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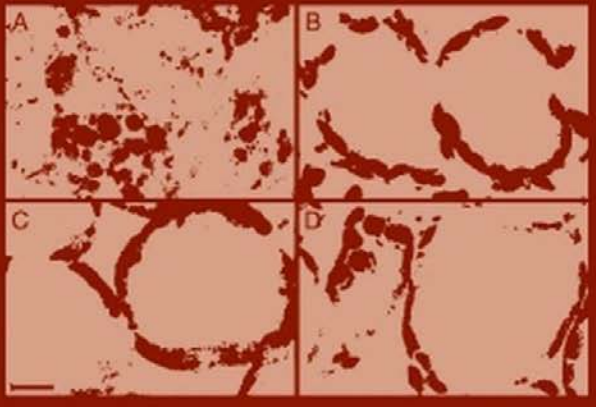

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CHAPTER ONE

MicroRNAs in *Drosophila* Development

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Contents

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Abstract

Micro-ribonucleic acids (miRNAs) are small (21–24 nucleotide), endogenously expressed, noncoding RNAs that have emerged as important posttranscriptional regulators of gene expression. MiRNAs have been identified and cloned from diverse eukaryotic organisms where they have been shown to control important physiological and developmental processes such as apoptosis, cell division, and differentiation. A high level of conservation of some miRNAs across phyla further emphasizes their importance as posttranscriptional regulators. Research in a variety of model systems has been instrumental in dissecting the biological functions of miRNAs. In this chapter, we discuss the current literature on the role of miRNAs as developmental regulators in *Drosophila*.

Key Words: microRNA, miRNA, Transcriptional regulation, *Drosophila*, Development, dsRNA.

1. Introduction

Development is a complex process that depends on the precise spatial and temporal control of gene expression. In addition to transcriptional regulation of gene expression, a significant proportion of regulation at the posttranscriptional level is increasingly being attributed to micro-ribonucleic acids (miRNAs; Ambros, 2004; Bartel, 2004; Chen and Rajewsky, 2007; Lai et al., 2003; Plasterk, 2006; Stark et al., 2007a; Staton and Giraldez, 2008). These are an abundant class of small regulatory RNAs that direct the inhibition of target messenger RNA (mRNA) expression via complementary base pairing interactions. miRNA-mediated regulation results in target repression, by reducing mRNA stability and/or inhibiting translation (Fabian et al., 2010).

The founding members of the miRNA family, *lin-4* and *let-7*, were identified in genetic screens for genes that control timing of larval development in the worm, *Caenorhabditis elegans* (Lee et al., 1993; Reinhart et al., 2000). The discovery of *let-7* homologues displaying conserved expression patterns in other bilateral animals led to the realization that these "short

temporal RNAs" might be playing orthologous roles in other species (Pasquinelli et al., 2000). Subsequently, *lin-4* and *let-7* were reported to represent a very widespread class of small endogenous RNAs found in worms, flies, and mammals, which were named miRNAs (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001). A combination of molecular cloning and computational approaches has led to the discovery of hundreds of miRNAs in animals, plants, and viruses (Griffiths-Jones et al., 2008).

Drosophila melanogaster is one of the model organisms that have made large contributions to the understanding of the basic principles and molecular mechanisms of heredity and animal development. Taking advantage of the powerful genetic tools offered by the fruit fly, the functional roles of a number of animal miRNAs have been deciphered in *Drosophila*. Gain-of-function screens undertaken in the 1990s identified a number of genetic loci that were recognized to be miRNAs several years later (Brennecke et al., 2003; Li and Carthew, 2005; Teleman et al., 2006). In addition, analysis of various hypermorphic alleles led to the discovery of regulatory motifs in the 3′UTRs of several genes that were subsequently identified as miRNA binding sites (Lai, 2002; Lai and Posakony, 1997, 1998). The sequencing of *Drosophila pseudoobscura* (Richards et al., 2005) set the stage for comparative genomic studies in *Drosophila* that led to the identification of novel miRNAs (Lai et al., 2003) and the prediction of miRNA target genes (Enright et al., 2003; Grun et al., 2005; Stark et al., 2003). These were followed up with studies leading to refinement of rules for miRNA–target recognition (Brennecke et al., 2005) and the description of global patterns of miRNA regulatory networks (Stark et al., 2005). The availability of 12 closely related *Drosophila* species combined with high-throughput sequencing has substantially increased and revised our understanding of miRNA–target relationships and lead to deeper insight into the functions and biogenesis of miRNAs (Ruby et al., 2007b; Stark et al., 2007a). These powerful approaches are geared toward attaining a complete knowledge of all miRNAs, which is extremely crucial, especially in *Drosophila*, where the study of mutants from genetic screens heavily relies on accurate genome annotation and where powerful reverse genetics tools allow the systematic analysis of miRNA functions. In this chapter, we have presented an overview of the literature on the biogenesis, mechanism of action, discovery, target prediction, and functions of some miRNAs known to play roles in different aspect of fly development.

2. Biogenesis of miRNAs

MiRNA sequences are encoded as single units (monocistronic) or as clusters of sequences generated from a single locus (polycistronic; Du and Zamore, 2005; Lagos-Quintana et al., 2001; Lau et al., 2001; Lee et al., 2002; Reinhart et al., 2002; Zeng, 2006). The largest cluster in the fly

genome consists of eight miRNAs: *miR-6.1*, *miR-6.2*, *miR-6.3*, *miR-3*, *miR-4*, *miR-5*, *miR-286*, and *miR-309* (Lagos-Quintana et al., 2001; Lai et al., 2003). These miRNA sequences are located within a compact ~500 base pair (bp) region in *D. melanogaster*, *D. pseudoobscura*, and *D. ananassae*, but are spaced further apart in *D. simulans*, *D. yakuba*, *D. erecta*, *D. mojavensis*, and *D. grimshawi* (Behura, 2007). About half of the miRNA genes in *D. melanogaster* are clustered and transcribed from a single polycistronic unit (Lagos-Quintana et al., 2001; Lai et al., 2003). Many miRNAs have been shown to be present in intergenic regions and function as independent transcription units (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001). miRNAs have also been shown to reside within the introns of protein-coding genes, or within either the introns or exons of noncoding RNAs. More than a quarter of the canonical miRNAs in *D. melanogaster* are intronic (Ruby et al., 2007b). In these cases, the miRNAs are often coordinately expressed with the host gene (Baskerville and Bartel, 2005; Rodriguez et al., 2004). However, those intronic miRNAs that are present in the antisense orientation may be transcribed from their own promoters. In some cases, both sense and antisense sequences have miRNA like characteristics and can be processed into mature miRNAs (*miR-iab-4* and *miR-iab-4AS*; Stark et al., 2008). A few human miRNAs have been found to be present in the 3′UTRs of protein-coding mRNAs, raising the possibility that they may be found in flies as well (Cai et al., 2004; Cullen, 2004).

2.1. Transcription of miRNA genes

The first step in the biogenesis of miRNAs is the transcription of the miRNA gene. The resulting primary transcripts, also referred to as pri-miRNAs, are often several kilobases (kbs) long and undergo extensive processing during the formation of the functional ~21 nt (nucleotide) miRNA (Fig. 1.1; Cai et al., 2004; Lee et al., 2004a). Several pri-miRNA transcripts have been studied in organisms ranging from mammals to flies (Aukerman and Sakai, 2003; Bracht et al., 2004; Cai et al., 2004; Houbaviy et al., 2005; Kurihara and Watanabe, 2004; Lee et al., 2004a; Sokol and Ambros, 2005; Tam, 2001). The majority of the miRNA genes are transcribed by RNA polymerase II. They contain the characteristics of RNA polymerase II transcripts including 5′ 7-methyl caps and 3′ poly-A tails. A minor group of miRNAs are transcribed by RNA polymerase III. miRNAs belonging to the human Chromosome 19 cluster, for example, have been shown to be transcribed by RNA polymerase III (Borchert et al., 2006).

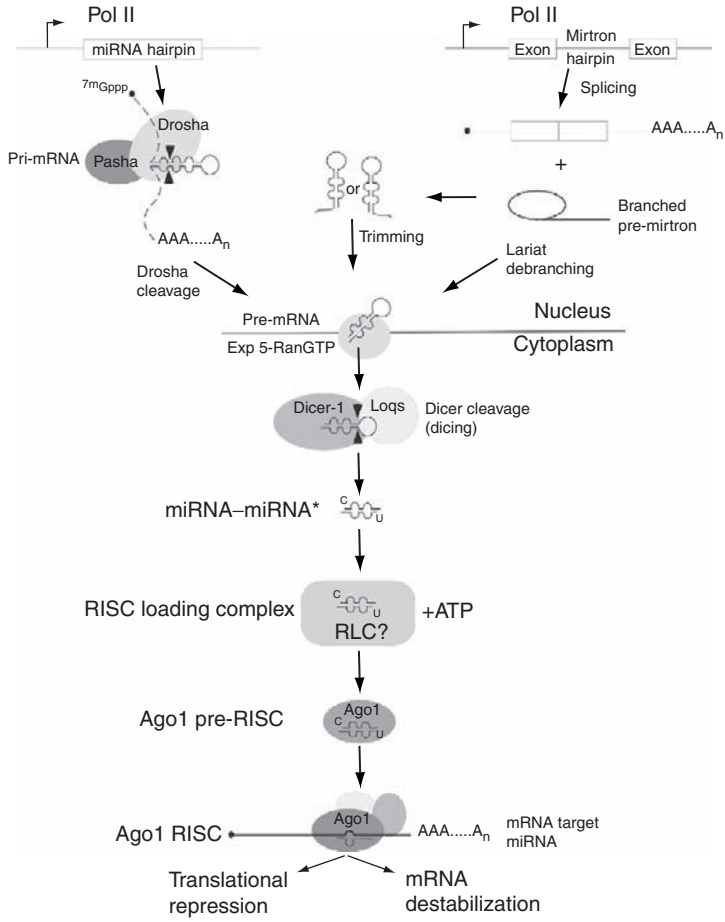

Figure 1.1 *miRNA biogenesis pathway*. Canonical microRNA (miRNA) genes are transcribed by RNA polymerase II (Pol II) to generate the primary transcripts whose hairpin structures are cleaved by the Drosha-Pasha complex to release ~65 nucleotide pre-miRNAs. Mirtrons are derived from small introns that are spliced and then debranched, yielding pre-miRNAs. Some introns have tails at either the 5′end or 3′end, so they need to be trimmed before pre-miRNA export. Pre-miRNAs have a short stem plus ~2 nucleotide 3′ overhang, which is recognized by the nuclear export factor Exportin 5-Ran-GTP. Pre-miRNAs are exported from the nucleus to the cytoplasm, where they are processed by Dicer-1/Loquacious complex to generate a duplex containing two strands, termed the miRNA and miRNA*. In flies, most miRNA:miRNA* duplexes are loaded into Ago1–RISC loading complexes (RLC). Ago1 pre-RISC-loading favors mismatches at guide positions 8–11 and U at guide position 1. The efficient unwinding of the miRNA:miRNA* duplex by Ago1 requires mismatches in the seed region (guide positions 2–8) or the middle of the 3′region (guide positions 12–16). The passenger strand is discarded and undergoes degradation. The functional strand of the miRNA guides RISC to silence target mRNAs through translational repression or mRNA destabilization. ^{7m}Gppp, 7-methylguanosine.

2.2. Nuclear processing of pri-miRNAs

The pri-miRNAs contain local stem-loop structures and undergo an endonucleolytic cleavage at the stem of the hairpin structure, releasing a 60–70 nt hairpin that is termed the pre-miRNA (Fig. 1.1; Lee et al., 2002). This step is catalyzed by a nuclear ribonuclease III (RNase III) enzyme known as Drosha. Drosha requires the activity of a double-stranded RNA (dsRNA) binding protein named Pasha (Partner of Drosha) in *Drosophila* and DGCR8 (DiGeorge syndrome critical region gene 8) in humans (Denli et al., 2004; Gregory et al., 2004; Han et al., 2004; Landthaler et al., 2004; Lee et al., 2003b). Drosha and Pasha form a complex referred to as the "Microprocessor complex," which is ~500 kiloDaltons (kDa) in *D. melanogaster* and ~650 kDa in humans (Denli et al., 2004; Gregory et al., 2004; Han et al., 2004). The microprocessor complex recognizes the pri-miRNA substrates through the Pasha/DGCR8 component of the holozyme and facilitates Drosha cleavage 11 nt away from the base of the hairpin stem (Han et al., 2006; Zeng and Cullen, 2005). Drosha-mediated cleavage of the pri-miRNA substrate occurs cotranscriptionally and precedes splicing of the host RNA in cases where miRNAs are located in introns. Drosha cleavage does not inhibit splicing, as a continuous intron is not required for splicing (Fortin et al., 2002; Han et al., 2006).

Recent deep sequencing analysis of mRNAs in *D. melanogaster* and *C. elegans* has led to the identification of a distinct class of miRNAs, referred to as mirtrons (Fig. 1.1; Berezikov et al., 2007; Okamura et al., 2007; Ruby et al., 2007a). These miRNAs are located within short introns and their biogenesis does not require Drosha cleavage. They are processed by the spliceosomal machinery to generate lariat mirtrons, which undergo debranching to generate linear mirtrons. The linearized mirtron forms a hairpin structure that resembles a pre-miRNA. Some pre-mirtrons possess extended 5′ or 3′ tails that need to be exonucleolytically cleaved before these can serve as substrates for nuclear export. Analysis of cloned small RNAs in *D. melanogaster* has led to the identification of additional 3′ tailed mirtrons. These recently identified mirtrons may form a subfamily that transits an exosome-mediated biogenesis pathway (Flynt et al., 2010). Though best studied in *Drosophila*, mirtrons have been shown to exist in other genomes (Babiarz et al., 2008; Berezikov et al., 2007; Glazov et al., 2008; Ruby et al., 2007a; Zhu et al., 2008). Several conserved mirtrons have been identified within Drosophilids, nematodes, and mammals, but so far no mirtron appears to be shared by these animals suggesting that mirtrons have been acquired independently during the evolution of different animal clades. miRNAs derived from other noncoding RNAs, such as snoRNAs, have also been described in humans (Ender et al., 2008). These studies point toward the existence of multiple Drosha-independent processing events that feed pre-miRNAs into the miRNA pathway.

