relative amount of Eph-A–ephrin-A signaling in neighboring RGC axons. The results of Brown *et al.* (2000) are consistent with classic studies in which half of the SC was ablated; in those experiments, a complete (albeit compressed) retinotopic map still formed (e.g., Marotte and Mark, 1987)—demonstrating there is no strict addressing of RGCs to specific retinotopic coordinates. At the same time, recent work in zebra fish argues that RGC axons project to specific locations in the tectum irrespective of other RGC axons (Gosse *et al.*, 2008). These contrasting results could relate to differences in the precision of retinotopic maps across species. Nevertheless, the double SC maps in ki/ki mice (Brown *et al.*, 2000; Triplett *et al*, 2009) provide strong evidence that relative signaling between Eph receptors and ephrin ligands is an important factor for establishing orderly retinotopic maps in the mammalian brain. An important goal now is to understand how RGC axons "read out" the relative levels of ephrin-As. Recent studies suggest these interactions are mediated at least in part by the p75 and/or TrkB neurotrophin receptors (Lim *et al.*, 2008; Marler *et al.*, 2008) but how those receptors drive the axonal changes required for TZ formation remain unknown.

2.3.3. The dorsal–ventral map

Thus far we have only discussed how RGCs establish maps along the N–T axis. Less is known about the formation of the dorsal–ventral (D–V) retinotopic map. Based on their complementary expression in the retina and target, gradient matching of Eph-Bs and ephrin-Bs have been implicated in D–V mapping. Indeed, when multiple Eph-Bs are knocked out, RGCs exhibit retinotopic D–V targeting errors in the SC (Hindges *et al.*, 2002). However, molecules other than Eph/ephrin-Bs also contribute to D–V mapping. In chicks, wingless (Wnt) signaling acting through Ryk receptors is crucial for D–V mapping (Schmitt *et al.*, 2006). Whether Wnts play a role in retinotopic mapping in mammals is yet to be tested. Another consideration is that axons from RGCs situated along the D–V axis of the retina are "preordered" in the optic tract before they reach their targets (Plas *et al.*, 2005). Whether this order is due to axon–axon recognition cues or whether it reflects differences in the timing of outgrowth for dorsal- versus ventral-RGCs, is not known. Clearly, more work is needed to understand

the mechanisms that establish D-V maps and some of those mechanisms appear to engage before RGC axons even reach their targets.

2.3.4. Activity and ephrins: Separate paths toward the same goal

We have described how neural activity and molecular guidance cues contribute to retinotopic map development. A key question therefore is: do activity and ephrins operate in the same pathway or do they operate in parallel? *In vitro* studies show that cAMP oscillations can influence RGC responsiveness to ephrin-As (Nicol *et al.*, 2007). Given that retinal waves are strongly dependent on cAMP levels (Stellwagen *et al.*, 1999), crosstalk between waves and ephrin-As could impact retinotopic mapping. However, *in vivo* evidence indicates that altering waves does not impact Eph-A expression in RGCs, nor does altering ephrin-As perturb wave activity (Huberman *et al.*, 2005; Pfieffenberger *et al.*, 2005). Indeed, the influence of activity, ephrin-As, and D–V mapping are strikingly separable by different experimental manipulations. As mentioned previously, altering retinal waves disrupts the precision of retinotopic TZs, but the approximate position of the TZ is normal (Chandrasekaran *et al.*, 2005; Grubb *et al.*, 2003; McLaughlin *et al.*, 2003; Fig. 8.3). Conversely, genetic removal of Eph-As or ephrin-As alters retinotopic mapping, but the ectopic TZs that form are normal in size (Feldheim *et al.*, 2000; Frisen *et al.*, 1998; Fig. 8.3). Finally, if both wave activity and ephrin-As are disrupted, the N–T retinotopic map is completely abolished, but the D–V map is spared (Cang *et al.*, 2008; Fig. 8.3). Thus, the current model of retinotopic mapping is based in the idea that patterned activity and mapping molecules operate in parallel: RGC axons are delivered to their grossly appropriate location by ephrin-As, ephrin-Bs, and Wnts and then neural activity drives those axons to cluster into a focal TZ (Feldheim and O'Leary, 2010; Huberman *et al.*, 2008b; McLaughlin and O'Leary, 2005). A major emphasis in the field now is to understand the signaling pathways that act downstream of activity and ephrins to mediate RGC axon–axon remodeling.

2.4. Segregating into eye-specific territories

After they establish retinotopic maps, mammalian RGCs are faced with a unique challenge. Because mammals have eyes positioned toward the front of their skulls, the visual fields viewed by each of the two eyes will overlap to some degree. As a result, some RGCs in the two eyes (i) view the same portion in visual space, (ii) project to the same side of the brain, and (iii) innervate the same retinorecipient targets. Ephrins in turn direct a subset of RGC axons from the two eyes to identical locations in their targets. Indeed, in developing carnivores, axons from the two eyes are retinotopically aligned and they overlap within their targets (Jeffery, 1985). This overlap does not persist, however; right and left eye axons

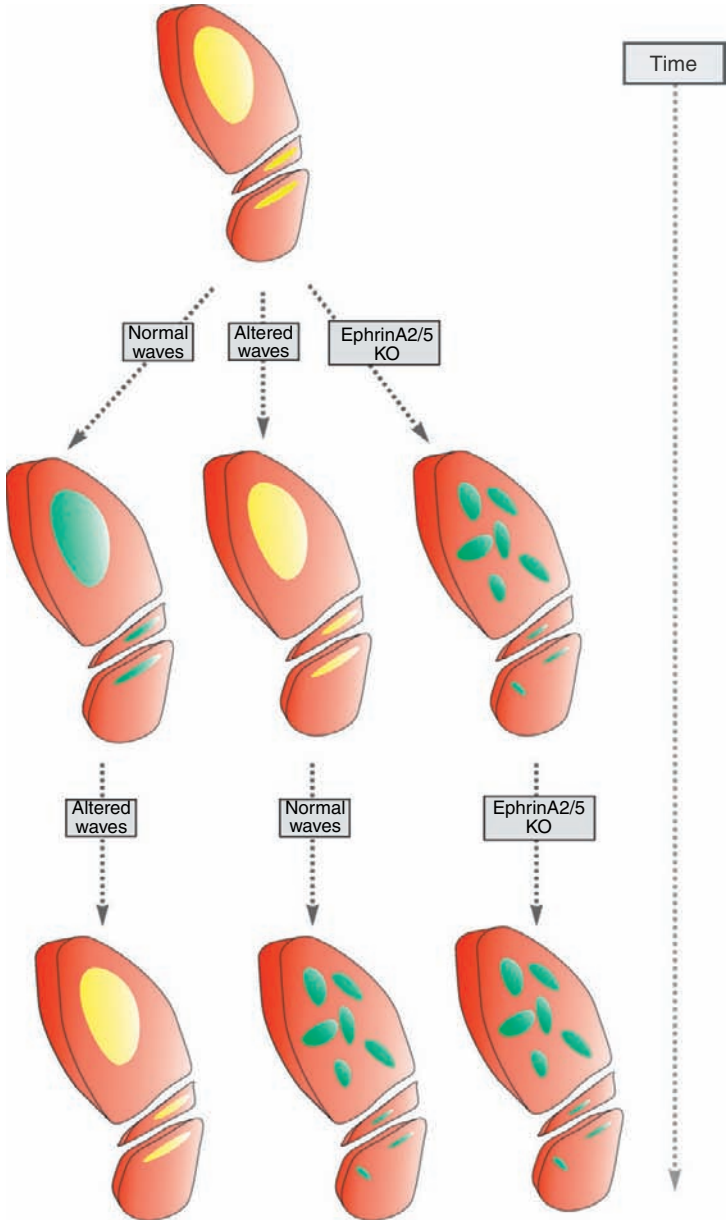

Figure 8.4 Eye-specific segregation in normal, ephrin-A, and wave-deficient mice. RGC axons from the contralateral (red) and ipsilateral (green) eyes and the regions where they overlap (yellow) are shown in the visual thalamus of the mouse (see Fig. 8.1 for description of these targets). (Top row) Axons from the two eyes overlap early in development. (Middle row) If normal retinal waves are present, over time axons from

always refine into contralateral and ipsilateral domains—a process referred to as eye-specific segregation (Fig. 8.4). How RGC axons progress from an overlapping to a segregated state has been a major focus of visual neuroscience for more than three decades (Godement *et al.*, 1984; Linden *et al.*, 1981; Rakic, 1976; Shatz, 1983), and remains a premiere model system for studying CNS circuit refinement (reviewed in Huberman *et al.*, 2008b).

2.4.1. Cellular changes that drive eye-specific segregation

Eye-specific segregation has mostly been studied in the dLGN where axons from the right and left eyes occupy territories of relatively stereotyped shape, size, and position. In an elegant series of now-classic studies, Shatz and coworkers explored the cellular rearrangements that RGC axons undergo as they progress from an intermingled to eye-specific state. They labeled individual RGC axons in fetal cats of different ages and analyzed the morphology of the labeled terminals in the dLGN (Sretavan and Shatz, 1984, 1986). Overall, they observed that RGC axons undergo dramatic growth and remodeling to achieve eye-specific segregation. During the overlap stage, RGC axons extend across the full width of the dLGN and display multiple "side branches" along their length. EM studies later showed those side branches are the substrate by which right and left eye axons form synapses onto the same dLGN neurons (Campbell and Shatz, 1992). The progression from an overlapping to an eye-specific state occurs as all the side branches are removed—except one—which in turn expands to form dense terminal arborizations in the correct eye-specific territory (Fig. 8.4; reviewed in Shatz, 1996). Although the precise cellular rearrangements that occur may somewhat vary across species (e.g., Snider *et al.*, 1999), the classic studies of Shatz and co-workers provided the basis for understanding how RGC axons remodel in order to achieve an eye-specific state.

2.4.2. Spontaneous activity is essential for eye-specific targeting

What forces drive eye-specific segregation in the dLGN? One thing is certain: it is not visual experience, because this process is completed before photoreceptors are capable of responding to light (reviewed in Huberman *et al.*, 2008b). Rakic (1976, 1977) showed that if one eye is removed during

the two eyes segregate into nonoverlapping, "eye-specific" domains. If early retinal waves are altered, RGC axons remain overlapping. In ephrin-A2/5 KO mice, axons from the two eyes segregate but into retinotopically misplaced patches. (Bottom row) Blocking waves after eye-specific segregation is completed causes desegregation. Conversely, if waves are blocked but then wave activity is allowed to recover, eye-specific patches form, but at retinotopically misplaced locations in the target. In ephrin-A2/5 KO mice, the ectopic eye-specific patches are stable over time.

the early overlap stage, axons from the intact eye remain throughout the target. Thus, eye-specific segregation is dependent on competitive interactions between axons from the two eyes.

Sretavan *et al.* (1988) proposed that spontaneously generated activity mediates binocular competition leading to ocular segregation in the dLGN. They tested that hypothesis by infusing TTX into the brain of fetal cats, starting at the time when right and left eye axons overlap. TTX prevented eye-specific segregation by inducing dramatic growth of RGC axons. That eventually led to the idea that retinal waves are the source of activity that drives binocular competition in the dLGN (Shatz, 1996). The retinal waves that occur during eye-specific segregation are driven by acetylcholine (Feller *et al.*, 1996; Torborg and Feller, 2005a), so Penn *et al.* (1998) used the cholinergic drug, epibatidine, to perturb retinal waves in neonatal ferrets. The results of that manipulation were clear: when spontaneous wave activity was reduced in one eye, axons from the contralateral eye expanded within the dLGN and axons from the activity-manipulated eye shrank their overall TZ. By contrast, when wave activity was reduced activity in both eyes, RGC axons failed to segregate and remained diffuse throughout the target (Penn *et al.*, 1998). Those results have now been confirmed many times over in ferrets and mice (Fig. 8.4; reviewed in Huberman *et al.*, 2008b; also see Koch and Ullian, 2010). The evidence is therefore strong that spontaneous retinal activity is necessary for eye-specific segregation in the dLGN. Indeed, the need for retinal waves is ongoing throughout early development; if the retinal waves are first eliminated or altered starting *after* eye-specific segregation is complete, axons from the two eyes desegregate in the dLGN (Chapman, 2000; Demas *et al.*, 2006; Fig. 8.4). Together these studies show that retinal waves play a fundamental role in both establishing and consolidating the basic architecture of eye-specific connections in the mammalian visual system.

2.4.3. What forms of activity drive segregation?

Retinal waves induce correlated firing in RGCs. A key question is whether those correlations are the parameter underlying eye-specific segregation or whether waves play a more "permissive" role in shaping visual connections. One could imagine, for example, that correlated RGC firing induced by retinal waves directly mediates Hebbian refinements at retino-dLGN synapses in a manner similar to how visual experience influences OD plasticity during the critical period (reviewed in Feller, 2009; Hensch, 2005; Smith *et al.*, 2009). Computational modeling based on the spatial temporal properties of retinal waves supports that idea (Butts *et al.*, 2007; Feller, 2009). Alternatively, eye-specific segregation may be dictated by guidance molecules that are only capable of exerting their effects on RGCs that have normal levels and patterns of activity (Chalupa, 2009; Crowley and Katz, 2000). Indeed, there is evidence that neural activity can modulate

RGC axon outgrowth, branching, and guidance (Goldberg *et al.*, 2002b; Nicol *et al.*, 2007). To distinguish among these possibilities, it is necessary to alter waves without silencing RGC firing altogether and then evaluate the consequences of that on eye-specific refinement. Stellwagen and Shatz (2002) were the first to accomplish that feat. They used cAMP-augmenting drugs to increase wave size and frequency in one or both eyes of developing ferrets. When they increased waves in one eye, they saw that axons from the normal unmanipulated eye lost territory in the dLGN. By contrast, when they increased waves in both eyes, binocular connections formed normally (Stellwagen and Shatz, 2002). Those results show that the relative level of activity in the two eyes is critically important for eye-specific segregation and that normal activity levels do not necessarily lead to normal patterns of visual connections. Together those data challenge the idea that activity is merely "permissive" for RGC axon growth and targeting.

The correlated firing of neighboring RGCs is the property of retinal waves that most models consider important for retinotopic and eye-specific refinement (Butts *et al.*, 2007). That makes sense because RGC firing is what actually drives the spiking and synaptic plasticity of dLGN and SC target neurons (Mooney *et al.*, 1996; Shah and Crair, 2008). However, to test if RGC firing patterns are in fact crucial for eye-specific segregation, it is necessary to somehow eliminate the "correlated" component of spontaneous retinal waves while retaining overall levels of activity. The first study to accomplish this used an immunotoxin directed against the interneurons that generate early retinal waves. That reduced the correlated firing of neighboring RGCs but did not change the overall levels of spiking activity in the retina. Surprisingly, eye-specific segregation proceeded normally under these conditions (Huberman *et al.*, 2003). Those findings, as well as a study showing that intraocular TTX injections do not prevent eye-specific segregation (Cook *et al.*, 1999), supported the idea that activity plays a permissive role in shaping binocular visual circuits. However, it is important to note that calcium wave activity persisted for some time in the immunotoxin-treated retinas and that TTX does not prevent calcium waves (Cook *et al.*, 1999; Huberman *et al.*, 2003; Stellwagen *et al.*, 1999). It therefore remains possible that broader scale correlations (i.e., larger waves) and/or calcium waves were present at levels sufficient to drive eye-specific segregation. More rapid and complete ablations of correlated activity, combined with large-scale recordings of retinal neurons (e.g., Stafford *et al.*, 2009), are needed to better understand if wave-like patterns of activity are necessary for eye-specific segregation.

A large number of studies make one thing certain: *something* in the pattern of spontaneous retinal activity is critically important for eye-specific segregation. As mentioned above, beta2 knockout mice can, under some recording conditions, exhibit waves that are abnormally large and fast and that exhibit altered directionality (Sun *et al.*, 2008; Stafford *et al.*, 2009).

Beta2 mice also exhibit defects in eye-specific segregation (Huberman *et al.*, 2008b; Muir-Robinson, 2002; Pfieffenberger *et al.*, 2005; Rossi *et al.*, 2001; Fig. 8.4). Still, it remains unclear exactly which parameters of retinal waves directly relate to eye-specific refinement. The ultimate experiment would be to systematically *control* the patterns of RGC spiking in the two eyes and thereby isolate which patterns of RGC activity drive eye-specific segregation—something that is now possible with the advent of optogenetic tools to modulate neural activity (e.g., Boyden *et al.*, 2005; Zhang *et al.*, 2007).

2.4.4. A potential role for guidance molecules in eye-specific targeting

Any review of eye-specific targeting would be remiss if the concept of activity-independent factors was not addressed. In a purely activity-dependent model, right and left eye axons should sort into salt and pepper like patterns. In reality, however, the basic pattern of eye-specific projections is essentially invariant for a given species. This stereotypy argues that factors other than neural activity help shape eye-specific connections. Differences in the timing of ingrowth for contralateral versus ipsilateral eye axons were hypothesized to ensure the stereotyped ordering of eye-specific territories (Shatz, 1996). Unfortunately, timing of axon growth is a difficult variable to manipulate *in vivo* and therefore has never been tested. Another hypothesis is that molecular cues pattern the regular spatial layout of eye-specific domains (Chalupa, 2009; Crowley and Katz, 2000). A naturally occurring experiment that indirectly supports that idea is the Belgian achiasmatic sheepdog—a spontaneously occurring mutant in which all RGCs project the ipsilateral side of the brain. In this remarkable dog, axons from the temporal retina form a domain in the dLGN that is separate from the domain formed by the axons arising from the nasal retina—even though both sets of axons arise *from the same eye (*Williams *et al.*, 1994). Binocular competition cannot underlie this segregation because axons from the two eyes never had the chance to interact. Similar observations have been made in ferrets, cats, and mice with altered RGC pathfinding at the optic chiasm (Guillery, 1969a,b; Rebsam *et al.*, 2009). In those animals, axons from RGCs in different parts of the retina segregate from one another, even though they originate from the same eye.

What sort of molecules might contribute to eye-specific patterning? In considering this question, it is worthwhile to note that contralateral versus ipsilateral eye-specific domains correspond to inputs from RGCs in the nasal versus temporal retina, respectively. Since Eph-A levels distinguish RGCs along the N–T retinal axis, they are good candidates to test in the context of eye-specific mapping. In mice, the story is very straightforward: ephrin-A2/5 are expressed in gradients suitable for a role in retinotopic mapping along the N–T axis of the dLGN (Feldheim *et al.*, 2000). If ephrin-A2/5 are knocked out, axons from the two eyes form patches scattered throughout the dLGN. However, ipsi- and contra-axons still segregate

from one another in ephrin-A2/5 mutants (Fig. 8.4; Pfieffenberger *et al.*, 2005). In ferrets, the role of ephrins is slightly more complicated and suggests they play a more direct role in segregating axons from the two eyes. If Eph-As are misexpressed in the retinas of neonatal ferrets, many axons from the two eyes fail to segregate from one another. Indeed, altering Eph-As in newborn ferret causes right and left eye RGC axons to overlap almost as much as they do following epibatidine-induced activity blockade (Huberman *et al.*, 2005). It is not entirely clear why ephrins mediate eye-specific segregation in ferrets but not in mice. This discrepancy may relate to the fact that ferrets have eye-specific projections that are mirrored by distinct cellular layers (Linden *et al.*, 1981), whereas mice do not (Godement *et al.*, 1984; but also see Reese, 1988).

Recent experiments show that contra- versus ipsi-projecting RGCs are molecularly distinct in a way that is independent of their different trajectories at the optic chiasm because in albino or ephrin mutants, eye-specific zones cluster into mini-islands of purely contra- or purely ipsi-eye axons (Pfieffenberger *et al.*, 2005; Rebsam *et al.*, 2009). Screens for molecules that are differentially expressed in contralateral versus ipsilateral domains of the dLGN have not yet revealed any candidate eye-specific patterning molecules (Kawasaki *et al.*, 2004), but as the sensitivity of genomic and proteomic screens improve, such cues may eventually be identified. In the meantime, the dominant model of eye-specific segregation is that spontaneous retinal activity helps cluster inputs from the same eye, and ephrin-As position those eye-specific projections into stereotyped retinotopically appropriate locations in the dLGN.

2.5. Picking a depth: Laminar-specific targeting

At this point in development, RGC axons have found their overall targets, arrived at the correct retinotopic zone and segregated into the appropriate eye-specific domain. Next they face the task of finding and forming synapses with the correct target neurons. One way that nature has simplified this task is by positioning different types (or portions) of postsynaptic neurons at different depths within their targets, creating parallel "layers" for different aspects of visual processing. By directing RGC axons to particular layers, functionally specific synaptic connections are maintained (Nassi and Callaway, 2009; Sanes and Yamagata, 1999).

One of the more salient examples of laminar specificity in the eye-to-brain pathway is the division of magnocellular (M), parvocellular (P), or koniocellular (K) layers in the primate dLGN (reviewed in Callaway, 2005; Nassir and Callaway, 2005). Generally speaking, the three different types of dLGN laminae receive axons from RGCs that encode motion (M), color and form (P), or yellow/blue color opponency (K). It should be noted, however, that

the number of different RGC subtypes that are known to project to the dLGN is ever-expanding (Crook *et al.*, 2008; Dacey *et al.*, 2003).

Despite the fact that laminar specificity is a salient and functionally relevant aspect of visual circuit organization, how it develops is not well understood. The main reason for this gap in understanding is that, until recently, there was no way to distinguish axons rising from functionally distinct RGCs until they achieved their adult patterns of connectivity. Work in hamsters suggested that RGC axons initially project broadly across the depth of the retinorecipient SC before refining to their correct lamina (Sachs *et al.*, 1986). By contrast, a study in the macaque showed that magnocellular and parvocellular projecting RGCs diverge early on to target separate dLGN regions (Meissirel *et al.*, 1997). The interpretation of those studies is complicated, however, by the fact that without markers to distinguish RGC axons destined to project to particular SC or dLGN laminae, it is impossible to know if a given axon is making correct versus incorrect targeting choices.

Recently, the discovery of a RGC subtype-specific marker was used to study the development of laminar specificity. In calbindin2 (CB2)–green fluorescent protein (GFP) mice, Off-alpha RGCs selectively express GFP and project specifically to the deeper layer of the retinorecipient SC (Huberman *et al.*, 2008a; Fig. 8.5). By monitoring the GFP-labeled alpha axons across development, it was observed that laminar-specific targeting

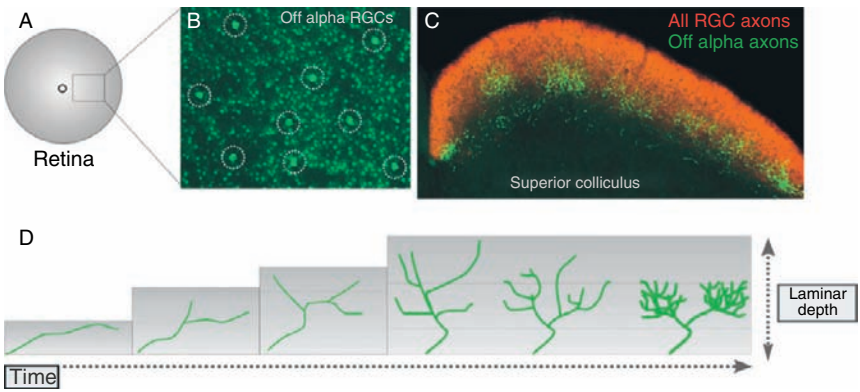

Figure 8.5 Laminar specificity of Off-alpha projections to the superior colliculus. (A, B) Off-alpha RGCs (outlined dashed circles) selectively express GFP (green fluorescent protein) in CB2–GFP mice. Amacrine cells (smaller cell bodies) also express GFP in these mice. (B) The axons of Off-alpha RGCs terminate at a specific laminar depth of the retinorecipient SC (superior colliculus). The axons from all RGCs are shown in red, whereas Off-alpha axons are shown in green. The green axons also form patches or "columns" within their layer of the SC. (D) Schematic diagram of Off-alpha RGC axons during the stage they pick their correct laminar depth in the SC (see main text and Huberman *et al.*, 2008a for details).

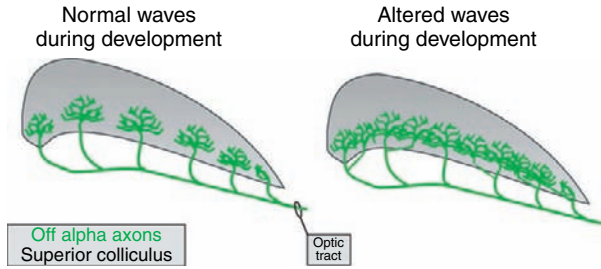

Figure 8.6 **Altering retinal waves prevents columnar but not layer-specific targeting. Normally, Off-alpha RGC axons refine to the deep layer of the SC and aggregate into columns by the time of eye-opening. If retinal waves are altered, Off-alpha axons still refine to the correct layer, but columnar specificity fails to emerge. (See main text and Huberman *et al.*, 2008a for details).**

occurs through broad-scale axonal refinement; Off-alpha axons initially project across the entire depth of the retinorecipient SC before removing their input from the superficial lamina (Fig. 8.5; Huberman *et al.*, 2008a). A subsequent study replicated that finding for a different RGC subtype, and showed that some other RGCs target their correct lamina from the outset (Kim *et al.*, 2010). Ultimately, however, labeling of specific RGC subtypes *and their postsynaptic neurons* is needed to determine if laminar-specific refinements reflect axonal retraction, synapse loss, or degeneration. In the meantime, one can conclude that laminar specificity often emerges from initially imprecise connections.

As with the other aspect of eye-to-brain connectivity described above, laminar specificity occurs prior to vision and during the period when waves propagate across the retina. Whether retinal waves help establish laminar-specific connections was tested by crossing CB2–GFP mice with beta2 knockout mice. The phenotype of those mice was highly consistent; laminar specificity of Off-alpha RGC axons developed normally in both the SC and dLGN (Fig. 8.6; Huberman *et al.*, 2008a). That result is consistent with studies in fish and chickens that completely silenced RGCs or tectal cells and no impact on laminar-specific targeting of RGC axons (Inoue and Sanes, 1997; Nevin *et al.*, 2008). No study has yet examined the impact of removing all activity on laminar specificity in mammals, but the bulk of evidence points to the idea that RGCs establish layered axonal connections on the basis activity-independent cues.

2.5.1. Molecular cues that direct laminar specificity in mammals: The search continues

Despite numerous lines of evidence pointing to the idea that adhesion molecules promote laminar specificity of RGC dendrites and axons in mammals, the identity of those molecules has remained elusive. In chickens, the

sidekicks and the DSCAMs (down syndrome cell adhesion molecules) are expressed in subsets of RGCs and amacrine cells where they regulate dendritic targeting (Yamagata and Sanes, 2008). A recent study from the Burgess lab (Fuerst *et al.*, 2009) asked if DSCAMs perform a similar function in mammals. They generated DSCAM and DSCAM-like (DSCAML1) knockout mice. Surprisingly, removal of one or both DSCAMs caused dramatic alterations in RGC soma spacing (a feature called "mosaicism") but it did not prevent laminar-specific targeting of RGC dendrites. Indeed, even functional specificity of synaptic connections was preserved in DSCAM mutant retinas (Fuerst *et al.*, 2009). Whether DSCAM mutant axons maintain laminar specificity of their connections is still unknown, but overall, the results of Fuerst *et al.* suggest that DSCAMs are unlikely to mediate laminar synaptic specificity in the mammalian visual system.

If DSCAMs are dispensable for laminar specificity in mammals, then what sorts of molecules might perform this role? Cadherins are a large class of cell adhesion molecules that typically promote homophilic attraction. Given their established role in promoting laminar specificity of the fly and chick visual systems (Inoue and Sanes, 1997; reviewed in Clandinin and Zipursky, 2002), the cadherins are exciting candidates to mediate RGC laminar specificity in mammals. At the same time, axon repellants could establish laminar-specific connectivity through graded expression across the depth of the target (reviewed in Huberman *et al.*, 2010). Given that not a single molecule has yet been identified as critical for establishing laminar-specific RGC connections in the mammalian brain, the search for laminar cues is going to be an intense and exciting area of research in the next few years.