2.3. Nuclear export of pre-miRNAs

Following nuclear processing, pre-miRNAs are exported to the cytoplasm (Kim, 2004). Export of pre-miRNAs is mediated by exportin 5 (Exp 5) and its Ran-guanosine triphosphate (GTP) cofactor (Fig. 1.1; Bohnsack et al., 2004; Lund et al., 2004; Shibata et al., 2006; Yi et al., 2003). Exp 5 cooperatively binds to its cargo and Ran-GTP in the nucleus and releases the cargo following GTP hydrolysis in the cytoplasm. Cells depleted of Exp 5 display reduced levels of mature miRNA. Additionally, pre-miRNAs do not accumulate in the nucleus, indicating that pre-miRNAs might be inherently unstable and that interaction with Exp 5 stabilizes pre-miRNAs (Yi et al., 2003). Exp 5 also functions as an export factor for tRNAs, human Y1 RNA, and adenoviral RNA VA1, a 160 nt long noncoding RNA (Gwizdek et al., 2003). However, Exp 5-Ran-GTP exhibits a very high affinity for pre-miRNAs, and this RNA–protein interaction has been demonstrated *in vitro*. Pre-miRNAs consist of $\sim$22 bp stem, a terminal loop and a 3′overhang of $\sim$2 nt (Basyuk et al., 2003; Lund et al., 2004; Zeng and Cullen, 2004). A RNA stem of $>$16 bp and a short 3′ overhang were defined as the significant structural requirements for pre-miRNA export by mutational analysis of *pre-miR-30a* (Zeng and Cullen, 2004).

2.4. Cytoplasmic processing of pre-miRNAs

In the cytoplasm, pre-miRNAs are processed into mature $\sim$22 nt miRNA duplexes by another RNase III type enzyme, Dicer (Dcr; Fig. 1.1; Bernstein et al., 2001; Grishok et al., 2001; Hutvagner et al., 2001; Ketting et al., 2001; Knight and Bass, 2001; Provost et al., 2002; Zhang et al., 2002). The Dcr protein includes multiple domains. In addition to two RNase III and a dsRNA binding domain, it contains a long N-terminal segment that comprises a Dead box RNA helicase domain as well as a DUF 283 domain and a PAZ domain. The PAZ domain is also a component that is characteristic of another group of highly conserved miRNA-interacting proteins known as Argonaute (Ago) proteins. Structural and biochemical studies of the PAZ domain from *D. melanogaster* Ago1 and Ago2 indicate that this domain preferentially binds to 3′ single-stranded ends of dsRNAs (Lingel et al., 2003; Ma et al., 2004; Song et al., 2003; Yan et al., 2003). Dcr recognizes the 2 nt 3′ overhang in pre-miRNAs generated by Drosha through its 130 amino acid long PAZ domain and cleaves the double-stranded region $\sim$20 nt away. This cleavage results in the generation of a miRNA duplex (miRNA:miRNA*) with 2 nt overhangs on both ends (Macrae et al., 2006; Zhang et al., 2004). The roles of other domains in Dcr remain unknown.

Dcr is a highly conserved protein, and some organisms contain multiple homologues suggesting that different isotypes have distinct roles (Lee et al., 2004b; Xie et al., 2004). In *D. melanogaster*, Dcr-1 is required for miRNA

biogenesis and Dcr-2 functions in siRNA synthesis (Lee et al., 2004b). Dcr proteins associate with several other proteins, including RDE-4 in *C. elegans*, R2D2, Loquacious (R3D1) and FMR1 in *D. melanogaster*, TRBP (TAR RNA binding protein; TARBP2) and PACT (PRKRA) in humans, and Ago family proteins in various organisms (Carmell et al., 2002; Caudy et al., 2002; Chendrimada et al., 2005; Forstemann et al., 2005; Haase et al., 2005; Hammond et al., 2001; Ishizuka et al., 2002; Jiang et al., 2005; Jin et al., 2004; Lee et al., 2006; Liu et al., 2003; Saito et al., 2005; Stark et al., 2007b; Tabara et al., 2002). The Dcr-interacting proteins are not required for the cleavage reaction, but play various roles in miRNA stability and effector complex formation and action (Chendrimada et al., 2005; Haase et al., 2005; Knight and Bass, 2001). They enhance the affinity of Dcr for RNAs and participate in the selection of mature miRNA strands and the transfer of miRNAs to Ago proteins.

2.5. Formation of miRNA-containing RNA-induced silencing complex

The Dcr-mediated cleavage is followed by the formation of an effector complex that is known as the miRNA-containing RNA-induced silencing complex (miRISC, see Fig. 1.1; Kawamata and Tomari, 2010). The Ago family proteins form the core of the miRISC and the association of a small RNA with a specific Ago protein determines its function. In mammals, all four Ago subfamily members (Ago1–4) function in the miRNA pathway, but only Ago2 functions in the siRNA pathway. In flies, Ago1 functions in the miRNA pathway and Ago2 functions in the RNAi pathway (Forstemann et al., 2007). In the first step of RISC assembly, the miRNA: miRNA* duplexes are inserted into Ago proteins to form a pre-RISC complex. The second step involves strand dissociation or unwinding of the two strands of the miRNA duplex to form the active RISC complex. The RNA strand retained in the RISC is called the "guide strand" and the other discarded strand is referred to as the "passenger strand." Such an asymmetric strand selection where one of the strands is preferred to serve as the guide (referred to as the "asymmetry rule") depends on the relative thermodynamic stability of the first one to four bases at each end of the RNA duplex, where the strand whose 5′ end is less stable serves as the guide whereas the other strand is discarded (Khvorova et al., 2003; Schwarz et al., 2003).

The incorporation of small RNA duplexes into Ago proteins requires other components of the RISC-loading machinery (Liu et al., 2004; Yoda et al., 2010). The RISC-loading complex (RLC) for *D. melanogaster* Ago2 includes Dcr-2 and its dsRNA binding protein partner R2D2 (Lee et al., 2004b; Liu et al., 2003; Pham et al., 2004; Tomari et al., 2007). Mutational analysis of *D. melanogaster* Dcr-2 indicates that its role in the dicing of dsRNA precursors into siRNAs is different from its function in

Ago2–RISC loading. A single amino acid change in the helicase domain separates dicing from RISC loading: *dcr-2* ^{G31R} mutant flies are incapable of dicing long dsRNAs, but can load siRNA duplexes into Ago2 (Lee et al., 2004b). The Dcr-2/R2D2 heterodimer gauges the thermodynamic asymmetry of the small RNA duplexes; R2D2 binds to the more stable end of the duplex, whereas Dcr-2 is found at the less stable end. This determines the polarity of small RNA duplexes upon RISC loading (Tomari et al., 2004b). *In vitro* reconstitution of fly Ago2–RISC has demonstrated that Dcr-2 and R2D2 comprise the minimal fly Ago2-RLC (Liu et al., 2009). In contrast to Ago2, much less is known about RISC-loading of Ago1. Dcr-1 and its partner Loquacious, both of which are required for processing of pre-miRNAs into the miRNA:miRNA* duplexes, were presumed to function analogously to the Dcr-2/R2D2 complex and constitute the RLC for fly Ago1 (Forstemann et al., 2005; Jiang et al., 2005; Saito et al., 2005). However, neither fly Dcr-1 nor Dcr-2 is required to load Ago1–RISC *in vitro* (Kawamata et al., 2009). Likewise, immunodepletion of human Dcr does not affect cleavage activity of siRNAs *in vitro* (Martinez et al., 2002), and *dcr* knockout mouse embryonic cells (ES) cells support siRNA-directed gene silencing (Kanellopoulou et al., 2005; Murchison et al., 2005). Thus, other than fly Ago2, Ago proteins apparently do not require Dcr for RISC-loading of small duplexes. However, the factors required for Dcr-independent Ago1–RISC-loading of small RNA duplexes and the mechanisms for sensing the thermodynamic asymmetry of small RNA duplexes in the absence of Dcr are not well understood.

In *D. melanogaster*, miRNAs are generally sorted into the Ago1-complexes and the siRNAs and miRNAs* are sorted into Ago2-complexes. The structural features of the small duplexes, mainly the presence or absence of central mismatches around positions 9–10, are important determinants in *D. melanogaster* small RNA sorting (Czech et al., 2009; Ghildiyal et al., 2010; Okamura et al., 2009; Tomari et al., 2007). In addition to the duplex structure, the identity of the 5′ nt and the thermodynamic asymmetry of the duplex are the features that are evaluated by the sorting machinery. Consequently, a typical miRNA:miRNA* duplex tends to present its miRNA:miRNA* orientation to the Ago1 pathway and its inverted miRNA*:miRNA configuration to the Ago2 pathway (Czech et al., 2009; Ghildiyal et al., 2010; Okamura et al., 2009). Notably, ambiguity in pre-miRNA dicing can produce several varieties of miRNA:miRNA* duplexes with distinct termini, switching not only the guide positions 2–8 (miRNA seed sequence) essential for target recognition (see below) but also the Ago species that are loaded (Ghildiyal et al., 2010; Kawamata and Tomari, 2010). Thus, a single pre-miRNA can generate many distinct RISC species that recognize different sets of target mRNAs and act in different modes (Kawamata and Tomari, 2010).

Two models have been proposed that explain how small RNA duplexes are unwound. In the first model, unwinding occurs in a slicer-dependent manner and results in the cleavage of the passenger strand. "Slicing" is the cleavage of target mRNAs by Ago proteins during RNA interference (RNAi). Several groups have demonstrated that fly Ago2 and human Ago2, both of which possess efficient slicer activities, cleave the passenger strand just as they cleave target RNAs (Leuschner et al., 2006; Matranga et al., 2005; Miyoshi et al., 2005; Rand et al., 2005). This mechanism functions only with the siRNA-like highly complementary small RNA duplexes, as target cleavage requires extensive base pairing. The cleaved passenger strand is released from Ago proteins and degraded to liberate the single-stranded guide. Recent studies have identified C3PO (Component 3 Promoter of RISC) as a novel activator of RISC in flies that functions in the removal of the products of the passenger strand cleavage from the RISC. C3PO is a multimeric complex of Translin and Trax, which acts as an Mg^{2+}-dependent endonuclease that specifically removes the products of siRNA passenger strand cleavage (Liu et al., 2009).

According to the second model for unwinding, Ago protein complexes unwind the duplex by a cleavage-independent mechanism. This model accounts for the fact that most miRNA:miRNA* duplexes contain central mismatches, rendering them refractory to passenger cleavage. Cleavage-independent unwinding is slow, but can be accelerated by internal mismatches in the seed region and the middle of the 3′ region of the small RNA duplexes (Kawamata et al., 2009; Matranga et al., 2005; Tomari et al., 2007; Yoda et al., 2010). This model also accounts for the fact that human Ago1, 3, and 4 as well as the fly Ago1 have poor or no slicer activity (Forstemann et al., 2007; Liu et al., 2004; Meister et al., 2004b). Interestingly, the two regions where mismatches are required for this unwinding mechanism coincide with the regions where base pairing is important for target recognition by miRNAs (see below). Moreover, G:U wobble base pairs are favorable for slicer-independent unwinding but unfavorable for target recognition (Doench and Sharp, 2004; Grimson et al., 2007; Kawamata et al., 2009). Both processes measure the positions of mismatches from the 5′ end of the guide and both are ATP-independent (Elbashir et al., 2001; Haley and Zamore, 2004; Kawamata et al., 2009; Yoda et al., 2010). Consistent with these observations, the slicer-independent unwinding appears to be a "mirror-image" process of target recognition, and hence this model has also been named the "mirror image" model (Kawamata et al., 2009). After the unwinding occurs, the discarded single-stranded passenger strands are immediately degraded by nucleases.

Although RISC assembly has been shown to require ATP (Nykanen et al., 2001; Tomari et al., 2004a), recent analysis of fly Ago1–RISC assembly has also shown that ATP hydrolysis is required for RISC-loading but not for unwinding of miRNA:miRNA* duplexes (Kawamata

et al., 2009). A similar requirement has also been shown for the human Ago2–RISC assembly (Yoda et al., 2010).

3. Regulation of miRNA Biogenesis

miRNA biogenesis is stringently controlled at several levels and is often subject to feedback regulation. As with any class of genes, transcriptional regulation is one main control mechanism, and transcription factors are known to affect miRNA expression positively or negatively in a tissue-specific or a developmentally specific manner.

3.1. Transcriptional regulation of miRNAs

Transcriptional regulation is a major level of control responsible for the tissue-specific or development-specific expression of miRNAs. For example, myogenic transcription factors Twist and Mef2 are involved in the muscle-specific expression of *D. melanogaster miR-1* (Sokol and Ambros, 2005). Other examples include the transcriptional regulation of *bantam* by Yorkie (Huang et al., 2005; Nolo et al., 2006; Thompson and Cohen, 2006).

Some miRNAs have been shown to be transcribed from opposite DNA strands of a single locus, giving rise to two miRNAs with distinct seed sequences (Stark et al., 2008; Tyler et al., 2008). In *D. melanogaster*, sense and antisense transcripts generated from the homeobox (Hox) miRNA locus *miR-iab-4* are expressed in nonoverlapping embryonic segments, and these mature miRNAs (*miR-iab-4 and miR-iab-8*) regulate development by targeting distinct sets of homeotic Hox genes expressed in specific embryonic domain (Stark et al., 2008; Tyler et al., 2008).