2.6. Dividing into columns: Functional modules

The arrival of an RGC axon to its appropriate layer in the brain is analogous to the arrival at a particular "zip code"—it represents a specific and defined territory but not necessarily a final destination. After RGC axons arrive at the appropriate layer, they still have to distinguish among the various cell types that reside there. This aspect of RGC wiring specificity relates to different aspects of visual circuit function. As such, it is not revealed unless the entire population of one RGC subtype is selectively labeled—something that has only recently become possible as genetic tools for labeling specific RGC subtypes have become available. For example, in the above-mentioned CB2–GFP mice, the entire mosaic of Off-alpha RGCs not only projects to a specific layer in the dLGN and SC, but within the SC, those connections are also arranged into regular alternating patches or "columns" of synaptic terminals (Huberman *et al.*, 2008a; Fig. 8.5). These columns do not correspond to right versus left eye connections so one idea is that they are modules representing specific aspects of the visual scene. In this sense, RGCs that view the same location in visual space and encode the same

quality of visual information will send that information to specific target neurons. Indeed, closer inspection of the columns formed by Off-alpha RGC axons in the SC revealed that (i) ~3–4 Off-alpha RGCs project to the same column and (ii) individual RGC axons often branch to innervate multiple columns (Huberman *et al.*, 2008a). Other RGC subtypes also can form columns in the SC (Huberman, unpublished data). Synaptic columns arising from the retina therefore represent an intriguing case of microcircuitry that elevates the demands for developmental mechanisms that can distinguish among RGC subtypes.

How do RGC axonal columns develop? Analysis of postnatal CB2–GFP mice revealed that Off-alpha columns emerge from an initially imprecise state; shortly after birth, there is a crude semblance of columnar specificity but only once RGCs begin to elaborate their arbors and form synapses do these columns become readily apparent (Huberman *et al.*, 2008a). Thus, like many of the other forms of eye-to-brain connectivity (Simon and O'Leary, 1992; Sretavan and Shatz, 1986), RGC subtype-specific columns emerge through axonal refinements and directed synapse formation (Fig. 8.5).

The columns formed by Off-alpha RGCs emerge during the early postnatal period when retinal waves occur. CB2–GFP:beta2 knockout mice thus provide a direct test of whether waves play an important role in establishing axonal columns. As we noted above, Off-alpha RGCs still refine into the correct synaptic layer in the dLGN and SC in the presence of altered waves. However, those abnormal waves completely prevent Off-alpha axons from achieving columnar specificity (Fig. 8.6; Huberman *et al.*, 2008a). This cannot be due to loss of beta2 nicotinic AChRs in target SC neurons because the phenotype is mimicked by injecting a cholinergic blocker into the eyes of otherwise wild-type CB2–GFP mice. Collectively these results tell us that waves heavily influence columnar-specific axon targeting, presumably by inducing distinct firing patterns in different RGC subtypes (Kerschensteiner and Wong, 2008).

2.7. Subcellular targeting and microcircuitry

As one looks closer and closer at RGC wiring specificity, it becomes increasingly clear that there will be additional developmental programs to ensure precision of RGC axonal connections. For example, fine-scale anatomical studies revealed that RGC synapses are selectively concentrated on the soma and proximal dendrites of dLGN neurons, whereas cortical input to dLGN cells resides elsewhere on the dendritic tree (Bickford *et al.*, 2010; Sherman, 2004). How RGCs achieve this immensely precise synaptic targeting is not known. It could be that RGC axons are the first afferents to arrive in the dLGN and therefore synapse onto dLGN neurons whose dendrites are still small and immature. Alternatively, portions of the dLGN neuron's dendritic tree may be molecularly distinct and thus reserved

for retinal versus nonretinal sources. The formation of perisomatic connections onto cortical neurons is regulated by adhesion molecules belonging to the IgG superfamily (Ango *et al.*, 2004), so there is a prescendent for this idea. Given that where synapses reside on the dendritic tree is critical for local circuit computations and output (Poirazi *et al.*, 2003; Sherman, 2004), probing how subcellular targeting develops in the visual system is an important and relatively untapped area of study.

2.8. Forming and eliminating a synapse

As we have reviewed the various stages of visual circuit wiring, we have periodically mentioned "synapses"—which of course are the basis by which RGCs communicate with the brain. With the exception of RGC pathfinding out of the eye and through the chiasm, essentially all the wiring milestones we have discussed involve synapse formation and/or elimination to some degree or another. Thus, the critical question remains—how do RGCs establish actual synaptic connections with their target neurons?

Several of the molecules that promote glutamatergic synapse formation have been identified including neuroligins, SynCAMs, thrombospondins, FGFs, and SynDigs (for review, see McAllister, 2007; Waites *et al.*, 2005; also see Kalashnikova *et al.*, 2010). Indeed, the thrombospondins were isolated as astrocyte-derived factors capable of promoting synapse formation in mammalian RGCs *in vitro* (Christopherson *et al.*, 2005; Ullian *et al.*, 2001). Most synaptogenic molecules are not thought capable of distinguishing among different neuronal subtypes to promote synaptic specificity. However, there is evidence that different neuroligin family members control excitatory versus inhibitory synaptogenesis in hippocampal neurons (Chih *et al.*, 2005; Chubykin *et al.*, 2007; Graf *et al.*, 2004), so the possibility cannot be ruled out that different RGC subtypes employ different synaptogenic molecules to connect with their specific targets, layers and postsynaptic cells in the brain.

Throughout this review, we also described RGC axonal "refinement"—some of which (e.g., eye-specific refinement) involve the elimination of functional synapses (Campbell and Shatz, 1992; Chen and Regehr, 2000; Jaubert-Miazza *et al.*, 2005). Recent experiments have greatly enhanced our understanding of the molecular signals that promote synapse elimination. Indeed, many of these were discovered for their impact on the stability of RGC synapses. For example, in order to understand how TTX prevents eye-specific refinement (Sretavan *et al.*, 1988), Shatz and co-workers screened for molecules whose expression is regulated by spontaneous activity and is altered by TTX. They discovered that the immune family of major histocompatibility complex (MHC) I proteins are strongly regulated by spontaneous activity (Corriveau *et al.*, 1998). They went on to show that the MHC receptors are expressed in the developing visual system where they are required for eyes-specific segregation (Huh *et al.*, 2000). Recently, experiments from the Shatz

lab also identified the ligands that mediate MHC-dependent synapse elimination in the dLGN (Datwani *et al.*, 2009). The MHCs belong to the adaptive immune system but molecules of the innate immune system are also important for removal of excessive RGC synapses leading to circuit refinements. Stevens *et al.* (2007) discovered that the complement protein C1q is expressed by RGCs during development, and is required for eye-specific segregation and fine-scale elimination of retino-dLGN synapses (Stevens *et al.*, 2007). Recent studies also point to the neuronal pentraxins as crucial for translating activity into structural refinements at developing RGC synapses by affecting the conversion of synapses from a "silent" to an "active" state (Bjartmar *et al.*, 2006; Koch and Ullian, 2010). Others have proposed that immune proteins act upstream of activity by regulating glutamate transmission and dendritic dynamics (Xu *et al.*, 2010). Regardless of mechanism, the emerging theme is that immune genes are important regulators of RGC synapse elimination during development. Given their widespread expression throughout the developing CNS, these genes are likely to regulate developmental refinement of diverse CNS circuits.

The discovery of new molecules that influence RGC targeting is ongoing. Culican *et al.* (2009) also recently identified a ubiquitin-ligase related molecule that is capable of modifying RGC synapses that is independent of activity or ephrin-As and that does not appear directly linked to the immune system. This underscores the idea that diverse molecular pathways will converge to direct proper synapse formation and refinement in the developing visual system.

2.9. Modifying synapses in response to experience

By the time of eye opening, RGC axonal connections are essentially adultlike. A hallmark principle of critical period visual plasticity is that RGC projection patterns are not strongly impacted by visual experience. This appears true for eye-specific projections in the dLGN (Wiesel and Hubel, 1963). However, other aspects of RGC axonal connectivity appear susceptible to visual experience. For instance, Hooks and Chen (2006) showed that visual deprivation alters the fine-scale retinotopic mapping in the dLGN by causing RGC inputs to revert to a poly-innervated state. Other features of RGC connectivity could be malleable in response to experience as well, but surprisingly few studies have examined this.

3. Conclusions and Future Directions

We have now reviewed the complete journey that an RGC axon takes in order to achieve its precise circuit connections. In doing so, we hope to have made apparent that each milestone—exiting the eye,

recognizing a target, selecting a layer, etc.—further constrains the number and type of synapses that a RGC can make and ultimately leads to highly precise circuitry. Two broad themes were intended to emerge from our sequential description of eye-to-brain wiring. First, guidance molecules demarcate correct versus incorrect territories at progressively finer scales over time. Second, neural activity plays an ongoing and critical role in honing the precise location and size of RGC arbors. We hope to have also made clear that many fundamental discoveries remain to be made in this area. Indeed, huge gaps remain in our understanding of how RGCs achieve overall target, laminar, and subcellular wiring specificity. The recent advent of genetic markers for functionally distinct RGC subtypes, combined with the rich set of tools to manipulate neural activity and gene expression, make this a truly unprecedented and exciting time for probing how the eyes wire up with the brain and ultimately, how that wiring influences visual perception and behavior.

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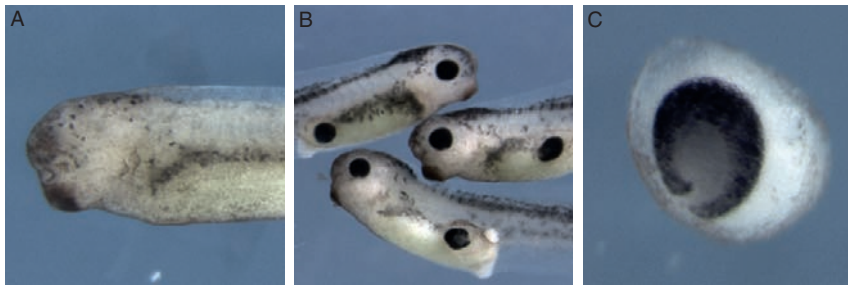
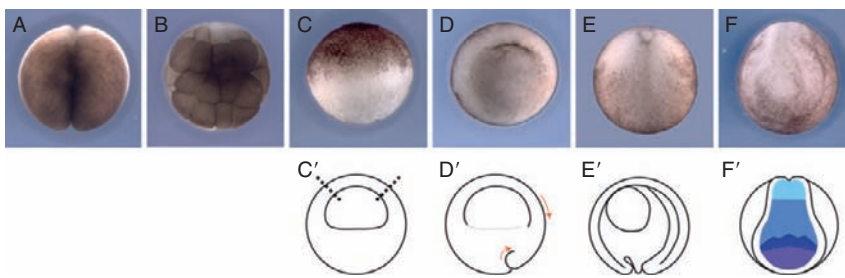
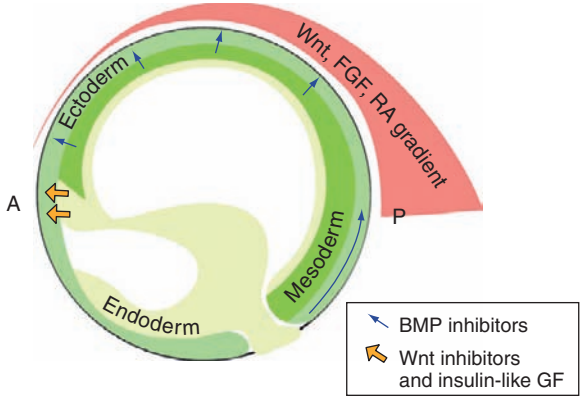


Michael E. Zuber, Figure 2.1 Eye field removal, transplantation, and culturing are easily accomplished using *Xenopus laevis*. One-half of an eye field can be removed from a stage 15 embryo. (A) The tadpole develops normally, but lacks an eye on the operated side. (B) If transplanted to the flank of host embryos, eye fields form eyes (shown here approximately 2 days after transplantation). (C) This eye formed from an eye field explant cultured for 3 days. These simple experiments show the remarkable, self-organizing nature of the eye field.

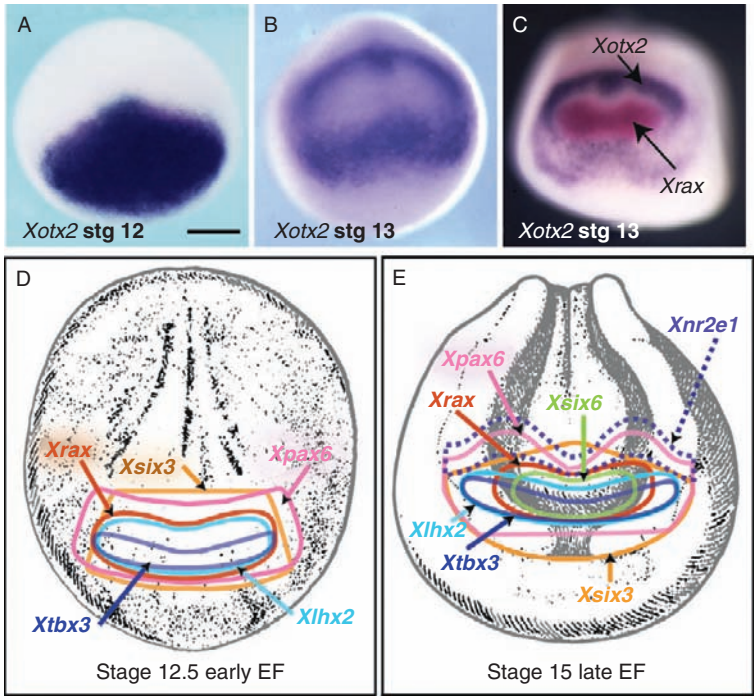


Stage 2	Stage 6	Stage 9	Stage 10	Stage 12.5	Stage 15
2-Cell (animal)	32-Cell (animal)	Blastula (lateral)	Early gastrula (vegetal)	Late gastrula (dorsal)	Neurula (anterior)
1.5 hpf	3 hpf	7 hpf	9 hpf	14.5 hpf	17.5 hpf

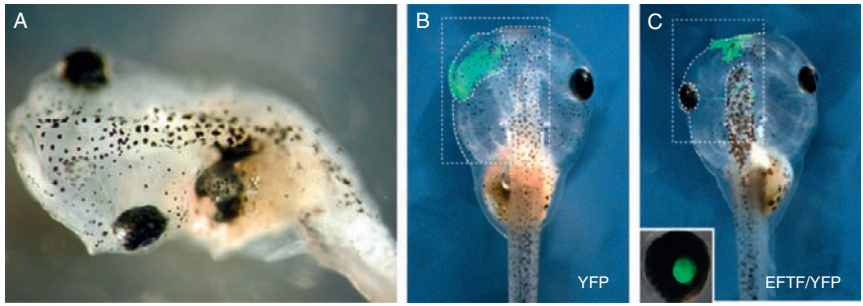
Michael E. Zuber, Figure 2.2 Early development of *Xenopus laevis*. (A–F) Bright field images of embryos from stages 2 to 15. (C′) Schematic cross section at stage 9 showing the tissue collected for animal cap assays. (D′) Stage 10 embryo at the start of gastrulation. Arrows indicate the direction of cell movements. (E′) Stage 12.5 embryo illustrating the layers of internal tissues that form. (F′) By stage 15 the anteroposterior axis of the neural plate is specified into the forebrain (purple), midbrain (dark blue), hindbrain (blue) and spinal cord (light blue). The table shows staging per Nieuwkoop and Faber (1994), and hours postfertilization (hpf).



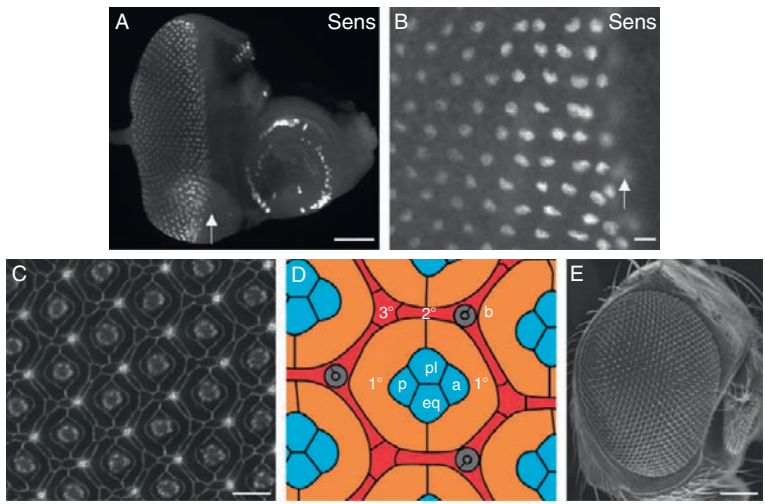
Michael E. Zuber, Figure 2.3 Signaling systems regulating neural patterning. Schematic transection of a *Xenopus* gastrula shows in red gradients of caudalizing signaling systems in the embryo (Wnt, FGF, and retinoic acid, RA). Wnt inhibitors (e.g., Cerebrus, Frzb, Dickkopf) and insulin-like growth factors (GF; yellow arrows) are expressed in the anterior endomesoderm, causing head formation. Purple arrows indicate BMP inhibitors (e.g., Follistatin, Noggin, Chordin) that neuralize the ectoderm. A, anterior; P, posterior.



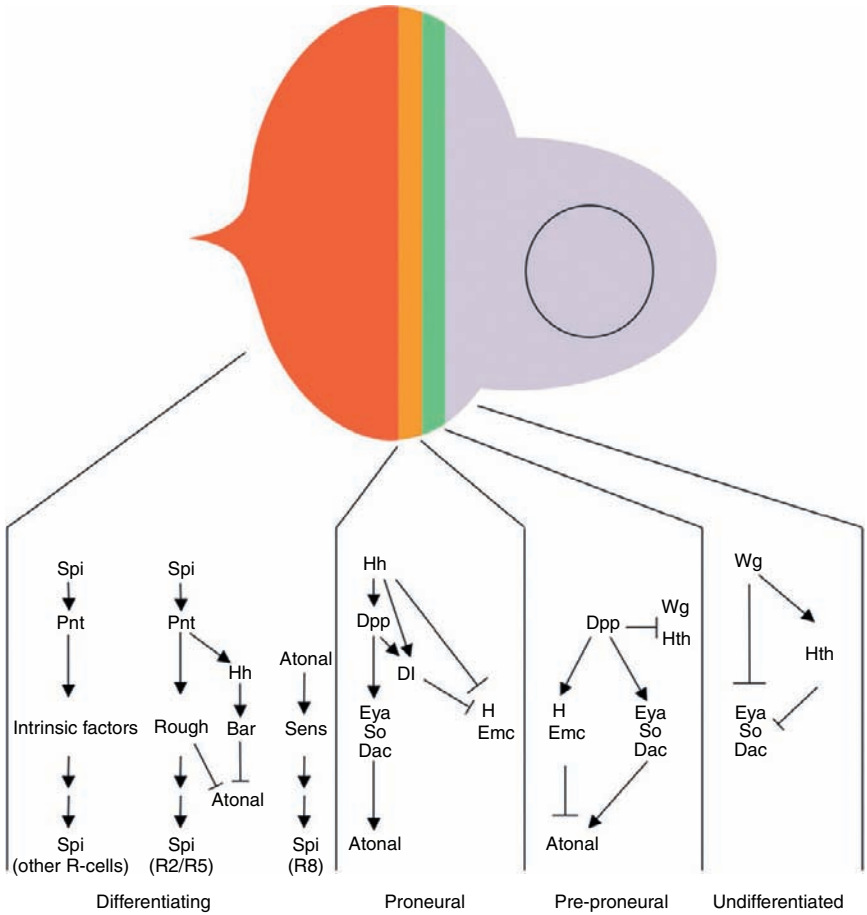
Michael E. Zuber, Figure 2.4 *Xotx2* and EFTF expression during eye field specification. (A) *In situ* hybridization of *Xotx2* shows it is expressed in the anterior neural plate prior to eye field specification. (B) Central *Xotx2* expression is rapidly repressed as EFTF expression is first detected in the 90 min between stages 12 and 13. (C) Double *in situ* hybridization for *Xotx2* (purple) and *Xrax* (red) illustrates their mutually exclusive expression domains. (D–E) Illustrations adapted from Nieuwkoop and Faber (1994), showing the dynamic, nonidentical overlapping expression patterns of the EFTFs at early and late stages of eye field specification. The expression domains of these genes outside the eye field at these stages have not been included for clarity.



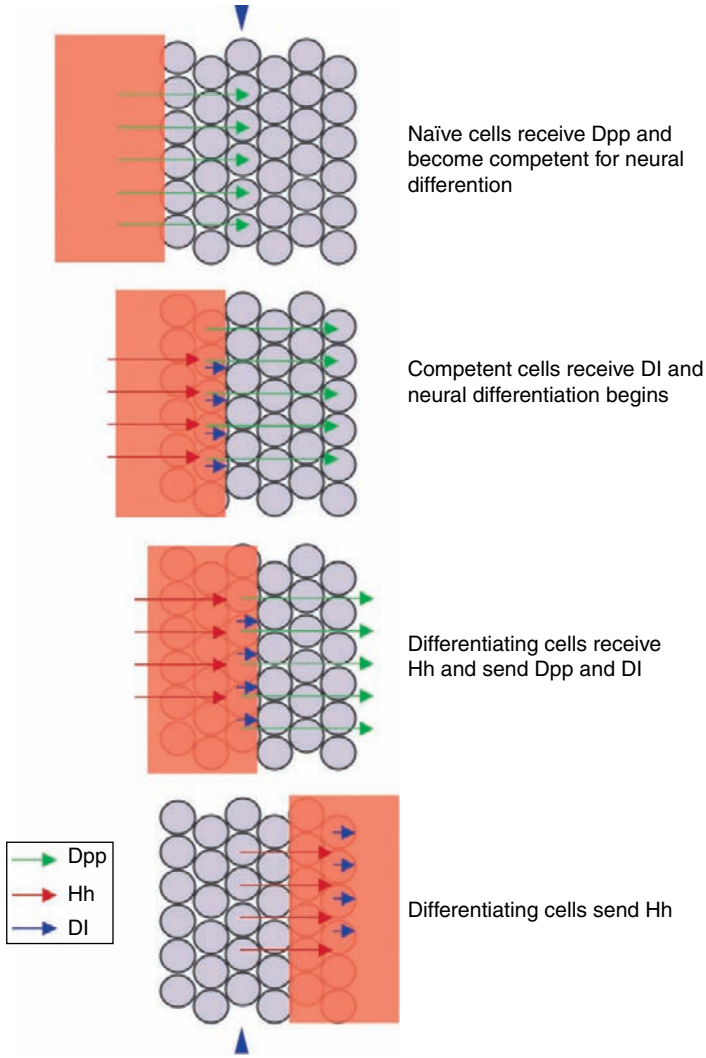
Michael E. Zuber, Figure 2.5 XOtx2 and the EFTFs are sufficient to induce eye formation from pluripotent cells. (A) RNAs coding for XOtx2 and the EFTFs is sufficient to induce eye formation when injected directly into one cell of a two-cell embryo. (B) Replacement of an eye field with YFP-only expressing animal cap cells generates only epidermis. (C) In contrast, cells expressing the EFTFs with XOtx2 form a functional eye.



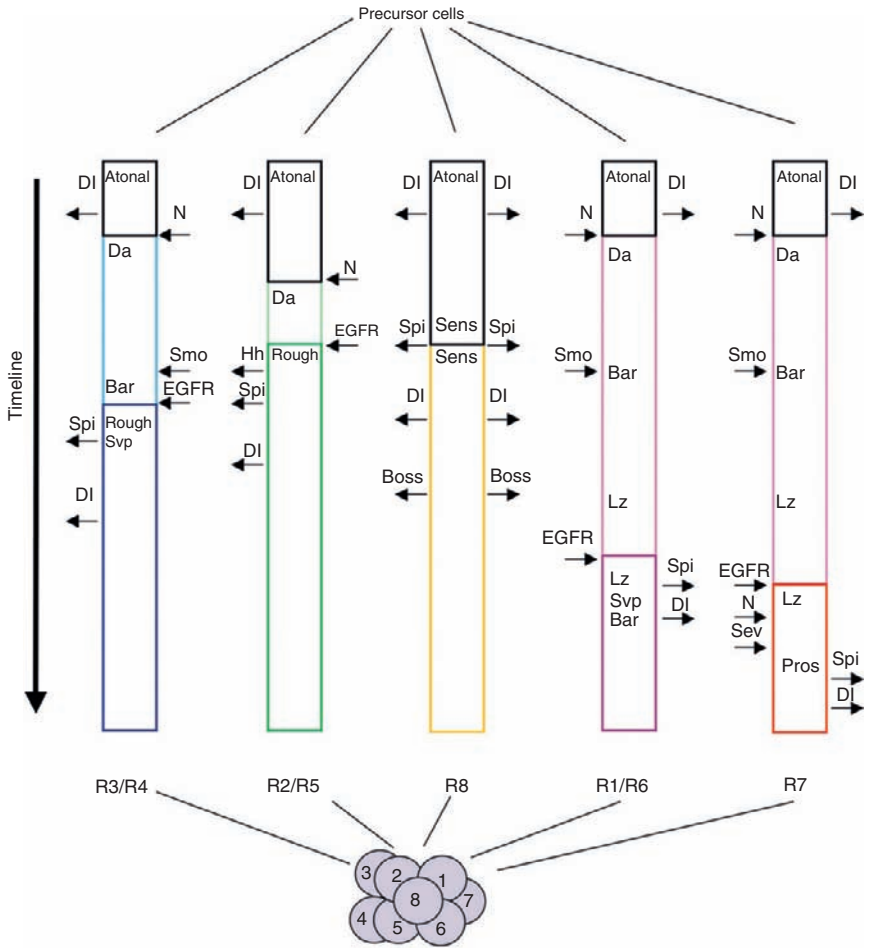
Sujin Bao, Figure 4.1 The *Drosophila* eye. (A) The R8 photoreceptor neurons marked by Senseless (Sens) are evenly spaced in the eye disc. A third instar larval eye disc was stained with an anti-Senseless antibody. Scale bar, 100 μm. A high magnification view is shown in (B). Bar, 10 μm. The morphogenetic furrow is indicated by an arrow. (C) Ommatidia are separated by interommatidial cells (IOCs) and form a precise hexagonal array in the pupal eye. An eye at 40 h APF was stained with an anti-E-cadherin antibody. Bar, 10 μm. Cell types visible on the apical surface are indicated in (D). Anterior (a), posterior (p), polar (pl), and equatorial (eq) cone cells are marked in blue. 1°, 2°, and 3° are primary (yellow), secondary (pink), and tertiary pigment cells (pink), respectively. b, bristle. (E) Regular spacing of ommatidia is maintained in the adult eye. A scanning electron microscopic (SEM) micrograph shows an adult eye. Ommatidia form a precise hexagonal array. Bar, 100 μm. Anterior is to the right in this and all subsequent figures.



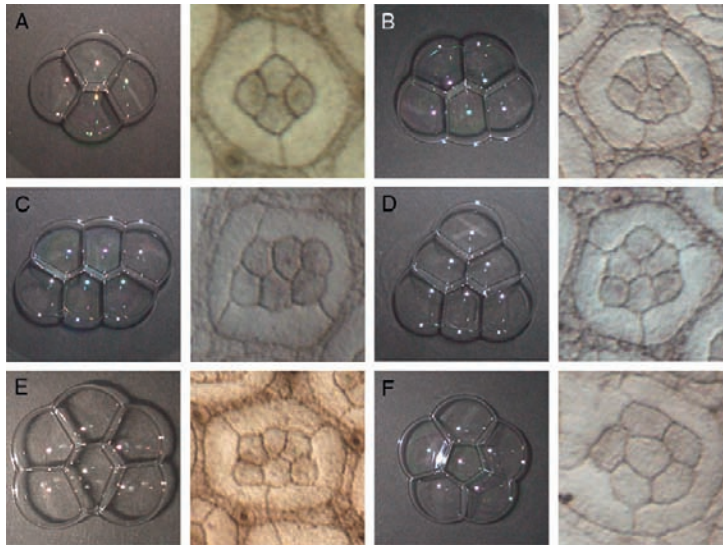
Sujin Bao, Figure 4.3 The interplay between cell signaling and competence determines the position of the morphogenetic furrow. After the furrow (yellow) is initiated, the eye disc can be roughly divided into four zones, corresponding to four different states of competence for neural differentiation. In front of the furrow, Wg signaling along with intrinsic inhibitors keeps cells in the undifferentiated state (gray). Cells receiving Dpp signaling undergo transition from the undifferentiated to pre-proneural state (green). However, these cells gain new inhibitors and Atonal is not yet expressed. Within the proneural zone (the furrow), cells receive both Dpp and Dl and gain full competence for neural differentiation marked by expression of Atonal. In the differentiating zone, Atonal promotes production of Spitz in R8s. Spitz triggers EGFR signaling, which induces Hh. EGFR and Hh via intrinsic regulators turn off Atonal. Emc, Extramacrochaete; H, Hairy; Pnt, pointed; Spi, Spitz. See text for a more detailed description of relevant genes.