3.2. Feedback motifs in miRNA regulation

miRNAs and transcription factors are frequently components of regulatory networks, in which these two classes of genes regulate each other's expression (Carthew and Sontheimer, 2009; Herranz and Cohen, 2010; Li et al., 2009a). Computational analysis has suggested that miRNAs are overrepresented in such gene regulatory networks, indicating that they confer useful regulatory properties (Martinez et al., 2008; Tsang et al., 2007). *Drosophila* miRNAs have been shown to play a role in both feedback and feedforward loops, and some of these are discussed briefly here (Fig. 1.2A).

miR-9a is involved in a positive feedback loop during sense organ specification in the peripheral nervous system. Small groups of cells that express a set of "proneural" transcription factors of the Hairy-Enhancer of

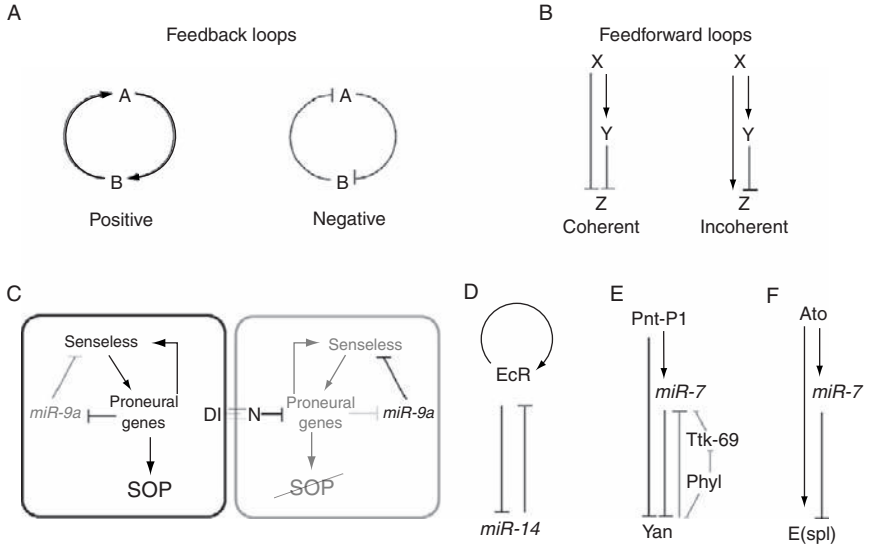

Figure 1.2 *Drosophila miRNAs involved in regulatory networks*. (A) Simple positive and negative feedback loops. (B) Feedforward motifs, where X regulates Z directly and indirectly through regulation of Y. Coherent motifs have the direct and indirect paths from X acting on the target Z in the same direction. Incoherent motifs have opposite signs for the two paths. (C) *miR-9a* in a positive feedback loop in SOP specification. The boxes represent neighboring cells within the proneural cluster. In the black cell, the *senseless*–proneural gene positive feedback circuit dominates and leads to SOP differentiation. Senseless activates *delta*, increasing Notch activity in adjacent cells. Notch inhibits proneural genes, allowing *miR-9a* repression of *senseless* to dominate in these cells. (D) *miR-14* acts in a negative feedback loop modulating EcR activity. EcR positively autoregulates itself. *miR-14* limits the expression of EcR, which in turn negatively regulates *miR-14*. (E) *miR*-7 acts in two coherent feedforward motifs. In one motif (black), Yan represses *miR*-7 directly and indirectly. In the indirect arm, Yan represses Phyllopod and thereby alleviates the repression of Ttk69, a repressor of *miR*-7. In the second motif (gray), Pnt-P1 directly activates *miR*-7, which represses *yan*. Pnt-P1 represses Yan directly. (F*) miR*-7 acts in an incoherent feedforward motif. Ato activates *E(spl)* directly. Ato also represses *E(spl)* via *miR*-7. Ato activity results in a pulse of *E(spl)* followed by a lower level of steady-state expression due to Ato-induced *miR*-7 activity.

split (E(spl)) or HES family form the sense organ primordial. One cell from the proneural cluster is selected by chance to become a sense organ precursor (SOP). This selection occurs as a consequence of fluctuation in the levels of a transcription factor Senseless (Nolo et al., 2000). Higher Senseless (Sens) levels lead to an increase in proneural gene expression in the SOP, which feedback to increase Sens expression (Fig. 1.2B). In addition, Sens increases expression of the Notch ligand Delta, which in turn leads to increased Notch signaling in the adjacent cells. Notch activity represses proneural gene expression in these cells and prevents the adjacent cells

from becoming SOPs (Jafar-Nejad et al., 2003). Thus, the positive feedback loop, together with lateral inhibition within the proneural cluster, promotes differentiation of a single cell into SOP. The role of *miR-9a* in SOP differentiation is to ensure that random fluctuations in Sens levels do not trigger this feedback loop inappropriately. *miR-9a* functions to set a threshold that *sens* transcription must overcome in order to trigger the feedback loop (Herranz and Cohen, 2010). Initially, all proneural cells express uniform levels of *miR-9a*. As Sens activity increases proneural expression in the SOP, *miR-9a* levels are reduced. *miR-9a* levels remain high in the neighboring cells, where proneural activity is kept low through Notch activity (Fig. 1.2B). The outcome is a situation where initially overlapping expression domains resolve into spatially reciprocal domains of the miRNA and its target. This data indicates that the expression of *miR-9a* is negatively regulated by Sens and positively regulated by Notch. Thus, *miR-9a* contributes to the robustness of the noise-dependent switch.

Drosophila miR-14 has been implicated in a regulatory switch, which serves to buffer against noise. In this regulatory loop, the nuclear hormone Ecdysone Receptor (EcR) autoregulation is linked to a negative feedback loop involving *miR-14* (Fig. 1.2C). *miR-14* acts to posttranscriptionally repress EcR levels while EcR reciprocates by negatively regulating *miR-14* transcription (Varghese and Cohen, 2007).

miR-7 has been implicated in two coherent feedforward motifs involved in photoreceptor specification (Li et al., 2009a). This loop involves the transcription factor Yan, whose expression is regulated by *miR-7*. In one circuit, Yan represses *miR-7* transcription directly as well as indirectly. The indirect arm involves three components: Yan represses Phyllopod (Phyl), which in turn derepresses Tramtrack69 (Ttk69), which is a direct repressor of *miR-7*. The presence of two branches to regulate *miR-7* makes the system less sensitive to transient fluctuation in the levels of either Yan or Ttk69. The second feedforward motif takes place in the same cells where the transcription factor Pointed-P1 (Pnt-P1) directly activates *miR-7*, which in turn represses Yan. Pnt-P1 also acts directly to repress *yan* transcription (Fig. 1.2D). This circuit buffers Yan expression to variations in Pnt-P1 or *miR-7*. The two interlinked motifs have been shown to provide stability to the process of photoreceptor specification (Li et al., 2009a).

miR-7 is also implicated in an incoherent feedforward motif that contributes to sensory organ specification. In this system, the transcription factor Atonal (Ato) acts directly to activate expression of the proneural gene *Enhancer of split* (*E(spl)*). In parallel, Ato acts via *miR-7* to repress *E(spl)* genes (Fig. 1.2E). In this context, activation of Ato results in a pulse of *E(spl)* expression followed by a lower level steady-state expression (Li et al., 2009a).

3.3. Posttranscriptional regulation of miRNAs

A number of proteins have been shown to regulate miRNA processing either by interacting with Drosha or Dcr, or by binding directly to miRNA precursors (Krol et al., 2010b; Siomi and Siomi, 2010; Winter et al., 2009). Most of these studies have been carried out in other model systems, so these regulatory relationships still need to be verified in flies. The best-studied negative regulator of miRNA biogenesis is the Lin-28 RNA-binding protein (Viswanathan and Daley, 2010). Repression by Lin-28 is highly specific and affects members of the *let-7* family only (Heo et al., 2008; Viswanathan et al., 2008). Lin-28 binds to the terminal loop of *pri-let-7* transcripts, and this binding interferes with its Drosha cleavage (Viswanathan and Daley, 2010). Binding of Lin-28 to *pre-let-7* can also block its processing by Dcr. In this case, Lin-28 induces terminal polyuridylation of *pre-let-7* by recruiting terminal uridylyl transferase (Hagan et al., 2009; Heo et al., 2008, 2009; Jones et al., 2009; Lehrbach et al., 2009). Uridylation prevents Dcr processing and targets *pre-let-7* for degradation (Heo et al., 2008). Interestingly, *lin-28* mRNA is targeted by *let-7*, thus resulting in a negative feedback regulatory motif (Rybak et al., 2008). A Lin-28-*let-7* regulatory relationship is highly conserved in evolution and has been shown to play an important role in maintaining the pluripotency of embryonic stem cells and also in development and oncogenesis. Though both *let-7* and Lin-28 protein are conserved in flies, so far no study has demonstrated such a regulatory relationship in this system.

3.4. Autoregulation of the microprocessor machinery

The two components of the microprocessor complex, Drosha and DGCR8, have been shown to regulate each other in the mammalian system. DGCR8 stabilizes Drosha by binding via its conserved carboxy-terminal domain to the middle domain of Drosha (Yeom et al., 2006). In turn, Drosha cleaves two hairpin structures located in the 5′ UTR and the coding sequence of the *Dgcr8* mRNA resulting in its degradation (Han et al., 2009; Kadener et al., 2009b; Triboulet et al., 2009). This results in a double negative feedback loop, ensuring tight coupling of the microprocessor components. The 5′ UTR of the *Drosophila* orthologue of DGCR8, *pasha,* contains a hairpin structure although the sequences of this hairpin are not conserved with vertebrates. Knockdown of *Drosophila* Drosha in a cell culture system, however, resulted in the accumulation of Pasha (Han et al., 2004; Kadener et al., 2009b). Thus, the Drosha/DGCR8 regulatory relationship is likely conserved and may have played a fundamental role throughout animal evolution.

4. Mechanism of miRNA Action

Most animal miRNAs imperfectly base pair with sequences in the 3′ UTR of target mRNAs and inhibit protein synthesis by either repressing translation or promoting mRNA deadenylation and decay (Fabian et al., 2010; Krol et al., 2010b). Efficient mRNA targeting requires continuous base pairing of the mRNA with the second to eight nucleotides of the miRNA, a region known as the "seed" (Bartel, 2009). The mechanism of miRNA-mediated translational repression is not as clearly understood as the mechanisms of miRNA-mediated mRNA deadenylation (Chekulaeva and Filipowicz, 2009; Fabian et al., 2010). Deadenylation of mRNAs is mediated by GW182, a protein that physically interacts with Ago proteins and acts downstream of them. The amino-terminal region of GW182 interacts with Ago proteins whereas its carboxy-terminus (C-terminus) interacts with the poly(A) binding protein (PABP) and recruits the deadenylases CCR4 and CAF1 (Eulalio et al., 2009; Fabian et al., 2010). Interestingly, the PABP regions that are targeted by GW182 are also recognized by many other translational factors, suggesting that these interactions may be subject to extensive regulation (Fabian et al., 2010). In addition to the C-terminus, *D. melanogaster* GW182 contains two additional domains that function in translational repression. These three repressive domains maybe differentially regulated or may target distinct sets of mRNAs (Chekulaeva et al., 2009). It is also now clear that miRNAs lead to the degradation of target mRNAs. When RISC containing Ago2 in mammals or flies encounters mRNAs bearing sites near perfectly complementary to miRNA, these mRNAs are cleaved endonucleolytically and degraded (Bartel, 2009; Carthew and Sontheimer, 2009; Chekulaeva and Filipowicz, 2009; Fabian et al., 2010). Finally, although most reports have focused on the repression of mRNAs by targeting miRNAs, some reports have indicated that miRNAs also activate translation of mRNA targets (Henke et al., 2008; Orom et al., 2008; Vasudevan and Steitz, 2007; Vasudevan et al., 2007).

5. Identification of *Drosophila* miRNAs

miRNAs have emerged as important regulators of gene expression. They are abundantly expressed and comprise 1–5% of animal genes (Bartel, 2004; Bentwich, 2005; Bentwich et al., 2005). A single miRNA can regulate a large fraction of genes (Brennecke et al., 2005; Krek et al., 2005; Lewis et al., 2005; Xie et al., 2005). Moreover, the regulatory potential of miRNAs is further increased due to the presence of target

sites for several miRNAs within many genes, allowing for a combinatorial control of gene expression that is reminiscent of transcriptional control (Hobert, 2004). Finally, an ancient role for miRNAs similar to transcriptional regulators is suggested by their high level of conservation (Pasquinelli et al., 2000). Thus, an extensive analysis of all miRNAs and their targets has become indispensable to the understanding of gene regulatory networks in animals. Both high-throughput cloning and sequencing as well as computational approaches have contributed to the currently annotated set of fly miRNAs.

5.1. Cloning and sequencing of miRNAs

The current tally of *Drosophila* miRNAs is 171. Most of these miRNA genes were discovered by high-throughput cloning and sequencing of complementary DNAs (cDNAs; Griffiths-Jones, 2004). For cloning, small RNAs were size fractionated by electrophoresis and ligated to RNA/DNA hybrid adapters. The ligation products were amplified by reverse transcription polymerase chain reaction, concatamerized, cloned into bacterial vectors and sequenced (Aravin et al., 2003; Elbashir et al., 2001; Lagos-Quintana et al., 2001, 2002, 2003; Lau et al., 2001; Lee and Ambros, 2001). The miRNA sequences were identified from the cloned pool of small RNA sequences using bioinformatic tools. Genomic sequences flanking these sequences were then retrieved to confirm that they folded into the hairpin structures that are typical of pre-miRNAs (Bentwich, 2005). In addition to miRNAs, these cloning efforts identified other class of small RNAs, including tiny noncoding RNAs, which are small RNAs that do not arise from miRNA precursors but were expressed at high enough levels to be detected by Northern blots (Ambros et al., 2003). Other distinct classes of small RNAs such as repeat-associated siRNAs (rasiRNAs) and Piwi-interacting RNAs (piRNAs) have been reported in flies and mammals (Aravin et al., 2003, 2006; Girard et al., 2006; Lau et al., 2006; Vagin et al., 2006).