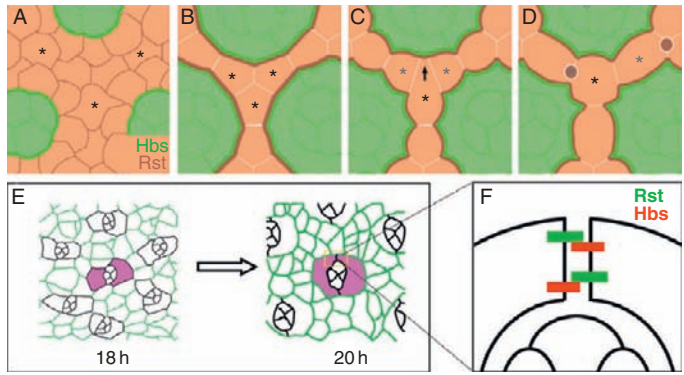
Sujin Bao, Figure 4.4 A relay of Hh signaling maintains progression of the furrow. The morphogenetic furrow is depicted by red blocks. An arrowhead points to the row of cells of which competence changes over time. Three major signaling molecules that act in different ranges are represented by arrows: Hh (red), Dpp (green) and Dl (blue).



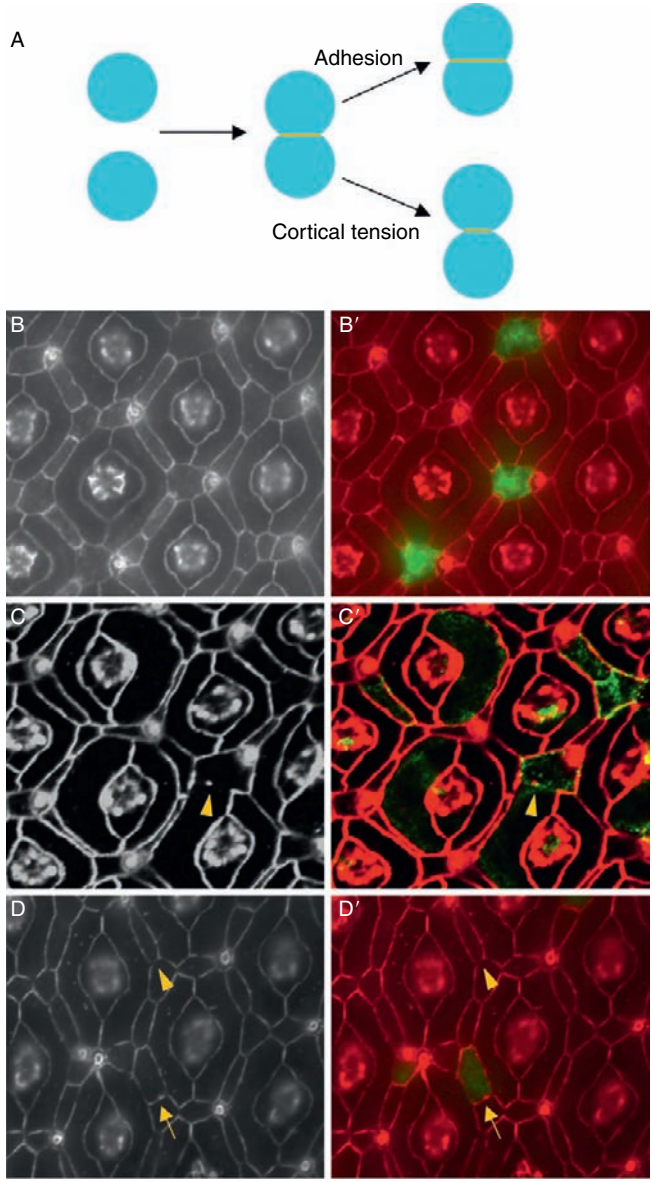
Sujin Bao, Figure 4.5 The interplay between cell signaling and competence generates diversity of photoreceptor neurons. Initially, precursor cells are equipotent and they all express Atonal. Early differences are generated by Scabrous, Spitz and Dl signals that cells receive. For simplicity, only Notch signaling is depicted. Without Notch input, cells retain Atonal, which biases the R8 fate (yellow). With Notch input, cells lose Atonal and gain high levels of Daughterless, and they are primed to adopt non-R8 fates. EGFR signaling triggers differentiation of all non-R8 neurons. Among them, cells receiving Notch early are directed to either R3/R4 or R1/R6/R7 fates while those receiving Notch later become R2/R5 (green). At the same time, intrinsic factors also bias cell fate choices: cells without Lz become R3/R4 (blue) while those with Lz favor R1/R6/R7 fates (magenta). Cells receiving additional Notch and Sevenless inputs besides EGFR singaling further become R7 (red). Hh contributes to this selection process by inducing Bar, a potent inhibitor of Atonal. The transition from an equipotent to a differentiating state is drawn in a lighter color. Da, Daughterless. Dl, Delta; Lz, Lozenge; N, Notch; Spi, Spitz; Svp, Seven-up; Smo, Smoothened. See text for detail.



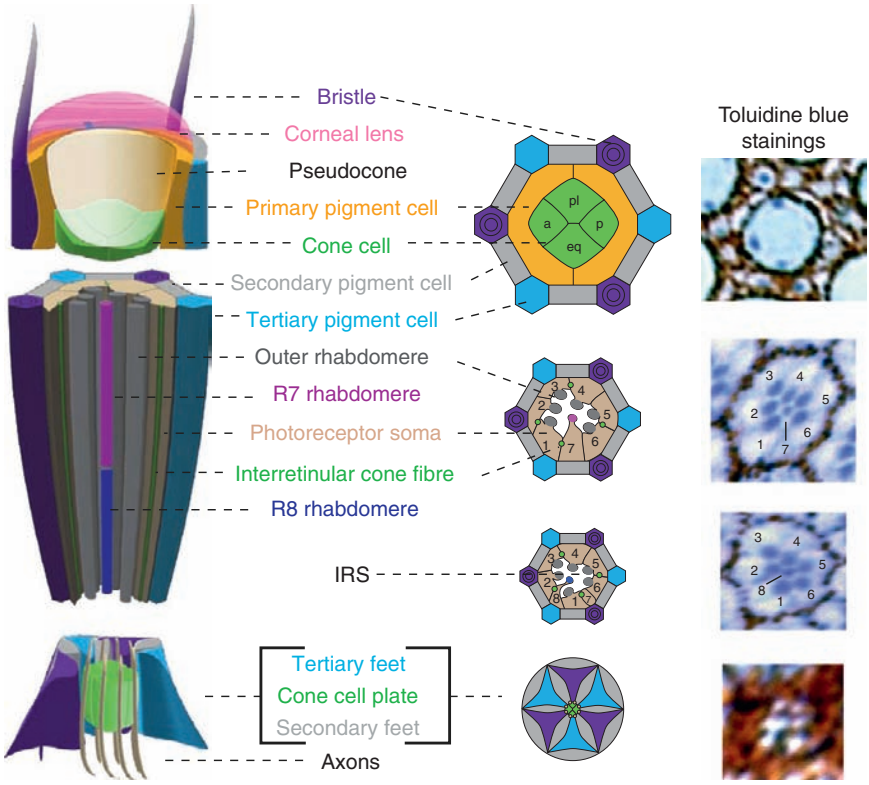
Sujin Bao, Figure 4.7 Configurations of cone cells in the Drosophila eye resemble patterns of soap bubbles in water. Patterns of four (A), five (B) and six (C-F) soap bubbles (left) are compared with those of cone cells of the same number (right). Pupal eyes from Roi/+ flies at 40 h APF were stained with cobalt sulfide (Images courtesy of R. W. Carthew).



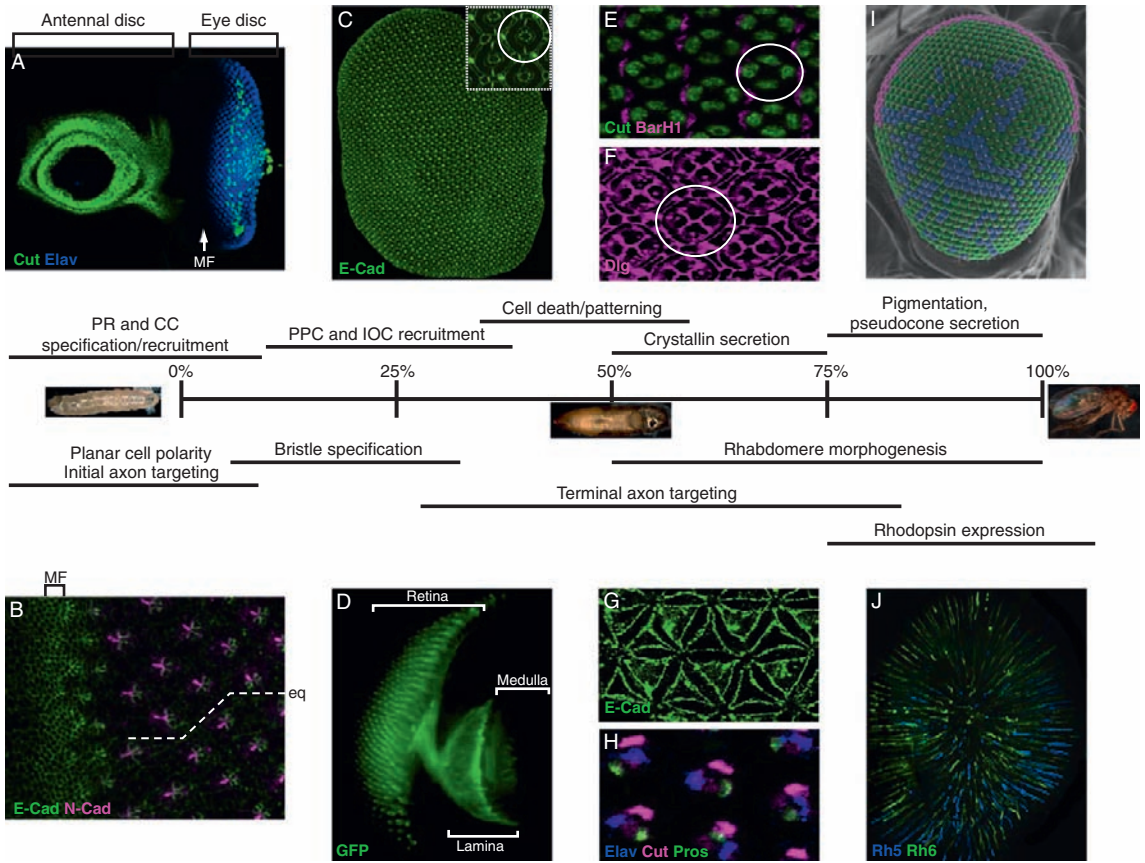
Sujin Bao, Figure 4.8 Preferential adhesion in spatial organization of pigment cells. (A) At the onset of cell rearrangement, multiple rows of interommatidial cells (IOCs, orange) are scattered between ommatidia (green). Three candidates for the tertiary pigment cell are marked by asterisks. (B) After 1°s are specified, adhesion molecules Hbs and Rst are expressed in complementary cell types: Hbs is expressed in 1°s and Rst in IOCs. Due to heterophilic interactions between Rst and Hbs, IOCs prefer to adhere to 1°s. This cell behavior is referred to as preferential adhesion. As a result, IOC–IOC contacts are reduced and multiple rows of IOCs sort into a single line. Note that IOC-IOC contacts are not yet minimized and three tertiary candidates are still competing for the tertiary niche. (C) After one IOC establishes junctions with three 1°s, the other competing IOCs join other IOCs to compete for survival. (D) A single secondary pigment cell is selected. Other IOCs are removed by cell death. IOC-IOC contacts are now minimized. (E) Emergence of 1°s. Tracing of pupal eyes is shown. Developing 1°s are highlighted in magenta and IOCs in green. (F) At an earlier stage, developing 1°s express both Hbs and Rst. Heterophilic interactions between Hbs and Rst promote formation of junctions between the two emerging 1°s. Panels A–D are adapted with permission from Bao and Cagan (2005).



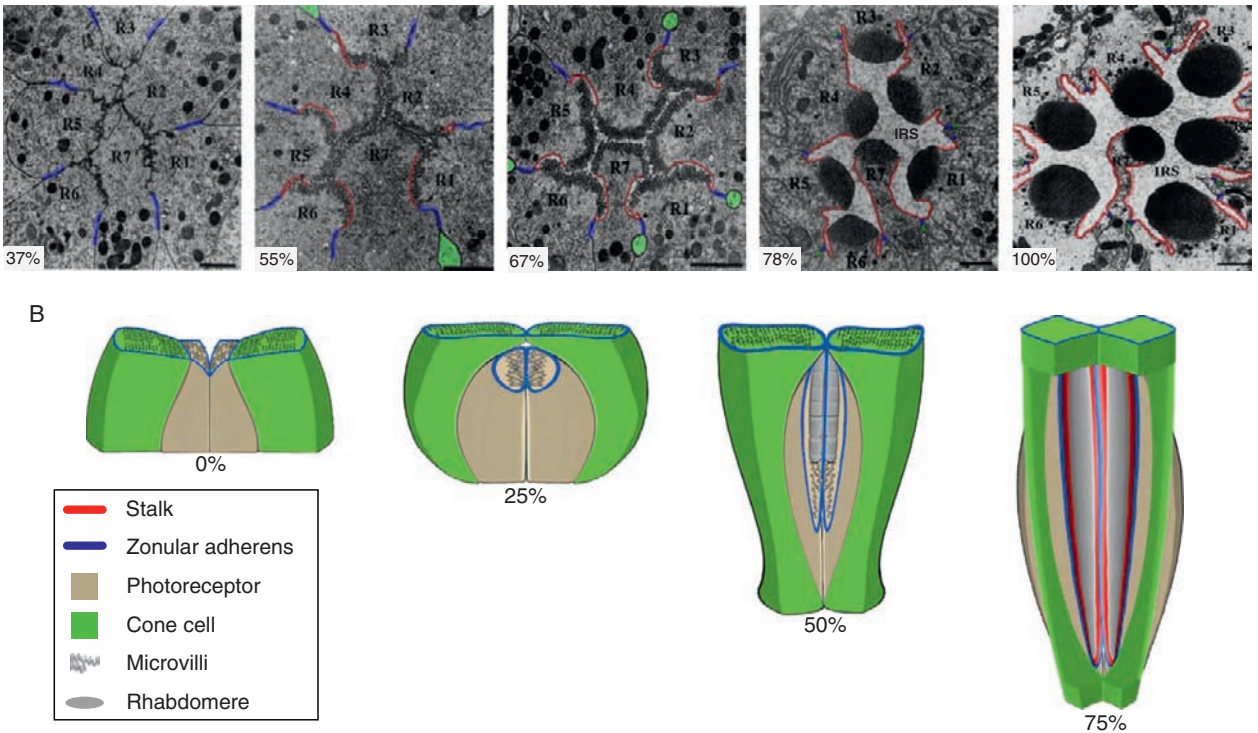
Sujin Bao, Figure 4.9 Cell adhesion and cortical tension control cell shape. Genetically manipulated cells are marked by GFP (green). Pupal eyes were stained with an anti-E-cadherin antibody (red) to visualize cell morphology. The single E-cadherin channel is shown in B–D and merged views in B′–D′. (A) Cortical tension counteracts adhesion. When two cells (light blue) are brought into contact, adhesion promotes and cortical tension reduces cell–cell contact. Size of cell–cell contact is correlated with formation of junctions (orange line). Modified from Lecuit and Lenne (2007). (B–B′) Preferential adhesion promotes cell–cell contact. Single IOCs (green) that receive extra Rst expand apical profiles. As a result, 1°-IOC contacts are increased. (C–C′) Loss of Rho1 leads to expansion of apical profiles. Arrowheads highlight a single-cell mutant for *rho1* (green). (D–D′) Heterophilic interactions between Hbs and Rst promote formation of cell junctions. Normally the E-cadherin level is low in-between IOCs at 36 h APF (arrowheads). Upon forced expression of Hbs (green) in single IOCs, robust junctions (arrows) form in-between IOCs. Panels C–C′, image courtesy of S. Warner and G. Longmore.



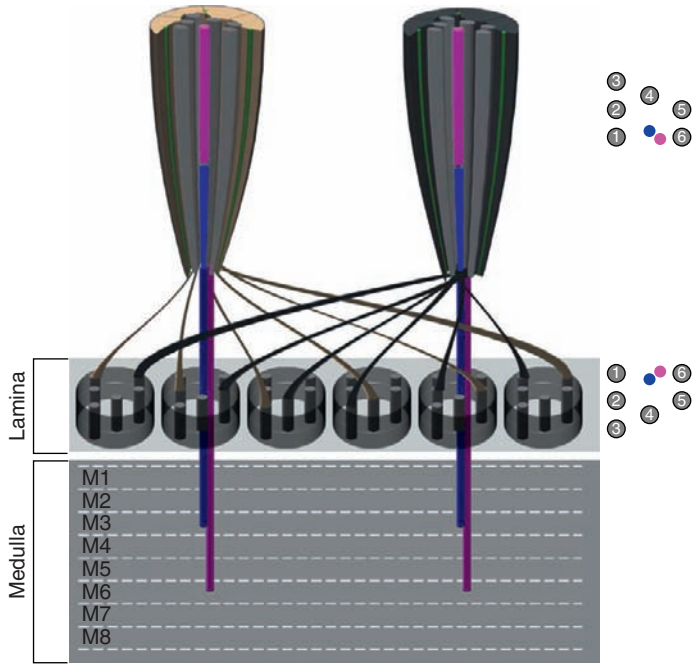
Mark Charlton-Perkins and Tiffany A. Cook, Figure 5.1 Structure of an adult *Drosophila* ommatidium. Schematic of different regions of an adult ommatidium: the corneal lens region (top), the neural retina (middle), and the retinal floor (bottom). Corresponding regions from toluidine blue-stained semi-thin sections of an ommatidium are provided at the right. Color scheme is as follows: photoreceptor (PR) cell bodies, beige; PR rhabdomeres, dark gray cylinders (outer PRs), dark magenta cylinder (R7), or dark blue cylinder (R8); cone cells, green; primary pigment cells, yellow; secondary pigment cells, gray; tertiary pigment cells, turquoise; mechanosensory interommatidial bristle, purple hexagon; eye unit, longitudinal. The cone cells and primary pigment cells secrete the corneal lens (translucent pink) and a gelatinous pseudocone (translucent white). Each cone cell also extends an "interretinular fiber" between the photoreceptors, eventually expanding just proximal to the rhabdomeres to create a CC feet "plate" at the base of the retina. Based on the position within the ommatidia, the four cone cells are referred to as the apical (a), posterior (p), polar (pl), and equatorial (eq) cone cells. Secondary and tertiary pigment cells and the bristle form a characteristic hexagon around each ommatidia, with the pigment granules easily observed in the toludine blue stainings as reddish-brown (pteridine-containing) and black (xanthommatin-containing) vesicular-like structures. The apical surfaces of the secondary and tertiary pigment cells are tightly restricted, but the basal surfaces of these cells expand at the base of the retina to form a fenestrated membrane through which the axons project into the brain. The six outer photoreceptor rhabdomeres (gray from cells R1 through R6) form a trapezoid at the top of the eye and extend the length of the retina, enwrapping the IPR rhabdomeres—the R7 rhabdomere (Magenta) extends through the top two-thirds of the retina and the R8 rhabdomere (Blue) occupies the bottom third. In addition, the cell body of the R7 is positioned between the R1 and R6 cell, whereas the R8 cell body is located between the R1 and R2 cell, seen by cross section (middle diagrams and thin sections). The interhabdomeric space (white) that is important for preventing rhabdomere fusion is also seen. The entire central portion of the ommatidia is encapsulated by the cone cells—distally, with the rhabdomeres attached by "hemidesmosome-like" contacts, and proximally, with the rhabdomeres attached to the cone cell feet just below the end of the rhabdomere.


Antennal disc
Eye disc
A
Cut Elav
MF
C
E-Cad
E
Cut BarH1
F
Dlg
I
PR and CC specification/recruitment
PPC and IOC recruitment
Cell death/patterning
Crystallin secretion
Pigmentation, pseudocone secretion
0%
25%
50%
75%
100%
Planar cell polarity
Initial axon targeting
Bristle specification
Rhabdomere morphogenesis
Terminal axon targeting
Rhodopsin expression
MF
B
eq
E-Cad N-Cad
D
Retina
Medulla
GFP
Lamina
G
E-Cad
H
Elav Cut Pros
J
Rh5 Rh6

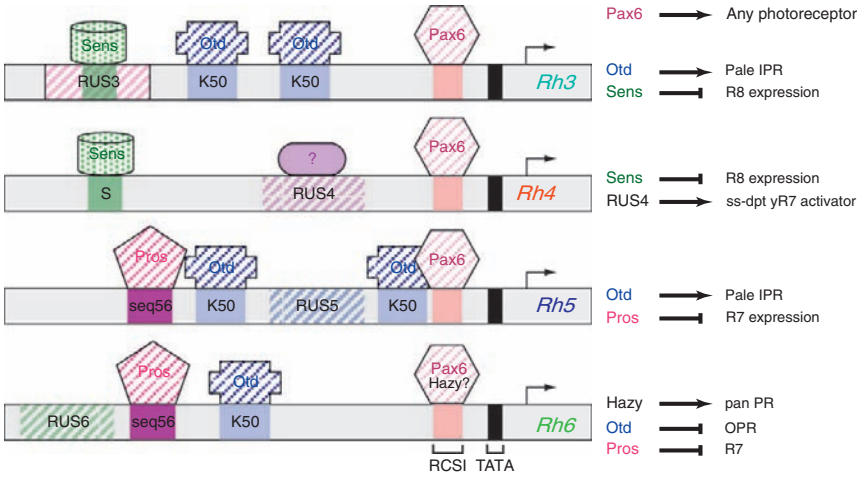
Mark Charlton-Perkins and Tiffany A. Cook, Figure 5.2 Time course of *Drosophila* eye development. A summary of various developmental processes that occur during *Drosophila* pupal eye development (0–100%). Prior to pupation, in late third instar larva, the antennal/eye disc (A) is easily recognized by strong Cut expression (green) in the antennal portion (anterior, left), and clusters of Elav-positive photoreceptor clusters (blue) in the eye portion (posterior, right) corresponding to individual ommatidial units. Cut-positive cells are also present in the eye-imaginal disc, which represent subretinal glia and CCs precursors. Nonstained cells anterior to the morphogenetic furrow (MF) are retinal progenitors that are still proliferating (see Chapters 1 and 4 for further description). (B) The constricted apical surface of cells within the MF is obvious with E-Cadherin staining (green). In addition, the boundary between the R3 and R4 cell, marked by intense N-cadherin staining (purple), reveals the rotation of the ommatidia relative to the equator that is important for establishing the chiral trapezoid of photoreceptors observed in the adult retina. (C) E-cadherin staining (green) of a whole retina isolated from pupa at ~50% pupation shows the highly regular organization of ommatidia. Inset: A single ommatidium is circled. (D) Photoreceptor-driven Moesin::GFP at 50% pupation shows outer PR axons projecting to the lamina and IPR axons projecting to the medulla. (E) Cut (green) and BarH1(Magenta) specifically recognize the four CC and two primary pigment cell (PPC) nuclei at 50% pupation. (F) Discs Large (Dlg, Purple) highlights the apical contacts of the CCs, PPCs and interommatidial cells in 50% pupal retinas. (G) E-cadherin (green) of the basal surface of the retina shows the petal-shaped distribution of the IOC feet. (H) The bristle cell lineage is composed of four cells which express the transcription factors Cut and Pros, and the neural factor Elav. These nuclei are present at the base of the retina during their development, and eventually move more apically. (I) A scanning electron micrograph of an adult eye pseudocolored to represent the distribution of the pale (blue), yellow(green) and Dorsal Rim Area (DRA; magenta) ommatidia in the eye. (J) Whole mounted adult retina immunostained with Rhodopsin 5 (blue) and Rhodopsin 6 (green) in R8 rhabdomeres. Note the enrichment of Rh6 in the dorsal portion of the retina, corresponding to the dy ommatidia (see text for more detail).



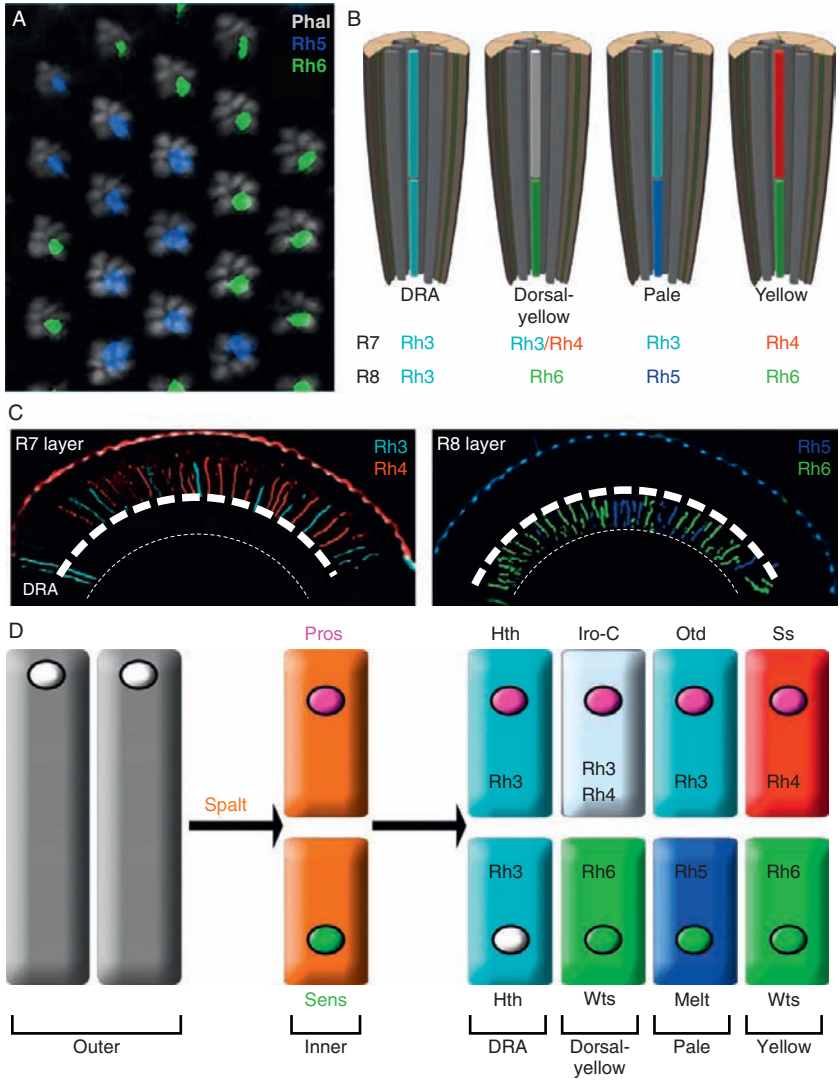
Mark Charlton-Perkins and Tiffany A. Cook, Figure 5.3 Rhabdomere morphogenesis. (A) Coronal TEMs showing the apical membrane elaborations of photoreceptors R1 through R7 at different stages of development. The zonula adherens are marked with blue, the stalk region is highlighted in red, and the interrhabdomeric space (IRS) is the clear space between rhabdomeres that are obvious by 78% pupation (modified from Longley and Ready, 1995, with permission from Elsevier). Some of the interretinular fibers from cone cells, found directly adjacent to the *zonula adherens* are highlighted in green. (B) Diagram of the 90° turn of the photoreceptor apical surfaces during early pupation and elongation of the rhabdomeres (gray), the stalk region (red), and *zonula adherens* (blue) at later stages of development. Only two cone cells (green) and two photoreceptors are shown for clarity.