The initial studies using these approaches led to the identification of 17 miRNAs in *D. melanogaster*. The expression of these miRNAs were analyzed in different stages of *D. melanogaster* development (embryo, larva, pupa, adult, and cultured cells) by Northern blot analysis (Lagos-Quintana et al., 2001). In a similar study, additional *Drosophila* miRNAs were identified by sequencing cDNA libraries generated from embryonic, larval, pupal, and adult stages (Aravin et al., 2003). Recent advances in high-throughput sequencing technology (Margulies et al., 2005) have greatly increased the sensitivity of these approaches, leading to discovery of many novel miRNAs that had escaped detection earlier due to a rare expression pattern (Berezikov et al., 2006b; Fahlgren et al., 2007; Lu et al., 2006; Rajagopalan et al., 2006; Ruby et al., 2006).

5.2. Computational identification of *Drosophila* miRNA genes

An approach for the identification of low abundance miRNAs that was successfully applied in *Drosophila* was to identify candidate miRNA hairpins computationally and then validate their expression by employing directed experimental methods (Lai et al., 2003). This informatic strategy (miRseeker) was employed to scan for miRNAs in the genomes of *D. melanogaster* and *D. pseudoobscura*. This analysis made use of previously identified pre-miRNA sequences (Lagos-Quintana et al., 2001; Pasquinelli et al., 2000) as a reference to define parameters that would specifically recognize miRNA genes within anonymous genomes. First, repeat masked *D. melanogaster* sequences were aligned to the corresponding *D. pseudoobscura* sequences. This was followed by the removal of sequences corresponding to exons, transposable elements, snRNAs, snoRNAs, tRNAs, and rRNAs. The aligned sequences were used to extract conserved sequences that could contain miRNA sequences. A total of 436,000 conserved nucleotide regions obtained from this analysis were used for identification of conserved stem loops. These structures were then evaluated and were ranked based on parameters like the length of the longest helical arm and the free energy of this isolated arm. The top ranking 25% of the stem loop candidates were evaluated based on their divergence patterns based on the assumption that miRNAs can be highly conserved during evolution (Tran et al., 2006). This approach led to the identification of 204 potential miRNA genes. The authors identified 48 novel miRNA candidates that were conserved in insect, nematode, and vertebrate genomes. The expression of a total of 24 miRNA genes was verified by Northern blot analysis. From this study, it was concluded that miRNAs constitute nearly 1% (110) of the genes in *Drosophila*, a percentage that is similar to the percentage of miRNAs predicted in other metazoan genomes (Lim et al., 2003a).

Subsequent studies have further increased the repertoire of *Drosophila* miRNAs (Berezikov et al., 2010; Chung et al., 2008; Lau et al., 2009; Lu et al., 2008; Ruby et al., 2007b; Sandmann and Cohen, 2007; Seitz et al., 2008; Stark et al., 2007a). These studies used computational methods to predict miRNAs conserved among *Drosophila* species and high-throughput sequencing of small RNAs from *D. melanogaster* to experimentally confirm and complement these predictions (Ruby et al., 2007b; Stark et al., 2007a).

One study identified novel miRNAs genes as hairpins with secondary structure and conservation patterns resembling those of previously annotated miRNAs (Ruby et al., 2007b). The computational approach used in this study was similar to "MiRscan," which had been described previously for nematodes and vertebrates (Lim et al., 2003a,b). Potential hairpins were predicted from the entire genome of six *Drosophila* species. The top 100 miRNA candidate loci from this approach included 55 previously annotated genes as well as 45 novel predictions. These miRNA predictions were

validated with more sensitive and reliable high-throughput pyrosequencing (Margulies et al., 2005; Ruby et al., 2006) on libraries of small RNAs isolated from 10 *D. melanogaster* tissues or stages. In order to identify miRNAs that were difficult to recognize computationally, the small RNA sequence reads were examined by several criteria. These included pairing characteristics of the hairpin, miRNA expression, evolutionary conservation, the absence of annotation suggesting non-miRNA biogenesis, and the presence of reads corresponding to the predicted miRNA* species. This analysis not only identified 59 additional miRNAs but also refined the description of about half of the previously annotated genes. With this study, the number of identified miRNA genes in *D. melanogaster* increased to 148. These included 74 of the previously annotated genes, 59 novel genes reported in this study, and another 15 novel genes whose transcripts bypass Drosha processing.

A parallel study provided independent predictions of miRNAs with even greater specificity (Stark et al., 2007a). The authors made use of 12 *Drosophila* genomes to discover novel miRNA loci. The structural and evolutionary patterns of the known *Drosophila* miRNAs were studied to derive rules for discriminating miRNAs from the more frequent nonfunctional hairpins in the genome. Some of the structural features that were considered in this study were also used in previous predictions (Bentwich et al., 2005; Berezikov et al., 2006a; Lai et al., 2003; Lim et al., 2003b). These characteristics included length of miRNA hairpins, length of the arms and the hairpin loop, secondary structures generated by folding of precursor sequences, and loop structures in the miRNA hairpins. The characteristic evolutionary profile of miRNAs in *Drosophila* genomes was determined by aligning the 60 previously cloned miRNAs and their flanking regions across all 12 species. The loop and the flanking regions showed abundant mutations, while the arms were very highly conserved. These structural and evolutionary parameters were used for the genome-wide identification of miRNAs from a set of 760,355 miRNA-like hairpins in the entire genome. The top ranking hairpins were strongly enriched in cloned miRNAs. This method predicted 101 hairpins in the fly genome, including 51 of the 60 cloned miRNAs and 41 novel miRNA candidates. Twenty-eight of these 41 predictions were experimentally validated by Solexa sequencing of *Drosophila* ovaries and testes. This approach resulted in very accurate predictions (at least 83% of all predictions were correct) owing to the features defined, the increased number of species in the comparative analysis and the large evolutionary distances they span.

This comparative genomic analysis has also enhanced the current understanding of miRNA function and biogenesis (Stark et al., 2007a). The authors showed that a single miRNA gene could give rise to several mature sequences by three different ways. First, both strands of the gene can be expressed and processed into miRNAs. Second, multiple mature products

can be obtained with small offsets from the primary miRNA product. Third, both arms of the miRNA hairpin can be processed into mature miRNAs, each with many potential mRNA targets.

6. Identification of miRNA Targets

The biological role of miRNAs is dictated by the mRNA targets that they regulate. Thus, the identification of miRNA targets is a critical step toward their functional characterization. The mRNA targets of the founding members of the miRNA family, *lin-4* and *let-7*, were identified through genetic approaches (Lee et al., 1993; Reinhart et al., 2000; Wightman et al., 1993). The role of these miRNAs in the regulation of developmental timing was deciphered by their loss-of-function phenotypes, and genetic interactions pointed toward the regulatory targets. These studies allowed identification of *lin-14*, *lin-28*, *lin-41*, and *hbl-1* as miRNA targets (Lin et al., 2003). Similar genetic approaches aided in the functional analysis of other miRNAs. The worm *lsy-6* miRNA was shown to regulate left/right neuronal asymmetry of chemosensory receptor expression by repression of *cog-1* (Johnston and Hobert, 2003). Another example was the identification of proapoptotic gene *head involution defective* (*hid*) as a biologically relevant target of the *Drosophila* miRNA *bantam* (Brennecke et al., 2003).

However, a very limited set of mRNA targets have been identified and validated by experimental approaches (Brennecke et al., 2003; Lee et al., 2003a; Reinhart et al., 2000; Slack et al., 2000). As miRNAs are short, and animal miRNAs have limited sequence complementarity with their targets, accurate prediction of target mRNAs has been a difficult task. Moreover, due to the laborious nature of experiments and the absence of high-throughput experimental approaches to identify targets, a number of sophisticated bioinformatic methods have been developed to allow reliable and testable predictions of miRNA target genes (Bentwich, 2005; Brown and Sanseau, 2005; Min and Yoon, 2010; Rajewsky, 2006; Yoon and De Micheli, 2006; Yue et al., 2009).

The first bioinformatic approach to identify miRNA targets in *Drosophila* was described in a study of gain-of-function mutant alleles that result in abnormal neuronal patterning. In this study, multiple families of conserved 3′UTR motifs (Brd boxes, GY boxes, and K boxes) that negatively regulate Notch target genes were discovered (Lai, 2002; Lai et al., 2005). This study showed that ~7 nt at the 5′ end of the miRNA is complementary to these 3′UTR regulatory motif. The relevance of the 5′ end of the miRNA for target recognition was supported by the previous findings that point mutations in *lin-4* and *let-7* loss-of-function alleles were found in their 5′ends (Lee et al., 1993; Reinhart et al., 2000).

6.1. Resources for analysis of miRNAs and their target genes

As increasing numbers of miRNAs and their target genes have been identified, web-based databases have been constructed and have served as registries for this information. These databases provide published miRNA sequences, their annotations, and their potential mRNA target genes. The miRNA registry, or miRBase, is one of the most well-known and widely used database of miRNA sequences that was initially designed to assign uniform names to miRNAs and also provided rules for classification of small RNAs as miRNAs (Griffiths-Jones, 2004; Griffiths-Jones et al., 2006, 2008). This database is also open for submission of newly identified miRNAs. Current release 15 contains 171 miRNA entries for *D. melanogaster*, 940 entries for *H. sapians*, and 175 entries for *C. elegans*. Subsequently, a number of other databases that house useful information about miRNAs and their targets have been constructed (see Table 1.1).

6.2. Features utilized in miRNA–target prediction

The first parameter used by computer predictions to identify potential miRNA binding sites is the base pairing pattern. Binding sites have been classified into the following three categories based on the level of complementarity of the miRNA to the target sequence: 5′-dominant canonical, 5′-dominant seed only, and 3′-compensatory (Fig. 1.3). The 5′-dominant canonical sites have perfect complementarity to the 5′ and 3′ end of miRNA with a characteristic bulge in the middle. The 5′-dominant seed-only sites have perfect complementarity to the 5′ seed, but poor base pairing at the 3′ end (Brennecke et al., 2005; Krek et al., 2005; Lewis et al., 2005). The 3′-compensatory have a mismatch or wobble in the seed region but compensate with complementarity at the 3′ end (Brennecke et al., 2005; Doench and Sharp, 2004).

The second feature used for target prediction programs is the thermodynamic stability of the miRNA:mRNA hybrid. This parameter is determined by calculating the free-energy (ΔG) of the putative binding. Multiple RNA folding programs such as the Vienna package (Wuchty et al., 1999), RNAfold (Hofacker, 2003), and Mfold (Mathews et al., 1999) have been used to calculate the approximate free-energy and secondary structure of the miRNA:mRNA hybrid. These programs require a single linear RNA as input: the 3′ end of the 3′UTR of the putative mRNA target and the 5′ end of the targeting miRNA are connected by a linker sequence. The linear sequence folded into a structure, and the minimum free energy of the structure is calculated. This is then used to calculate a threshold of free energy of binding. However, since datasets of identified miRNA:mRNA duplexes are very limited and a low free energy of hybridization does not guarantee accurate prediction of target genes, it is complicated to resolve

Table 1.1 Databases for miRNAs and their targets

Name of the database	URL	Main features	References
miRBase	http://microrna.sanger.ac.uk/	miRNA sequences, annotations, and computationally predicted targets	Griffiths-Jones (2004), Griffiths-Jones et al. (2006, 2008)
miRNAmap	http://miRNAMap.mbc.nctu.edu.tw/	Known and computationally predicted miRNAs and their targets	Hsu et al. (2008)
Tarbase 5.0	http://diana.cslab.ece.ntua.gr/tarbase	Database of miRNA targets with experimental support	Papadopoulos et al. (2009)
miRDB	http://mirdb.org/miRDB/	Target prediction (MirTarget2) and functional annotation	Wang (2008)
miRGator	http://genome.ewha.ac.kr/miRGator	Target prediction, functional analysis, gene expression data, genome annotation	Nam et al. (2008)

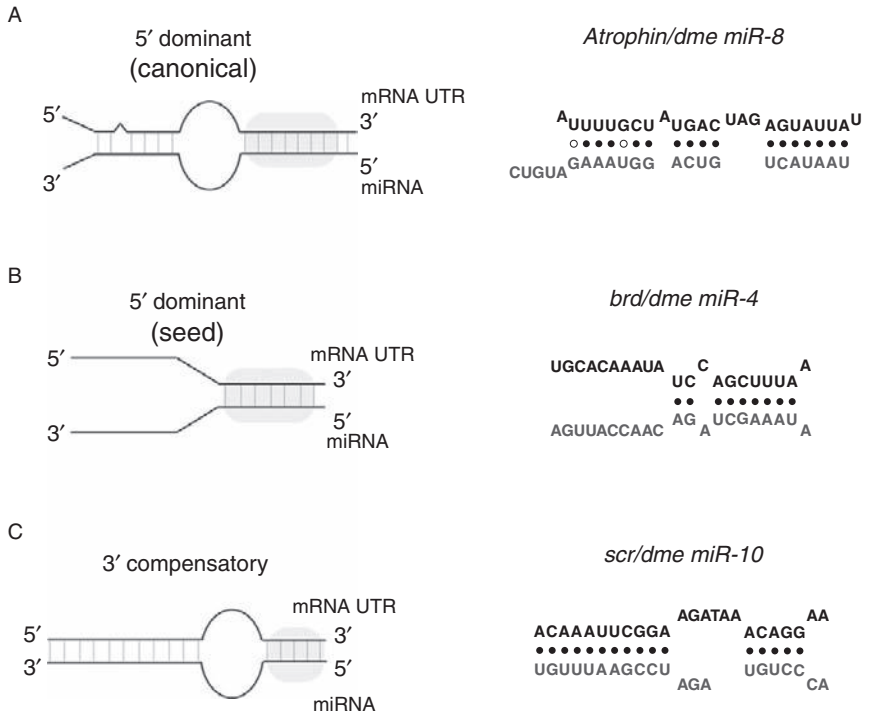

Figure 1.3 *Three classes of miRNA target sites*. Model of (A) canonical, (B) dominant seed, and (C) 3′compensatory target sites. The diagrams illustrate the mode of pairing between the target site (upper line) and the miRNA (lower line). The seed region is highlighted with gray shading. The right panel in each row displays examples of experimentally validated *Drosophila melanogaster* miRNA–target sites for each model (*atrophin/miR 8*: Karres et al., 2007; *bearded/miR-4*: Lai et al., 2005; Brennecke et al., 2005; *sex com reduced/miR-10*: Brennecke et al., 2005). The predicted structure of the duplex between the miRNA (gray font) and its target site (black font); canonical base-pairs are marked with filled circles, G:U base-pairs with open circles.

appropriate thresholds (Watanabe et al., 2007). Thus, additional parameters such as comparative sequence analysis have been employed for reliable prediction of target transcripts. A study that described the prediction of targets of vertebrate miRNAs showed that replacing the thermodynamic parameter with evolutionary conservation derived from multiple sequence alignment did not lower the specificity of the algorithm (Lewis et al., 2005).