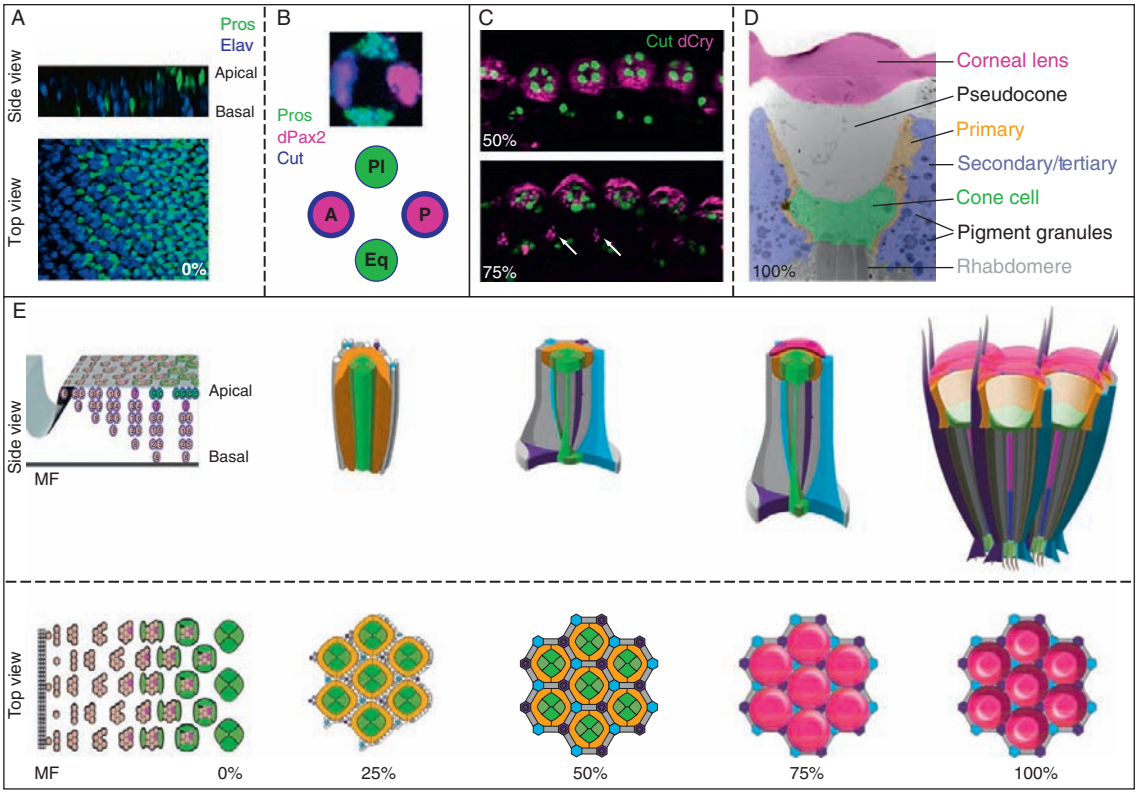
Mark Charlton-Perkins and Tiffany A. Cook, Figure 5.4 Axonal targeting differences between outer and inner photoreceptors. Diagram representing two ommatidia sharing lamina cartridges. The axons from the six outer PRs from each ommatidium turn 180° and project to six different cartridges present in the lamina neuropil present directly underneath the retina. R1–R6 positions within the lamina represent a mirror image of the outer photoreceptor arrangement found in the retina. The R7 (magenta) and R8 (blue) axons bypass the lamina and project to layers M3 and M6 respectively in the adult medulla.



Mark Charlton-Perkins and Tiffany A. Cook, Figure 5.5 Regulatory sequences of the inner photoreceptor Rhodopsin-encoding genes. Schematic of the minimal promoters for Rh3 through Rh6 that recapitulate expression of the endogenous genes. Senseless binding sites (S) are green, Otd binding sites (K50) are light blue, Pax6/RSCI sites (Rhodopsin Conserved Sequence I) are pale pink and Pros sites (seq56) are dark magenta. Rhodopsin Unique Sequences (RUS) 3, 4, 5, and 6 are represented by striped boxes. The summary of the role of each transcription factor is highlighted to the right. Otd activates Rh3 and Rh5, the two Rhodopsins expressed the pale ommatidia, and represses Rh6 in outer photoreceptors (Tahayato *et al.*, 2003). Pros represses the R8 Rhodopsins, Rh5 and Rh6, in R7 photoreceptors (Cook *et al.*, 2003), while Sens represses the R7 Rhodopsins, Rh3 and Rh4, in R8 photoreceptors (Xie *et al.*, 2007). A transcription factor that is predicted to be activated by Spineless in yellow R7 cells to activate Rh4 is indicated by a ? on the Rh4 promoter. In addition, Hazy has recently been shown to be necessary and sufficient for Rh6 expression and bind to the RCSI, making it possible that Hazy, and not Pax6, is responsible for activating the Rh6 promoter in the fly eye (Mishra *et al.*, 2010).



Mark Charlton-Perkins and Tiffany A. Cook, Figure 5.6 Ommatidial subtypes express different inner photoreceptor Rhodopsins. (A) A whole-mount staining of an adult retina stained with phalloidin (gray) shows the trapezoidal arrangement of the actin-rich rhabdomeres of the six outer photoreceptors and the random distribution of the pale and yellow ommatidia are revealed by immunostaining for Rh5 (blue) and Rh6 (green) that are expressed in the central R8 cells. (B) Diagram of the Dorsal Rim Area (DRA), dorsal yellow, pale, and yellow subsets found in the *Drosophila* eye, defined by the Rhodopsins expressed in the R7 and R8 inner photoreceptors. All outer photoreceptors express the same Rhodopin, Rhodopsin 1. (C) Transverse sections of adult eyes, dorsal left, stained with R7 Rhodopsins (left), Rh3 (cyan) and Rh4 (red), or R8 Rhodopsins (right), Rh5 (blue) and Rh6 (green). Note that two rows of ommatidia at the dorsal side of the eye express Rh3 in the R7 and R8 layers, representing the DRA ommatidia. Rh3 and Rh4 expression in the dy ommatidia are weaker than in the remainder of the eye. (D) Schematic representing the factors that direct inner photoreceptor identity, differentiation, and rhodopsin expression. The relative position of the nuclei that would be in the cell body for the different cell types are also indicated. See text for detail.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Pros
Elav
Side view
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Basal
Top view
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B
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dPax2
Cut
PI
A
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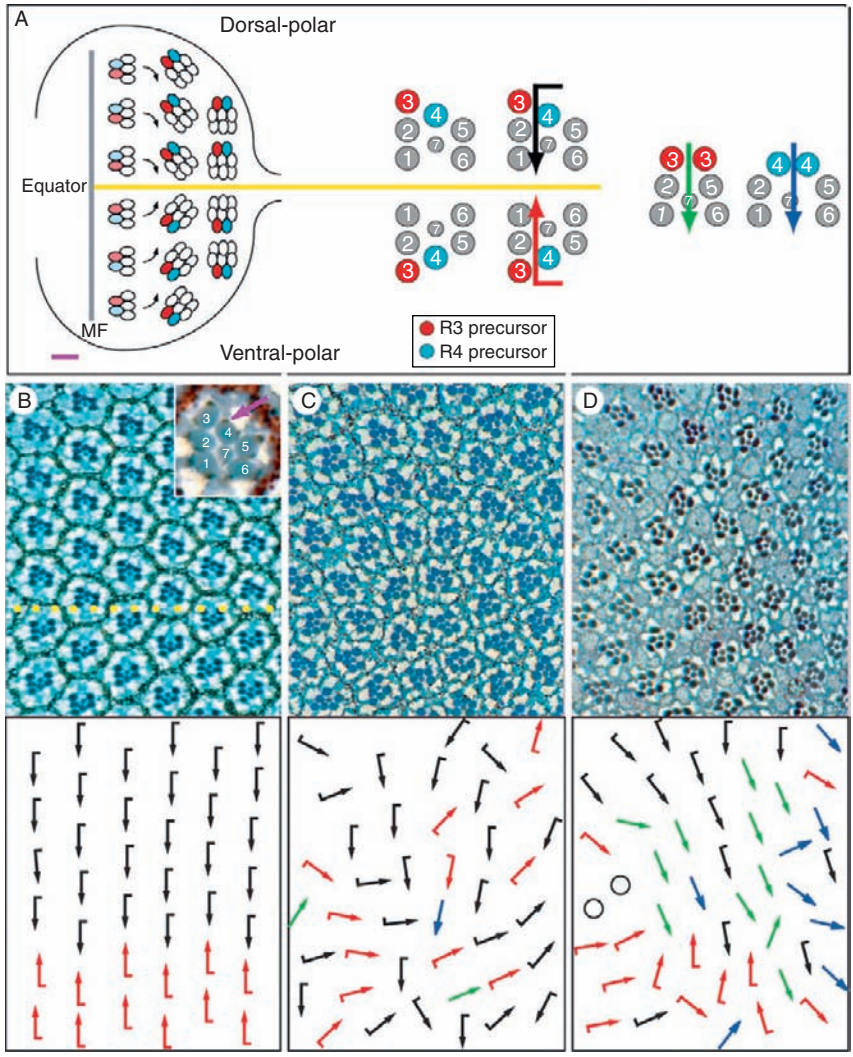
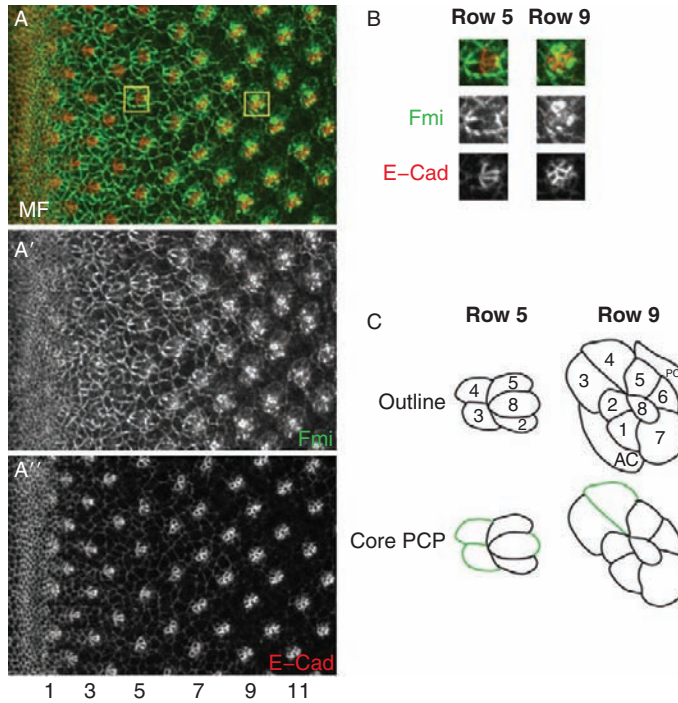
Eq
C
Cut dCry
50%
75%
D
Corneal lens
Pseudocone
Primary
Secondary/tertiary
Cone cell
Pigment granules
Rhabdomere
100%
E
Side view
Apical
Basal
MF
Top view
MF
0%
25%
50%
75%
100%

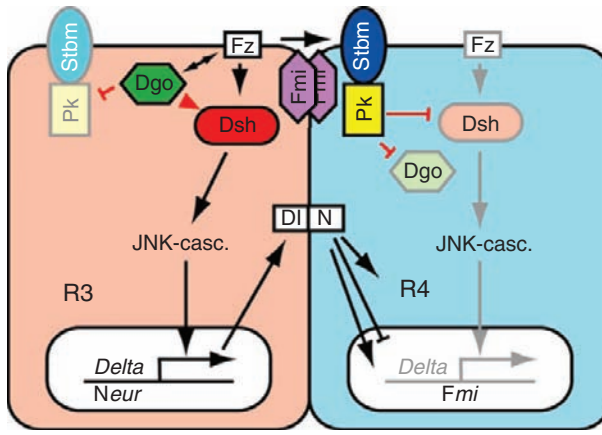
Mark Charlton-Perkins and Tiffany A. Cook, Figure 5.7 Events leading to *Drosophila* corneal lens formation. (A) A third instar imaginal disc, stained with Elav (blue) to mark specified photoreceptors and the transcription factor Prospero (green), to mark the R7 photoreceptor and the cone cell precursors. The side view shows that the nuclei of cell move from a basal to apical position as they are recruited. (B) A high magnification of the cone cell layer from a single ommatidium shows that distinct subpopulations of cells that express different levels of Prospero (green), dPax2 (magenta), and Cut (blue) exist. This also is represented diagrammatically, with high Pros expression in equatorial (eq) and polar(pl) CCs, and high dPax2/Cut expression in anterior (a) and posterior (p) CCs. (C) Drosocrystallin (magenta) begins to be made in CCs, marked with Cut (green) at 50% pupation and is secreted from the cells by 75%. Drosocrystallin is also expressed at lower levels in the interommatidial bristle lineage (arrows). (D) A transmission electron micrograph of an adult ommatidium, pseudocolored to highlight the striated corneal lens (magenta), the clear pseudocone (gray), the primary pigment cells (PPCs, yellow), the cone cells (CCs, green), and the secondary/tertiary pigment cells (IOCs, purple). Note the abundant, large pigment granules in the IOCs, that the PPCs outline the CCs and pseudocone, and that the CCs lie between the pseudocone and the tips of the photoreceptor rhabdomeres. (E) Top and side view schemata of lens development, beginning from the imaginal disc through different stages of pupation using the same color scheme as in Fig. 5.1. The apical surface contacts change between the a/pCCs and eq/pl CC during pupation, patterning, and pruning of the IOCs occur prior to 30% pupation, and the corneal lens is secreted by ~75%. Afterward, the pseudocone is secreted and pushes the cone cells away from corneal lens.