A crucial determinant in the identification of miRNA targets in a particular species is a complete and comprehensive collection of the 3′UTRs of its protein-coding mRNAs. This would include information about location and extent of splice variation of 3′UTRs. The Berkeley *Drosophila* genome project (BDGP) has provided high-quality transcript

information that has facilitated accurate determination of fly 3′UTRs. Thus, reliably annotated and verified 3′UTR datasets are extremely useful for target prediction.

Conservation of target sequences across related species is another important parameter used in prediction analysis. Comparative sequence analysis of predicted target binding sites across related species and filtering out non-conserved sites is considered more likely to reduce the number of false positives (Lewis et al., 2003; Stark et al., 2003; Watanabe et al., 2007). All of the known binding sites of *lin-4*, *let-7*, *bantam*, *mir-2*, *mir-7*, and *lsy-6* are conserved between closely related species. One point of consideration during conservation analysis is the set of species that are compared. Comparisons between evolutionary distant species might be more relevant and will yield less false negatives (e.g., comparison of human transcripts with mouse, rat, or dog rather than chimpanzee transcripts which are at least 99% conserved with human transcripts; Maziere and Enright, 2007).

Some algorithms search for the presence of multiple target sites and take the number of target sites per transcript into account for prediction (Stark et al., 2005). Though single target sites are also known to regulate target mRNAs, the presence of multiple target sites in the same 3′UTR may potentially increase the extent of translational repression. This hypothesis is supported by studies that have shown that multiple miRNAs are coexpressed and are likely to regulate the same mRNA coordinately (Rajewsky, 2006).

Finally, another useful feature considered for the identification of miRNA targets is the potential coordinate regulation of multiple genes in functionally related pathways (Lai, 2004). For example, *Drosophila mir-7* has been shown to regulate a number of Notch targets, multiple proapoptotic genes are *mir-2* targets, and a number of enzymes involved in amino acid degradation have predicted sites for *mir-277* (Enright et al., 2003; Lai, 2002; Stark et al., 2003). Incorporation of these criteria in prediction analysis increases one's confidence in selected candidates but can run the risk of overlooking some genuine candidates (Lai, 2004).

6.3. Algorithms for miRNA–target prediction

During the initial phase of development, the prediction algorithms were designed to recover the known targets and detect new ones that could be experimentally validated. The most commonly used parameter is complementarity and this has been applied for target prediction in vertebrates (Lewis et al., 2003), humans (Kiriakidou et al., 2004), flies (Brennecke et al., 2005; Stark et al., 2003), and worms (Lall et al., 2006; Watanabe et al., 2006). Other features such as evolutionary conservation (Grun et al., 2005; Lewis et al., 2003), secondary structure of the target transcript (Kertesz et al., 2007; Long et al., 2007), and nucleotide composition of target sequences (Grimson et al., 2007) were introduced to increase the

accuracy (Min and Yoon, 2010). Currently available miRNA–target prediction methods are described in Table 1.2, and some that are relevant to target prediction in flies are described in more detail.

The first large-scale prediction of *D. melanogaster* miRNA targets was reported by (Stark et al., 2003). A conserved 3′UTR database was generated by comparing 3′UTR regions in *D. melanogaster* and *D. pseudoobscura*. The approach combined a sensitive sequence database search with an RNA folding algorithm to evaluate the quality of predicted miRNA:target duplexes. The alignment tool HMMer (Eddy, 1995) was utilized to search for sequences complementary to the first eight residues of the miRNA, but allowing for G:U mismatches. The 3′UTRs obtained from the prediction algorithm were filtered for conservation in *D. pseudoobscura* and *Anopheles gambiae*. The identified sequences were evaluated for their ability to form thermodynamically favorable duplexes with the miRNA using the Mfold algorithm (Mathews et al., 1999). This method was able to detect all previously known targets, which ranked as top scoring candidates. Many new target sites were also predicted (e.g., *miR-7*: *hairy*, *HLHm3*; *miR-2*: *reaper*, *grim*, *sickle*; *miR-277*: enzymes involved in the amino acid metabolic pathway) and six of these were validated experimentally.

miRanda was the second method to be reported. It was initially designed to predict miRNA targets in *D. melanogaster* (Enright et al., 2003). miRanda uses sequence complementarity to identify potential target sites in 3′UTRs. The scoring matrix is designed so that complementary bases at the 5′ end of the miRNA are rewarded more than those at 3′ end. Hence, those binding sites that exhibit a near perfect match at the seed region display a better score. The potential binding sites were then evaluated thermodynamically, using the Vienna RNA folding package (Wuchty et al., 1999). The BDGP 3′UTRs dataset was used, and results were filtered to limit predictions to targets conserved in *D. pseudoobscura*. miRanda was able to predict 9 out of 10 currently characterized target genes, and its false positive rate was between 24% and 39%. This method was improved by incorporating a strict model for the binding sites that require almost perfect complementarity in the seed region allowing a single wobble pairing (John et al., 2004). In addition, it was applied to other genomes and predicted about 2000 human genes with miRNA target sites that were conserved among other mammals. Despite similar parameters and identical input datasets, the scoring and ranking strategies devised by Stark et al. and miRanda (Enright et al., 2003) are different: only 40% of the top 10 miRNA targets predicted by both methods overlap (Maziere and Enright, 2007).

PicTar is a prediction method that relies on comparative data from several species to identify common targets for miRNAs and has also been applied to target identification in flies (Grun et al., 2005; Krek et al., 2005). In addition, this algorithm also computes the maximum likelihood that a given sequence is bound by one or more miRNAs. The initial parameters

Table 1.2 Tools for predicting miRNA targets

Program	Clades[a]	Distinguishing features	Website URL	Reference
TargetScan	f, w	Considers seed match features and the free energy (thermodynamic based modeling) and target site conservation	http://targetscan.org	Ruby et al. (2006, 2007a)
EMBL	f	Considers stringent seed pairing, site number, overall predicted pairing stability, and conservation of target site	http://www.russell.embl.de/miRNAs/	Stark et al. (2005)
PicTar	f, w, m	Input for PicTar is a set of coexpressed miRNAs and a set of orthologous 3′UTRs. Additional features considered are miRNA–target binding energy. Uses cross-species comparisons to filter out false positives	http://pictar.mdc-berlin.de	Grun et al. (2005), Lall et al. (2006)
EIMMo	f, w, m	Considers seed match features and likelihood of preferential conservation. Infers the phylogenetic distribution of functional target sites for each miRNA	http://www.mirz.unibas.ch/ElMMo2	Gaidatzis et al. (2007)
Miranda	f, w, m,+	Considers nucleotide complementarity, binding energy of RNA–RNA duplexes, and conservation of target sites in related genomes. Also provides the expression of miRNA in various tissues	http://www.microrna.org	Betel et al. (2008)
MicroCosm Targets	f, w, m, +	Moderately stringent seed pairing, site number, overall pairing	http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/	Griffiths-Jones et al. (2008)
PITA Top	f, w, m	Considers seed pairing and accessibility energy of miRNA–target site interaction. The model of miRNA–target interaction is based on the	http://genie.weizmann.ac.il/pubs/mir07/mir07_data.html	Kertesz et al. (2007)

(*continued*)

Table 1.2 (*continued*)

Program	Clades[a]	Distinguishing features	Website URL	Reference
		observation that a strong secondary structure formed by a 3′UTR prevents the binding of miRNA		
Moving targets	f	Considers number of target sites in an mRNA, free energy of binding, number of nucleotides in 5′ region of miRNA involved in base pairing (consecutive), number of G:U base pairs in the 5′ region of miRNA	DVD available upon request	Burgler and MacDonald (2005)
RNA hybrid	f, w, m,+	Considers seed match and thermodynamic features. It finds the energetically most favorable hybridization sites of a small RNA within a large target RNA sequence, and base pairings between target nucleotides or between miRNA nucleotides are not allowed	http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/	Rehmsmeier et al. (2004)
RNA22	f, w, m	Moderately stringent seed pairing, matches to sequence patterns generated from miRNA set, overall predicted pairing, and predicted pairing stability. Eliminates the use of cross-species conservation filtering, and leads to putative target sites in 5′ UTRs and ORF	http://cbcsrv.watson.ibm.com/rna22.html	Miranda et al. (2006)

[a] Letters indicate predictions provided for the mammalian/vertebrate (m), fly (f), worm (w), or additional (+) clades.

used for target prediction include optimal binding free energy, and the resulting target set is tested statistically using genome-wide alignment. PicTar was the first method that utilized the criteria of coexpression of miRNA and its target. The false positive rate for PicTar has been estimated to be about 30%. This program was successfully used to predict vertebrate miRNA targets and suggested that, on average, approximately 200 transcripts are regulated by a single miRNA. Analysis of *Drosophila* targets suggested that, on average, 54 genes are regulated by a given miRNA and that *D. melanogaster* miRNAs regulate expression of target genes coordinately (Grun et al., 2005).

A reporter gene assay was developed to extract hybridization characteristics of miRNAs and targets for further computational analysis (Brennecke et al., 2005). This study used transgenic flies expressing GFP reporters modified in their 3′UTRs to be targeted by different miRNAs. These functional assays were used to define the minimal sequence required for a functional miRNA target site. This study confirmed that complementarity at the 5′end of miRNAs was sufficient to confer regulation; however, weaker base pairing at the 5′end could be compensated by complementarity at the 3′end. These characteristics were then applied to genome wide identification of miRNA targets in *D. melanogaster*. One of the important conclusions from these computational studies was that a given miRNA can have many targets and that the expression of more than 10% of *D. melanogaster* genes are regulated by miRNAs (Brennecke et al., 2005; Lewis et al., 2005; Robins et al., 2005).

6.4. Evaluation of computational predictions

Evaluation of computational prediction algorithms is crucial toward understanding the biological significance of the prediction results. The two most commonly used strategies used for evaluation of miRNA target prediction software include determining their success at predicting known miRNA target genes and calculating the false positive rate using negative control tests (evaluation of artificial miRNA-like sequences; Watanabe et al., 2007).

The performance of most miRNA prediction algorithms is evaluated by their ability to recover known miRNA–mRNA interactions. One major caveat of this method of evaluation is the lack of an extensive positive dataset. Due to the limited number of experimentally analyzed miRNA target interactions, it is also difficult to determine statistical significance. Moreover, the datasets used for evaluation become smaller due to the use of validated targets in generating training sets for the development of the prediction algorithm. This method of evaluation will lead to more accurate results as additional validated target sets become available (Papadopoulos et al., 2009; Sethupathy et al., 2006).

The second approach for the evaluation of miRNA target prediction is the comparison of prediction results calculated from real and artificial miRNA data inputs. Artificial miRNAs are randomly permutated sequences that resemble miRNAs in terms of base composition, sequence, length, and frequency of occurrence in the genome. By determining the differences between the results obtained from the two sets, a signal-to-noise ratio or a false-positive rate is estimated (Enright et al., 2003; John et al., 2004; Kiriakidou et al., 2004; Lewis et al., 2003; Stark et al., 2003).

6.5. Validation of computational predictions

The success of a computer prediction algorithm relies on experimental validation of novel miRNA targets. Since computer prediction systems are not always accurate and there is a risk of false positive prediction, target validation in a biological system is a crucial step in any target prediction study. The most commonly used methods to test interaction between an miRNA and its predicted target include reporter assays in which a putative target sequence is fused to a reporter gene (e.g., luciferase or green fluorescent protein) and reporter gene expression measured in the presence or absence of an miRNA (Kiriakidou et al., 2004; Lewis et al., 2003; Miranda et al., 2006; Robins et al., 2005). Other approaches that have been used for experimental validation include *in vivo* genetic analysis using transgenic flies expressing reporter constructs (Brennecke et al., 2003; Stark et al., 2003), 5′-RACE (Jones-Rhoades et al., 2006; Wang et al., 2004), and biochemical purification of miRNA:mRNA complexes (Bartel, 2009; Easow et al., 2007; Hafner et al., 2010; Hong et al., 2009). In addition, microarray analysis provides a high-throughput method for identifying cleaved target mRNAs (Giraldez et al., 2006; Lim et al., 2005; Rehwinkel et al., 2006; Wang and Wang, 2006). Techniques like Northern blot analysis, quantitative real-time PCR (qRT-PCR), ribonuclease protection assay, or *in situ* hybridization are performed to examine reciprocal expression of miRNA and its predicted mRNA target. Western blot analysis and immunocytochemistry are used to compare protein levels of the miRNA target in either the presence or the absence of miRNA.

For more detailed analysis, miRNA overexpression or knockout experiment under *in vitro* and *in vivo* conditions are used to validate miRNA–target predictions (Behm-Ansmant et al., 2006; Lim et al., 2005; Teleman et al., 2006). A particular miRNA can be specifically silenced by chemically modified oligonucleotides that are perfectly complementary to the mature miRNA. These modified antisense oligonucleotides include morpholinos, antagomirs, locked nucleic acids (LNA), or 2′-*O*-methyl oligonucleotides (2′OM-ORNs). Additionally, siRNAs have also been applied to knockdown miRNA expression (Li et al., 2009b). A more recently reported technique used to explore miRNA functions with temporal and spatial

specificity *in vitro* and *in vivo* is the expression of "miRNA sponges," which are decoy targets that function as competitive inhibitors of miRNAs (Cohen, 2009; Ebert et al., 2007). However, the relevance of a particular miRNA target interaction *in vivo* requires more stringent genetic tests. These would include analysis of loss-of-function mutants of specific miRNA for misregulation of their predicted targets, and testing whether mutations of the miRNA-binding sites of a predicted target gene partially phenocopy the genetic elimination of the miRNA.

7. Functions of miRNAs in *Drosophila*

A combination of approaches have provided evidence that individual miRNAs have target sites in hundreds of mRNAs (Baek et al., 2008; Farh et al., 2005; Krek et al., 2005; Lewis et al., 2005; Lim et al., 2005; Ruby et al., 2007b; Selbach et al., 2008; Sood et al., 2006; Stark et al., 2005; Xie et al., 2005). These studies have also provided evidence that abundant RNAs are under selective pressure to avoid regulation by coexpressed miRNAs and that genes involved in basic cellular processes encode shorter 3′UTRs that have been selected for the absence of miRNA binding sites whereas genes involved in developmental processes show evidence of enrichment for miRNA sites. One of the proposed mechanisms of miRNA action is that miRNAs function to ensure accuracy and confer robustness to gene expression programs. This idea is supported by studies reporting that many miRNAs and their targets appear to be expressed in a largely nonoverlapping manner, either temporally or spatially (Farh et al., 2005; Sood et al., 2006; Stark et al., 2005). This suggests that the role of miRNAs is to tune gene expression patterns imposed by transcriptional regulation.

Genetic analysis of general genes involved in miRNA biogenesis is another approach that has been used to examine the spectrum of possible functions of miRNAs in development (Bushati and Cohen, 2007). For example, the depletion of the Dcr-interacting protein Loquacious results in female sterility (Forstemann et al., 2005), whereas the elimination of *dcr-1* itself results in mutant germline stem cells (GSCs) that display cell division defects (Hatfield et al., 2005). Somatic elimination of *dcr-1* in eye tissue results in eyes that are small with disorganized ommatidial arrays and missing interommatidial bristles (Lee et al., 2004b). Although *dcr-1* and *ago-1* single mutants do not display embryonic patterning defects, *ago1 dcr-1* double mutants exhibit strong segmentation defects, suggesting that miRNAs regulate patterning of the *Drosophila* embryo (Meyer et al., 2006).

Genetic analysis of miRNAs in various organisms have suggested that there are a variety of distinct modes of miRNA action. In this section,

we describe the diverse functional roles and different modes of action of specific *D. melanogaster* miRNAs (Table 1.3). miRNAs can function in regulating developmental transitions, and an example is *Drosophila let-7*, which plays a role promoting stage specific developmental events during metamorphosis (Sokol et al., 2008). Some miRNAs function by "tuning" or "thresholding" mRNA targets that are functionally active in the miRNA expression domain. For example, *Drosophila miR-9* regulates tuning of its target *sens* during sensory organ precursor specification (Li et al., 2006). Other miRNAs function by repressing key individual targets as evidenced by the fact that phenotypes resulting from the removal of these miRNAs can be suppressed by partially reducing the expression of a single target gene. For example, *D. melanogaster miR-14* mutant phenotype is suppressed by heterozygous deletion of its target gene, *EcR* (Varghese and Cohen, 2007).

7.1. A miRNA that regulates developmental timing

The two founding members of the miRNA family, *lin-4* and *let-7*, were identified in genetic screens for heterochronic defects in *C. elegans* (Lee et al., 1993; Pasquinelli et al., 2000; Reinhart et al., 2000). Heterochronic genes function in regulatory pathways that specify the timing of cellular development in diverse cell types and thereby ensure a coordinated schedule of developmental events throughout the worm (Moss, 2007; Rougvie, 2005). The *Drosophila let-7-Complex* (*let-7-C*) is a polycistronic locus encoding three ancient miRNAs: *let-7*, *miR-100*, and *miR-125*, the fly orthologue of *lin-4* (Sokol et al., 2008). The sequences and developmentally regulated expression profiles of *let-7* and *lin-4* are conserved among diverse bilaterians (Pasquinelli et al., 2000; Sempere et al., 2003), with *Drosophila let-7-C* induction being tightly coupled with the onset of metamorphosis (Bashirullah et al., 2003; Pasquinelli et al., 2000; Sempere et al., 2002). To investigate whether *let-7-C* miRNAs regulate developmental timing in *Drosophila*, knockout strains for *let-7-C* were generated by gene targeting. The functional analysis of *let-7-C* loss-of-function mutants were independently reported by two groups (Caygill and Johnston, 2008; Sokol et al., 2008). *let-7-C* knockout flies appeared normal morphologically but displayed defects in adult behavior (e.g., flight, motility, and fertility) as well as juvenile features in their neuromusculature. In particular, dorsal internal oblique muscles (DIOMs), which are normally destroyed during adult maturation, persist in *let-7* mutant adults. Thus, *let-7-C* function is required for appropriate remodeling of the abdominal neuromusculature during the larval-to-adult transition (Sokol et al., 2008). The perdurance of juvenile features in adult *Drosophila let-7* mutants is analogous to the reiteration of larval cell fates in adult *C. elegans let-7* mutants (Reinhart et al., 2000) and is consistent with the idea that *let-7* might control developmental transitions in diverse bilateria (Pasquinelli et al., 2000; Sokol et al., 2008).

Table 1.3 Experimentally validated miRNA–mRNA interactions in *Drosophila melanogaster*

miRNA	Homologues	Transcriptional regulators	Target gene(s)	Process affected	References
bantam		Hippo pathway/Yorkie	*head involution defective*	Apoptosis/cell proliferation	Brennecke et al. (2003), Nolo et al. (2006), Thompson and Cohen (2006)
bantam		Hippo pathway/Yorkie	*Clock*	Circadian rhythm	Caygill and Johnston (2008), Sokol et al. (2008), Bashirullah et al. (2003), Kadener et al. (2009a)
let-7	Hs, Mm, Ce	Ecdysone receptor?	*abrupt*	Remodeling of neuromusculature	Sempere et al. (2002, 2003)
miR-1	Hs, Mm, Ce	Mef-2, Twist	*delta*	Muscle development and maintenance	Kwon et al. (2005), Sokol and Ambros (2005)
miR-8	Hs, Ce	Unknown	*atrophin*	Prevention of neurodegeneration	Karres et al. (2007)
miR-8	Hs, Ce	Unknown	*u-shaped*	Growth	Hyun et al. (2009)
miR-8	Hs, Ce	Unknown	*wntless, CG32767, TCF*	Repression of wingless pathway	Kennell et al. (2008)
miR-9a	Hs, Mm	Unknown	*senseless*	Sensory organ specification	Li et al. (2006)
miR-9a	Hs, Mm	Unknown	*lim-only*	Wing development	Bejarano et al. (2010), Biryukova et al. (2009)

(*continued*)

Table 1.3 (*continued*)

miRNA	Homologues	Transcriptional regulators	Target gene(s)	Process affected	References
miR-7	Hs, Mm, Ce	Atonal	*enhancer of split*	Notch signaling/Wing development, Chordotonal organ development	Li et al. (2009a), Stark et al. (2003)
miR-7	Hs, Mm, Ce	Yan, Pointed-P1, Tramtrack69	*yan*	Photoreceptor differentiation	Li and Carthew (2005), Li et al. (2009a)
miR-7	Hs, Mm, Ce	Maelstrom	*bag-of-marbles*	Differentiation of germline stem cells	Pek et al. (2009)
miR-2	Ce	Unknown	*reaper*, *grim*, *sickle*	Apoptosis during embryogenesis	Leaman et al. (2005), Stark et al. (2003)
miR-13		Unknown	*reaper, grim, sickle*	Apoptosis during embryogenesis	Leaman et al. (2005), Stark et al. (2003)
miR-11		Unknown	*reaper*, *grim*	Apoptosis during embryogenesis	Leaman et al. (2005), Stark et al. (2003)
miR-6		Unknown	*hid*, *reaper*, *sickle*	Apoptosis during embryogenesis	Leaman et al. (2005), Stark et al. (2003)
miR-308		Unknown	*grim*, *sickle*	Apoptosis during embryogenesis	Leaman et al. (2005), Stark et al. (2003)
miR-14		Ecdysone receptor	*ecdysone receptor*	Physiology and life span	Varghese and Cohen (2007)
miR-315		Unknown	*axin*, *notum*	Upregulates wingless pathway	Silver et al. (2007)
miR-iab-4-5p		Unknown	*ultrabithorax*	Wing/Haltere specification	Ronshaugen et al. (2005)

miR-iab-4AS		Unknown	*ultrabithorax*	Wing/Haltere specification	Stark et al. (2008)
miR263a/b	Hs, Mm, Ce	Unknown	*head involution defective*	Patterning of retina	Hilgers et al. (2010)
miR-278		Unknown	*expanded*	Insulin signaling	Teleman et al. (2006)
miR-279		Unknown	*nerfin*	Olfactory neuron fate determination	Cayirlioglu et al. (2008)
miR-184	Hs, Mm	Unknown	*saxophone*	Germline stem cell differentiation	Iovino et al. (2009)
miR-184	Hs, Mm	Unknown	*K10*	Dorsoventral patterning of the egg shell	Iovino et al. (2009)
miR-184	Hs, Mm	Unknown	*tramtrack69*	Anteroposterior blastoderm patterning	Iovino et al. (2009)

Homology with miRNAs in *Homo sapiens*, *Mus musculus*, *Caenorhabditis elegans* is indicated with Hs, Mm, and Ce, respectively. Homology information was retrieved from miRBase release 15.

In another study, *let-7* loss-of-function mutants were found to have smaller wings in addition to widespread defects in metamorphosis. This phenotype was attributed to a significant reduction in cell size, in spite of the presence of more cells than wild type (Caygill and Johnston, 2008). The wing develops from a population of progenitor cells, which proliferate throughout larval stages until cell divisions cease at the onset of metamorphosis in preparation for terminal differentiation. Analysis of *let-7* mutant wing discs revealed that the cells in these discs continued to divide 24 h after puparium formation, a time when divisions have largely ceased in wild-type animals. Conversely, ectopic expression of *let-7* during larval development caused wing disc cells to precociously exit the cell cycle. This data showed that *let-7* expression at puparium formation temporally restricts the period during which wing disc cells undergo division, which was very similar to the role of *C. elegans let-7* in temporally restricting specific larval cell divisions (Caygill and Johnston, 2008). In addition, this study also examined maturation of neuromuscular junction (NMJ), and both groups showed that adult-specific dorsal muscles (DM) and their associated NMJ appeared immature compared to wild-type controls (Caygill and Johnston, 2008; Sokol et al., 2008). Furthermore, Caygill and Johnston (2008) have identified a BTB-zinc finger transcription factor Abrupt (Ab) as a target for *Drosophila let-7*. The 3′UTR of *abrupt* mRNA contains five *let-7* binding sites (Burgler and Macdonald, 2005), and ectopic *let-7* expression in wing disc cells lead to a precocious downregulation of Ab protein levels (Caygill and Johnston, 2008). Moreover, this study also showed that the NMJ phenotype observed in *let-7* mutants was suppressed by a partial loss of *abrupt* function, suggesting that this phenotype is due to Ab overexpression (Caygill and Johnston, 2008). However, *abrupt* activity does not seem to account for the cell-cycle defect or shortened lifespan of *let-7* mutants, so other important targets might be deregulated in these settings.

7.2. miRNAs regulating neurogenesis and neurodegeneration

A group of *D. melanogaster* miRNAs including *miR-8*, *miR-7*, *miR-9*, and *bantam* regulate different aspects of neuronal function. *miR-8* is a highly conserved miRNA which is expressed as a single copy in flies. Mammals have five paralogues of *miR-8* distributed in two genomic clusters (*miR-200b*, *miR-200a*, *miR-429* clustered on chromosome 1 and *miR-200c* and *miR-141* on chromosome 12). The functional analysis of *miR-8* locus was made feasible due to the generation of its loss-of-function mutants. The *Drosophila miR-8* null mutants displayed reduced survival, morphological defects in legs and wings, and progressive neurodegeneration (Karres et al., 2007). A combination of computational approaches and expression profiling identified *atrophin* (CG6964) as one of the conserved target of *miR-8*. Potential binding sites for *miR-200b* and *miR-429* were also found in

the human homologue of Atrophin. Sensor constructs linked to the *atrophin* 3′UTR were responsive to *miR-8*, both in transgenic flies as well as in cell culture. Notably, overexpression of *atrophin* in *miR-8* cells resulted in a spectrum of defects resembling those in *miR-8* null mutants, while removal of a single copy of *atrophin* reduced the severity of the *miR-8* phenotype. These data indicated that misregulation of *atrophin* contributed substantially to the defects associated with loss of *miR-8* function (Karres et al., 2007).

A recently described role for *Drosophila miR-8* is the regulation of morphogenesis and function of NMJs (Loya et al., 2009). Third instar *miR-8* null mutants display phenotypes characterized by a decrease in the number of synaptic boutons, reduction in the presynaptic terminal arbors, and fewer axonal branches. Comparable NMJ phenotypes were observed in transgenic flies ubiquitously expressing *miR-8* sponges (*miR-8SP*). Moreover, tissue-specific knockdown using the *mir-8SP* confirmed that postsynaptic activity of *miR-8* was sufficient for NMJ function, as expression of *miR-8SP* with muscle-specific but not neuronal-specific GAL4 driver resulted in the same NMJ defects observed in *miR-8* null mutants as well as in flies containing the ubiquitously expressed *miR-8SP*. Several pieces of evidence suggest that *miR-8* functions in the NMJ to directly repress the expression of *enabled* (*ena*). For example, protein levels of Ena were significantly increased in *miR-8* mutants and Ena overexpression using a muscle-specific GAL4 driver elicited the same NMJ phenotype as *miR-8* null mutant flies. In addition, reducing levels of Ena protein either by using loss-of-function alleles or sequestering the endogenous protein in the mitochondria resulted in rescue of all *miR-8* NMJ phenotypes. This confirmed that *miR-8* promotes presynaptic growth by postsynaptically limiting Ena expression (Loya et al., 2009).