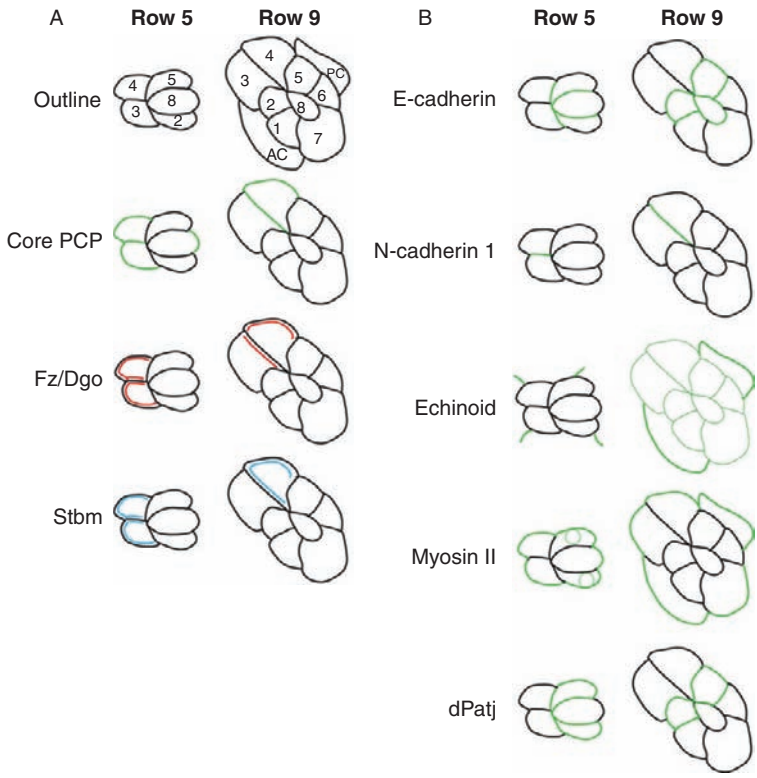
Andreas Jenny, Figure 7.1 Establishment of PCP in the *Drosophila* eye. Anterior is to the left, dorsal is up in all panels. (A) Schematic of a 3rd instar eye imaginal disk with the dorsoventral midline (equator) in yellow and the morphogenetic furrow (MF) in gray. Purple bar outlines the approximate region of nonautonomous/*dachsous* signaling phase. Initially, ommatidial preclusters are symmetrical with the precursors for R3 (pale red) and R4 (light blue) next to each other. The cell of the R3/4 pair closer to the equator is specified as R3 (red) upon Fz–PCP signaling. The neighbor becomes R4 (blue). Ommatidia rotate 90° in opposing directions on either half of the eye. The rhabdomeres of adult ommatidia are thus mirror symmetric (chiral) with the rhabdomere of R3 at the polar-anterior tip and the R4 more equatorial and posterior (schematic on the right). Far right: schematic representing symmetric ommatidia of the R3/3 and R4/4 type occurring in certain mutant situations. Colors of the flagged arrows correspond to the ones shown in the sections in B–D. (B–D) Tangential sections through wild-type (B), *fz* (C), and *dsh* (D) mutant adult *Drosophila* eyes. Note the randomized chirality and degree of rotation in the mutants. Schematic below the sections indicates the polarity of ommatidia (see (A) for arrows). Circles represent ommatidia with defects in the photoreceptor complement. Yellow dots represent the equator. Inset in (B): high magnification of a single ommatidium with numbered photoreceptors. Note that R8 is below R7 and thus cannot be seen. Purple arrow points to pigment granules associated with the rhabdomere of photoreceptors. The presence of these granules is used as a marker during genetic mosaic analysis. See text for details.



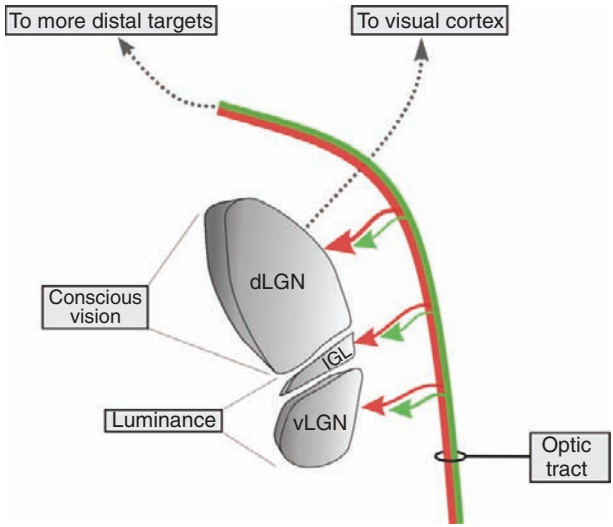
Andreas Jenny, Figure 7.2 Subcellular localization of core PCP genes during PCP signaling. (A) Dorsal area at the apical level of a 3rd instar eye disk stained for Fmi in green (single channel image in A′) and E-Cad in red (single channel image in A″). The morphogenetic furrow (MF) is on the anterior (left). Note the progressive rotation of anterior (young) to posterior (more mature) ommatidia. Ommatidial row numbers are indicated at the bottom. (B, C): (B) Higher magnification of the ommatidial clusters indicated with yellow squares in A. In row 5, Fmi, a representative core PCP protein, is enriched in apical membranes in a "double horseshoe" pattern in R3 and R4, but reduced where they are in contact with R2/5. After the initial phase of rotation (about row 9), Fmi enrichment is visible as a single "horseshoe" in R4. Colors as in (A). (C) Schematic drawing outlining the cluster cells and localization of typical core PCP proteins (as described in B). In the upper panel, PRs are numbered. AC, PC: the anterior and posterior cone cells. Note that based on localization at the cell membrane it is not possible to determine of which cells touching each other actually expresses the protein (see also Fig. 7.5). Images courtesy of K. Gängel and M. Mlodzik.



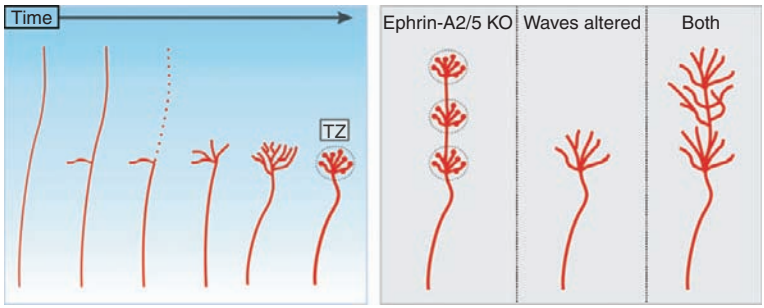
Andreas Jenny, Figure 7.4 Schematic summarizing PCP signaling during R/3/4 cell fate specification. Factors in bright colors are genetically required in the respective cell.



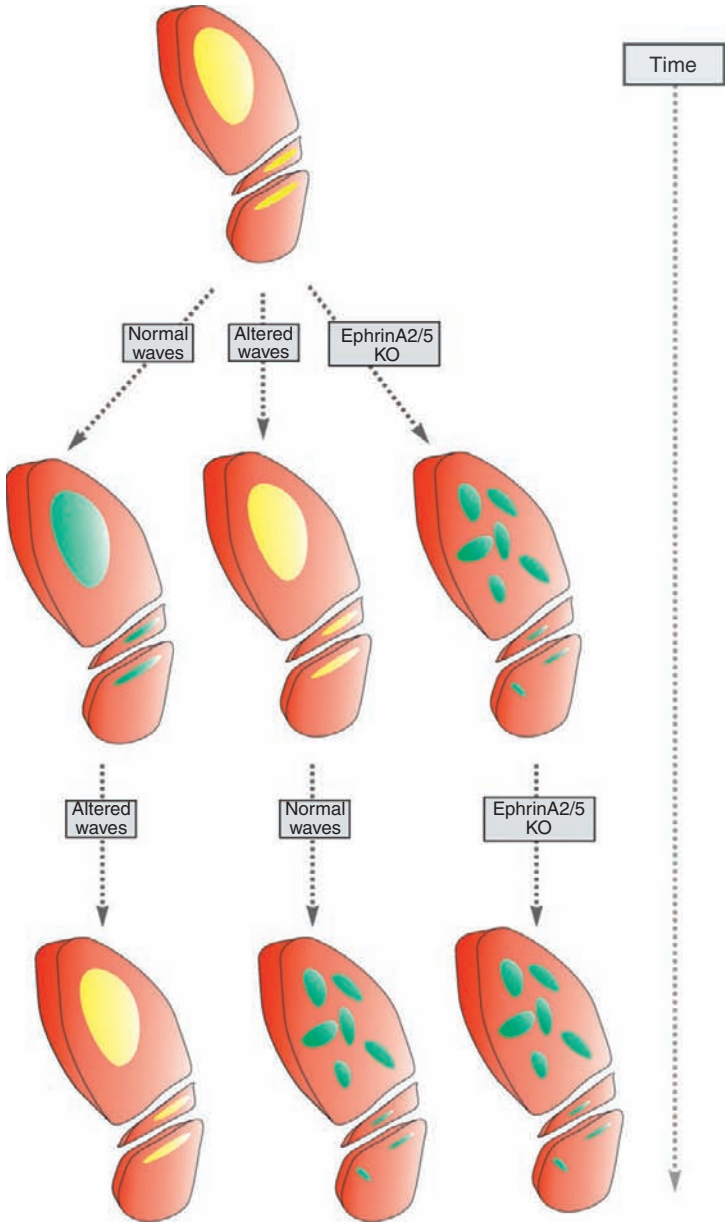
Andreas Jenny, Figure 7.5 Schematic of protein expression patterns during PCP signaling in ommatidial clusters. (A) Subcellular core PCP protein expression. Use of clonal expression of GFP fusion proteins of Fz, Dgo, and Stbm showed that the overall similar single horseshoe pattern (green) is actually due to asymmetric protein localization that is distinct for Fz/Dgo (red) and Stbm (blue). While Fz and Dgo localize to the polar border of R3 (and R4), but are excluded from the equatorial side of R4, Stbm localizes to the equatorial side of R4 (and its polar side). The situation at the R3/4 cell interface is thus equivalent to the proximal/distal border of wing cells, where Stbm/Pk on the proximal side of distal cells abut Fz/Dgo/Dsh on the distal side of the more proximal cell. (B) Schematic summarizing simplified expression patterns of E- and N-cadherin 1, Echinoid, Myosin II (Zip), and dPatj. See text for a more detailed description and references. Note that these proteins are expressed at lower levels in most cells and green color indicates a strong enrichment. In particular, Ed and Myosin II expression is highly simplified. Ed is absent from early preclusters and later enriched at the interface between the ommatidia and IOCs. Similarly, Myo II is also expressed within the cytoplasm of cluster cells (as indicated by pale green circles).



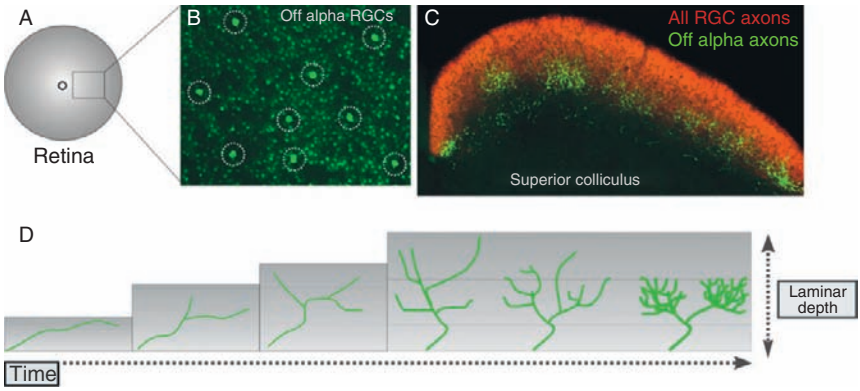
Nicko J. Josten and Andrew D. Huberman, Figure 8.2 RGC axons enter their targets by defasciculating from the optic tract. RGC axons from the two eyes (red and green) travel together in the optic tract. The axons of functionally distinct RGC subtypes innervate different retinorecipient targets, such as the vLGN (ventral lateral geniculate nucleus), IGL (intergeniculate leaflet), or dLGN (dorsal lateral geniculate nucleus), by leaving the tract. The neurons in those targets serve different functional roles in visual perception and behavior and have outputs to different brain areas (e.g., dLGN neurons project to visual cortex).



Nicko J. Josten and Andrew D. Huberman, Figure 8.3 Retinotopic mapping in normal, ephrin-A, and retinal wave-deficient mice. The blue box includes the normal developmental sequence that RGC axons (red) undergo to find their correct retinotopic termination zone (TZ) in their targets. (See text for detailed description of these events.) The dashed red line indicates axonal degeneration. The blue gradient reflects a typical distribution of ephrin-A ligands across the target. The gray box encompasses the phenotypes seen after knockout (KO) of ephrin-A2/5, when early retinal waves are altered, or when ephrin-As *and* waves are both disrupted (see text for full description).



Nicko J. Josten and Andrew D. Huberman, Figure 8.4 Eye-specific segregation in normal, ephrin-A, and wave-deficient mice. RGC axons from the contralateral (red) and ipsilateral (green) eyes and the regions where they overlap (yellow) are shown in the visual thalamus of the mouse (see Fig. 8.1 for description of these targets). (Top row) Axons from the two eyes overlap early in development. (Middle row) If normal retinal waves are present, over time axons from the two eyes segregate into nonoverlapping, "eye-specific" domains. If early retinal waves are altered, RGC axons remain overlapping. In ephrin-A2/5 KO mice, axons from the two eyes segregate but into retinotopically misplaced patches. (Bottom row) Blocking waves after eye-specific segregation is completed causes desegregation. Conversely, if waves are blocked but then wave activity is allowed to recover, eye-specific patches form, but at retinotopically misplaced locations in the target. In ephrin-A2/5 KO mice, the ectopic eye-specific patches are stable over time.



Nicko J. Josten and Andrew D. Huberman, Figure 8.5 Laminar specificity of Off-alpha projections to the superior colliculus. (A, B) Off-alpha RGCs (outlined dashed circles) selectively express GFP (green fluorescent protein) in CB2–GFP mice. Amacrine cells (smaller cell bodies) also express GFP in these mice. (B) The axons of Off-alpha RGCs terminate at a specific laminar depth of the retinorecipient SC (superior colliculus). The axons from all RGCs are shown in red, whereas Off-alpha axons are shown in green. The green axons also form patches or "columns" within their layer of the SC. (D) Schematic diagram of Off-alpha RGC axons during the stage they pick their correct laminar depth in the SC (see main text and Huberman *et al.*, 2008a for details).