Expression analysis of individual miRNAs in eye imaginal discs of *Drosophila* larvae localized *miR-7* to photoreceptor cells at the time when neuronal differentiation is first detected (Li and Carthew, 2005). *miR-7* loss-of-function mutants were generated to examine the role of *miR-7* in photoreceptor development. This study revealed a reciprocal negative regulation between Yan protein and *miR-7* RNA in retinal cells. Yan is a neural repressor expressed in undifferentiated progenitor cells, where it inhibits *miR-7* transcription by direct interaction with *cis*-regulatory elements upstream of *miR-7* gene. However, the cells undergoing photoreceptor differentiation express *miR-7*, which inhibits Yan protein synthesis (Li and Carthew, 2005). The switch in expression patterns is triggered by the epidermal growth factor (EGF) signaling pathway. EGF receptor (EGFR) signaling activates rapid turnover of Yan protein via extracellular-signal-related kinase (ERK)-mediated phosphorylation (Rebay and Rubin, 1995). The degradation of Yan allows activation of its competitor Pnt-P1, which in turn activates *miR-7* transcription (Li and Carthew, 2005). In an extension of this work, it was reported that *miR-7* and Yan both

participate in coherent feedforward loops (Li et al., 2009a). Coherent feedforward loops direct their desired output by both direct and indirect means. In one of the feedforward loops, Yan affects *miR-7* transcription by both direct repressing *miR-7* as well as the expression of Phyl, which encodes the Ubiquitin E3 ligase subunit that degrades the Ttk69 repressor of *miR-7* transcription (Li and Carthew, 2005). A second feedforward loop involving *miR-7* is the activation of *miR-7* by Pnt-P1, which in turn represses Yan. Pnt-P1 also directly represses Yan (Rohrbaugh et al., 2002). The two coherent feedforward loops are interlocked together to generate a double-negative feedback loop between *miR-7* and Yan that determines the fate of photoreceptor cells (Li et al., 2009a).

miR-7 also participates in regulatory networks that determine the fates of proprioceptor organs and olfactory organs (Li et al., 2009a). *yan* and *E(spl)* are direct targets of *miR-7* and these factors are essential for the development of insect sensory organs (Lai et al., 2005; Li and Carthew, 2005; Stark et al., 2003). *miR-7* transcription is activated by Ato and causes the repression of *E(spl)*. Ato also directly activates transcription of *E(spl)* (Cave et al., 2005; Cooper et al., 2000; Nellesen et al., 1999). Thus, Ato participates in an incoherent feedforward loop where it directly activates and indirectly represses *E(spl)*, imparting an accelerated and transient pulse of downstream gene expression. In addition, *E(spl)* feedbacks on Ato to create a double-negative feedback loop that is interconnected with the feedforward loop. In this network, fluctuating peaks of Ato would result in transient pulses of *Ato* repression by *E(spl)*, but sustained increase of Ato would result in sustained repression of *E(spl)* by *miR-7* and stabilization of Ato (Li et al., 2009a).

Though *miR-7* is expressed in developing sensory organs, loss-of-function mutants of *miR-7* displayed little or no detectable impact on the development of these organs under uniform laboratory conditions. Moreover, *miR-7* mutants had only minor defects in the protein expression of their target Yan and displayed normal Ato expression levels (Li and Carthew, 2005). Thus, it was proposed that *miR-7* participates in feedforward and feedback loops to buffer developmental programs against variation and imparts robustness to diverse networks. Consistent with this hypothesis, *miR-7* mutant, subjected to environmental perturbation using temperature cycling, displayed a reduction in Ato signal and a strong increase in Yan expression in the eyes, suggesting that under these conditions the mutant failed to activate Ato and repress Yan. The mutants also displayed defects in patterning of sensory organs that could be correlated to reduced Ato levels (Li et al., 2009a).

Another miRNA involved in neural specification is *miR-9a*. Flies lacking *miR-9a* exhibit mild ectopic sensory organ phenotype in the larva as well as on the adult anterior wing margin and thorax. These phenotypes are consistent with an inability to repress neural identity (Li et al., 2006). These

flies also exhibited a completely penetrant loss of posterior wing margin. The zinc finger proneural gene *sens* was reported as a critical target of *miR-9a*. Notably, heterozygosity for *sens* rescued the SOP phenotype on the anterior wing margin (Li et al., 2006). Based on the sensory organ phenotype of *miR-9a*, it was proposed that this miRNA helps prevent inappropriate induction of SOPs by keeping *sens* levels below a threshold needed to induce the SOP fate (Cohen et al., 2006).

In a recent report, the *bantam* miRNA has been shown to regulate sensory neuron development in the peripheral nervous system (Parrish et al., 2009). Dendrites of morphologically distinct classes of dendrite arborization (da) sensory neurons undergo an early, rapid growth phase to enable dendrite growth to synchronize with overall body growth as dendrite establish receptive field coverage, and a subsequent scaling phase in which dendrites grow in proportion with underlying epithelial cells and the larva as a whole to maintain receptive field coverage (Parrish et al., 2009). *bantam* was identified as a regulator of the scaling phase in a screen for growth defective mutants. *bantam* mutants displayed proper receptive field coverage; however, dendrite growth failed to coincide with the growth of the surrounding tissue, leading to a profuse dendrite growth in the late-stage (Parrish et al., 2009). Notably, overexpression of *bantam* in the overlying epithelial cells and not in the sensory neurons was sufficient to rescue this phenotype. Moreover, microarray experiments revealed that *akt* expression and activity was increased in neurons but reduced in the epithelia of *bantam* mutants. Consistently, ectopic expression of Akt, or a constitutively active form of PI3 kinase (PI3K) caused a significant increase in dendritic coverage that was similar to *bantam* mutants.

Finally, antagonizing neuronal Akt activity was able to suppress the dendrite overgrowth in *bantam* mutants. Although the biologically relevant *bantam* target has not yet been identified, these studies indicate that *bantam* regulates signaling between epithelial cells and neurons essential for scaling growth of dendrites (Parrish et al., 2009).

7.3. A miRNA involved in muscle differentiation and maintenance

miR-1 is one of the most highly conserved miRNAs and is specifically expressed in the muscle tissue (Mansfield et al., 2004; Sokol and Ambros, 2005; Wienholds et al., 2005; Zhao et al., 2005). The worm and fly genomes possess a single copy of *miR-1* while the zebra fish, mouse, and human genomes contain two *miR-1* loci (Sokol and Ambros, 2005).

The fly *miR-1* is initially expressed throughout the presumptive mesoderm and continues to be expressed in the larval somatic, visceral, cardiac, and pharyngeal muscles. Muscle-specific transcription factors control *miR-1* expression in flies. Early expression of *miR-1* was shown to be exclusively

regulated by the promesodermal factor Twist, while its later expression is controlled by Mef2 through an evolutionary conserved binding site (Sokol and Ambros, 2005). Null mutants of *miR-1* (Kwon et al., 2005; Sokol and Ambros, 2005) and antisense-mediated depletion using 2′OM-ORNs (Leaman et al., 2005) have been used to define biological role of *miR-1* in flies. Loss of *miR-1* during *Drosophila* development resulted in a highly penetrant phenotype; few *miR-1* mutants died as first instars but none progressed beyond second instar (Sokol and Ambros, 2005). Newly hatched *miR-1* larvae displayed normal muscle function and architecture, suggesting that *miR-1* does not participate in muscle patterning or differentiation. However, feeding triggered paralysis, growth arrest, disruption of body wall muscle, and death in second instar. Notably, muscle-specific expression of *miR-1* completely rescued all mutant phenotypes, indicating that the phenotypes were specific to *miR-1* and that *miR-1* function was required exclusively in the mesodermal cells. Thus, *miR-1* was shown to play a key role in maintaining muscle integrity during the dramatic, postmitotic growth in muscle mass that occurs during larval growth (Sokol and Ambros, 2005). Kwon et al., however, reported mesodermal patterning defects in some *miR-1* embryos, but the variability in phenotype between the two studies was attributed to a difference in genetic backgrounds in which the knockouts were generated or the contribution of maternal *miR-1* transcripts. This study also identified the Notch ligand Delta as a target for *miR-1* (Kwon et al., 2005). Several other studies have reported genome wide computational predictions for candidate target mRNAs of *miR*-1 (Enright et al., 2003; Farh et al., 2005; Griffiths-Jones et al., 2006; Grun et al., 2005; Legendre et al., 2006; Lim et al., 2005; Rajewsky, 2006; Sood et al., 2006; Stark et al., 2005; Tomancak et al., 2002). A common theme that emerged from these studies was that mRNAs targeted by *miR-1* are predominantly expressed in nonmuscle tissues. These observations suggest that *miR-1* could downregulate mRNAs that are specific for nonmuscle tissues and whose products need to be stringently excluded from muscle tissue (Nguyen and Frasch, 2006; Sokol and Ambros, 2005).

7.4. miRNAs that regulate cell growth and proliferation

The *Drosophila* miRNAs *bantam*, *miR-278*, and *miR-8* have all been implicated in regulating growth and proliferation. *bantam* is an example of a *Drosophila* specific miRNA with multiple biological roles. The *bantam* locus was initially identified in a gain-of-function screen for genes that affect tissue growth (Hipfner et al., 2002; Raisin et al., 2003) and was later shown to encode an miRNA involved in cell growth and apoptosis (Brennecke et al., 2003). The complete elimination of *bantam* results in lethality at early pupal stage, whereas flies homozygous for hypomorphic alleles of *bantam* survive to adulthood but are small and display female fertility defects.

Conversely, ectopic expression of *bantam* promotes growth of various tissues such as eyes and wings, due to an increase in the number of cells, with no increase in cell size. The mRNA targets through which *bantam* regulates growth are still unknown. However, there is evidence that *bantam* expression is regulated by pathways that promote cell growth. *bantam* was shown to interact genetically with the tumor suppressor *expanded* by its ability to modify the small blistered eye phenotype caused due to ectopic expression of *expanded* (Hipfner et al., 2002). Subsequently, *expanded* was shown to be a crucial component of the Salvador/Warts/Hippo (SWH) pathway, a key signaling pathway that controls tissue size in *Drosophila* (Cho et al., 2006; Hamaratoglu et al., 2006; Silva et al., 2006). Hippo signaling restricts tissue size by promoting apoptosis and cell-cycle arrest, and animals carrying clones of cells mutant for *hippo* develop severely overgrown structures. The Hippo pathway modulates gene expression through phosphorylation of the transcriptional coactivator Yorkie. *bantam* was shown to be a downstream target of Yorkie (Huang et al., 2005; Nolo et al., 2006; Thompson and Cohen, 2006). *hippo* mutant cells had elevated levels of *bantam* and *bantam* was shown to be required for Yorkie-driven outgrowth. Additionally, overexpression of *bantam* rescued the growth defects of *yorkie* mutant cells (Nolo et al., 2006; Thompson and Cohen, 2006). *bantam* has also been shown to be a target of the SWH pathway operating in progenitor cells of the eye imaginal disc where Yorkie functions in conjunction with other tissue-specific transcription factors (Peng et al., 2009). Thus, *bantam* forms a crucial component of the growth signaling pathways operating in diverse cellular as well as developmental contexts.

miR-278 is another miRNA that promotes growth and inhibits apoptosis, and has also been shown to play a role in controlling energy homeostasis in *Drosophila*. The insulin signal transduction pathway closely links the mechanisms that control energy homeostasis and tissue growth during development. *miR-278* locus was initially identified in a gain-of-function screen for genes that affect growth during *Drosophila* development (Teleman et al., 2006). Loss-of-function mutants of *miR-278* were generated by homozygous recombination and these displayed metabolic defects. *miR-278* mutants have elevated insulin levels and are correspondingly lean. However, in spite of elevated insulin levels, *miR-278* mutants have increased circulating levels of sugar mobilized from adipose-tissue glycogen stores, suggesting that the mutants are insulin-resistant (Teleman et al., 2006). *miR-278* was shown to act through regulation of its target mRNA *expanded*. Interestingly, *expanded* loss-of-function mutants cause tissue overgrowth (Blaumueller and Mlodzik, 2000), consistent with the expected effects of target repression by *miR-278*. Moreover, expanded mRNA levels increased several fold in *miR-278* mutant tissue and overexpression of *expanded* under *miR-278 GAL4* control in wild-type flies was sufficient to cause elevated expression of *Insulin-like peptide* (*ilp*) genes and to produce the lean phenotype. These data were consistent with

the idea that misregulation of *expanded* contributed to the lean phenotype of *miR-278* mutants (Teleman et al., 2006).

In a screen for miRNAs that affect cell proliferation, human *miR-200* family of miRNAs were shown to promote cell growth when transfected into several human cell lines (Park et al., 2009). Furthermore, increased levels of *miR-200* family members in certain cancers like ovarian cancer correlated with their role in promoting cell growth (Nam et al., 2008). *D. melanogaster* provided an ideal genetic system to decipher the role of *miR-200/miR-8* in cell proliferation as *miR-8* was expressed from a single locus (Hyun et al., 2009). Phenotypic analysis of *miR-8* null flies, $miR\text{-}8^{\Delta 2}$ (Karres et al., 2007), revealed a significant growth defect at larval stages, slightly delayed adult eclosion, and a smaller size and mass of adults which was a result of a reduced cell number and not cell size. Since *Drosophila* fat body is an important organ in the control of metabolism and growth (Delanoue et al., 2010; Edgar, 2006), the authors reexamined the expression of *miR*-8 in larvae. In addition to the previously observed expression pattern, *miR-8* was found abundantly expressed in the fat body. Thus fat body-specific expression of *miR-8* was sufficient to rescue the small body phenotype of *mir-8* null mutants, suggesting that *miR-8* in the fat body was important for systemic growth (Hyun et al., 2009). Since *miR-8* is also expressed in the central nervous system (Karres et al., 2007), the authors also examined whether neuronal expression of *miR-8* could rescue the growth defect of the null mutant. This genetic manipulation did not rescue the dwarf phenotype, demonstrating that the fat-body specific functions and the neuronal functions of *miR-8* are distinct. To identify biologically relevant targets of *miR-8* responsible for body size control, lists of *Drosophila miR-8* and the human *miR-200* family targets predicted using multiple computational methods were compared to identify homologous gene pairs. Out of 15 orthologous pairs that were either tumor suppressors or negative regulators of cell proliferation in at least one species, seven were found responsive to their cognate miRNAs in their respective cell culture luciferase reporter assays. To assess the physiological relevance, each of these candidates was specifically knocked down in the fat body by RNAi and examined for rescue of the dwarf phenotype. The most dramatic rescue of *miR-8* null flies was observed by the depletion of *Drosophila u-shaped* (*ush*). Importantly, *ush* knockdown did not increase the body weight of wildtype flies, indicating that the rescue was not due to an additive effect of *ush* knockdown and *miR-8* mutation. Consistent with these RNAi experiments, hypomorphic ush^{1513} (Cubadda et al., 1997) heterozygotes had larger adult bodies than control flies and *ush* RNA and protein levels were elevated in *miR-8* flies. Further analysis showed that insulin signaling was defective in the fat body of *miR-8* null flies, and that Ush and its ortholog FOG2 regulate the insulin signaling pathway by directly interacting with the regulatory subunit of PI3K (Hyun et al., 2009).

7.5. miRNAs that regulate apoptosis

The proapoptotic gene *hid* is a bona fide target of *bantam*. Bioinformatic searches identified five potential sites in the 3′UTR of *hid*. A sensor construct consisting of tubulin promoter-driven GFP linked to *hid* 3′UTR was responsive to bantam. Moreover, overexpression of *bantam* reduced the GFP signal from the sensor transgene and suppressed experimentally induced apoptosis caused by overexpression of *hid* (Brennecke et al., 2003). A recent report has addressed the significance of this posttranscriptional regulation under physiological conditions. In addition to the previously described growth defects (Brennecke et al., 2003), *bantam* mutants exhibit ectopic apoptosis, and animals homozygous for hypomorphic alleles of *bantam* show significantly increased levels of apoptosis in response to ionizing radiation (IR; Jaklevic et al., 2008). Moreover, *bantam* activity is strongly increased after exposure to IR, as measured by a decrease in *bantam* sensor fluorescence in the imaginal discs and brains of irradiated third instar larvae. However, these changes did not appear to correspond to significant changes in the levels of mature miRNA levels. The change in *bantam* sensor was dependent on the transcriptional activator p53, a known mediator of IR response, since no change in *bantam* sensor expression was observed in irradiated wing imaginal discs of *p53* mutant larvae. Since irradiated *p53* mutants display reduced and delayed apoptosis, the loss of *bantam* sensor activity may be attributed to a defect in induction of apoptosis. Consistent with this view, overexpression of a viral caspase inhibitor p35 that blocked radiation-induced apoptosis prevented the down-regulation of the *bantam* sensor, suggesting that cell death and not DNA damage is required for optimal activation of *bantam* after irradiation (Jaklevic et al., 2008). In another recent study, *bantam* has been shown to prevent Retinoblastoma family protein (Rbf) induced cell death in larval eye discs, demonstrating that bantam-mediated cell autonomous antiapoptotic activity is also present in the developing eye disc of flies (Tanaka-Matakatsu et al., 2009).

In a systematic loss-of-function analysis of miRNAs using antisense oligos, one of the largest family of miRNAs in *Drosophila* (*miR-2/6/11/13/308*) was shown to be required for suppressing embryonic apoptosis (Leaman et al., 2005). The activity of individual miRNAs was blocked *in vivo* by injecting antisense 2′OM-ORNs into embryos, and subsequent development was monitored for phenotypic abnormalities. Antisense 2′OM-ORNs irreversibly inhibit small RNA function *in vitro* and in intact cells in a sequence-specific manner, presumably by stoichiometric binding to RISCs containing the cognate miRNA targets (Hutvagner et al., 2004; Meister et al., 2004a). The phenotypes observed were caused by antisense and not sense or scrambled 2′OM-ORNs and were rescued by genomic overexpression of the cognate miRNAs, indicating the sequence-specificity

of the observed effects (Leaman et al., 2005). Amongst the 46 miRNAs expressed during the first half of *Drosophila* embryogenesis that were examined, 25 displayed either visible phenotypes.

For example, individual depletion of all the *miR-2* family members resulted in excessive cell death, but with a marked difference in the phenotypic strength. Embryos injected with 2′OM-ORNs for *miR-6* and *miR-2/13* failed to differentiate normal internal and external structures and fell apart on touch at the end of embryogenesis. Anticaspase-3 staining indicated an excessive and widespread apoptosis in *mir2/13* and *miR-6* depleted embryos. Interestingly, overexpression of the cognate miRNA together with the injection of the antisense 2′OM-ORN could partially rescue the observed phenotype (Leaman et al., 2005). Depletion of the other two *miR-2* family members, *miR-11* and *miR-308*, resulted in only moderate or mild increases in cell death, respectively. In *Drosophila*, the main control of caspase activity is mediated through proapoptotic factors Hid, Grim (Grm) Reaper (Rpr), and Sickle (Skl) that are transcriptionally activated in response to a range of natural and toxic events and promote caspase activation through inhibition of the caspase inhibitor *D*rosophila *i*nhibitor of *ap*optosis (Diap; Goyal et al., 2000; Yoo et al., 2002). Injection of *miR-2/13* and *miR-6* antisense 2′OM-ORNs into embryos mutant for the *hid*, *grim*, and *rpr* genes was unable to trigger apoptosis indicating that these miRNAs act through *hid*, *rpr*, and/or *grim*. Examination of expression levels of these proapoptotic genes in embryos depleted of *miR-2/13* or *miR-6* indicated no significant changes in mRNA expression levels. Hid protein levels, however, were dramatically higher in *miR-6*-depleted embryos and modestly increased in *miR2/13*-depleted embryos. This indicated a post-transcriptional regulation of the proapoptotic factor Hid by *miR-2/13* and *miR-6*. Furthermore, GFP-sensor assays (Brennecke et al., 2003) and transient dual-luciferase assays confirmed that the 3′UTRs of all four proapoptotic factors were subject to translational control by the *miR-2* family members, but each miRNA displayed a distinct interaction profile (Table 1.3). Notably, the interaction preferences correlated well with the observed differences in phenotype: *miR-6* had the most severe death phenotype and is the only family member to regulate *hid*, the factor with the broadest expression and the strongest proapoptotic effect. Rpr is under strong control by *miR-2/13* and *miR-6* and under modest control by *miR-11*, whereas Grim expression is affected by *miR-2/13*, *miR-11*, and *miR-308* and Sickle is regulated by all members of the *miR-2* family (Leaman et al., 2005). Interestingly, previous computational searches had predicted *miR-2* family member sites in the 3′UTRs of all of these pro-apoptotic factors (Enright et al., 2003; Stark et al., 2003). Moreover, these predicted miRNA–target interactions were validated by overexpressing *miR-2* along with sensor constructs in which GFP was linked to either *rpr*, *grm*, or *skl* 3′UTRs in wing discs (Stark et al., 2003). Together, these studies

demonstrate that the proapoptotic factors Hid, Grim, Rpr, and Skl are phenocritical mRNA targets of the *miR-2* family.

7.6. miRNAs regulating germ stem cell differentiation

miR-184 and *miR-7* are two *Drosophila* miRNAs that have been shown to play roles in germ stem cell differentiation. *miR-184* is a highly conserved miRNA that has been assigned multiple roles in *Drosophila* female germline development (Iovino et al., 2009). This miRNA was originally identified by expression cloning from the small RNA fraction of *Drosophila* embryos (Aravin et al., 2003). *miR-184* loss-of-function mutants appear normal and do not display any obvious defect in development and overall morphology. Among adult flies homozygous for a deletion of *miR-184*, male fertility is normal whereas females lay fewer eggs than wild type, and the eggs and embryos that are produced show severe abnormalities. The failure of egg production is the prevalent phenotype and this defect becomes progressively worse with time. However, its incomplete or delayed penetrance allowed a range of other distinct defects to be detected as well, indicating that *miR-184* function is required at multiple successive steps of oogenesis and early embryogenesis (Iovino et al., 2009). The earliest defect is observed in ovaries, where the daughters of GSCs are unable to differentiate. Differentiation occurs as the GSCs move away from the niche and express *bag-of-marbles* (*bam*) to become cystoblasts. Bam expression is suppressed and the stem cell character maintained by *decapentaplegic* (*dpp*) signaling that responds to ligand secreted from the somatic niche cells (Xie and Spradling, 1998). As the cystoblasts move away from the niche, they receive less Dpp signal, leading to the derepression of *bam* transcription and the initiation of differentiation (Casanueva and Ferguson, 2004). In loss-of-function mutants of *bam*, no cystoblast differentiation takes place and the germarium becomes filled with in differentiated GSC-like cells, referred to as the "bag of marbles" phenotype (McKearin and Spradling, 1990).

This phenotype can be mimicked by ectopic Dpp signaling activity. Interestingly, *miR-184* mutants displayed phenotypes similar to those observed for *bam* mutants, suggesting that target regulation by miR-184 is required for differentiation. Amongst the genes whose downregulation are known to promote cystoblast differentiation, only the 3′ UTR of the DPP receptor *saxophone* (*sax*) was predicted to contain an *miR-184* target site. Consistent with this, *miR-184* mutants displayed increased and mislocalized Sax protein expression as well as increased levels of downstream factors. Furthermore, removal of one maternal copy of the *sax* gene from *mir-184* mutants rescued the *bam*-like phenotype. These results indicated that *miR-184* regulates germline cell differentiation by tuning levels of the Sax receptor (Iovino et al., 2009).

Later in oogenesis, depletion of *miR-184* resulted in defects in the dorsoventral patterning of the eggshell. The key component known to regulate this dorsoventral patterning in the egg chamber is the transforming growth factor α (TGFα) homolog Gurken (Grk). The localization of Grk protein in the anteriodorsal corner of the oocyte is required to cause dorsalization of the nearby follicle cells (Nilson and Schupbach, 1999). *miR-184* was shown to affect Grk localization via K10, which is a nuclear protein required for the export of *grk* mRNA and the only known *grk* regulator that contains an *miR-184* binding site in the 3′UTR of its mRNA. Loss of *mir-184* leads to a premature increase in K10 expression, followed by a 50% reduction of its protein level in the oocyte nucleus at the time when it is required for *grk* mRNA transport. This partial loss of K10 protein at the critical stage is consistent with the observed mislocalization of Grk protein and thus explains the moderate dorsalization defect in egg shells of *miR-184* mutants (Iovino et al., 2009).

A significant proportion (85%) of the fertilized and morphologically normal eggs laid by 2 to 3-day-old *miR-184* females showed severe defects in anteroposterior patterning. In particular, the expression of pair rule genes was severely affected. The onset and development of the *fushi tarazu* (*ftz*) and *odd-skipped* (*odd*) pattern were delayed and some pattern elements, including *runt* expression, were missing. These phenotypes were similar to those observed for ectopic expression of the transcriptional repressor Ttk69, the repressor of *miR-7* expression mentioned above (Brown and Wu, 1993). Ttk69 is required for the proper timing and patterning of pair rule gene expression and has been shown to bind to the *ftz* promoter (Brown et al., 1991). Computational analysis predicted a conserved *miR-184* binding site in the *ttk69* 3′UTR, and this site was found to be functional using luciferase sensor constructs in S2 cell line. Furthermore, the depletion of maternally provided *miR-184* led to a 2.5-fold increase in the Ttk69 protein. Moreover, removal of one copy of the *ttk69* partially rescued the *miR-184* embryonic patterning defects, confirming that tuning of expression levels of Ttk69 by *miR-184* is required for the proper timing of pair rule gene expression in the blastoderm (Iovino et al., 2009).

Analysis of *maelstrom* (*mael*) mutants uncovered a role for *Drosophila miR-7* in the differentiation of GSCs (Findley et al., 2003; Pek et al., 2009). In male flies lacking *mael*, lack of differentiation leads to an accumulation of GSC-like cells in the testes and a progressive decline in fertility with age. This phenotype was similar to the *bam* phenotype, and Bam was shown to be reduced at the posttranscriptional level in *mael* testes (Pek et al., 2009). The *bam* 3′UTR was shown to contain binding sites for *miR-7*, and a *bam* sensor construct was sensitive to *miR-7* levels *in vivo*. *miR-7* levels were elevated in *mael* testes, concomitant with a reduction of a repressive histone mark at the *miR-7* locus. Thus, it was concluded that Mael regulates Bam via repression of *mir-7*. Consistent with this idea, a reduction in

miR-7 expression was able to rescue germline differentiation defects of *mael* mutants by alleviating Bam repression (Pek et al., 2009). Another recent study has implicated *miR-7* in the development of ovarian GSCs through the regulation of the cyclin-dependent kinase inhibitor *dacapo* (Yu et al., 2009).

7.7. miRNAs involved in wingless/Wnt signaling

The wingless/Wnt (wg) pathway is a highly conserved signaling system that directs cell specification, tissue patterning, and cell proliferation. *Drosophila miR-8* was identified as one of the candidates in a genetic screen for antagonists of the Wg signaling pathway (Kennell et al., 2008). *miR-8* was shown to regulate this pathway by directly targeting *wntless* (*wls*), a transmembrane protein required for secretion of Wg. A sensor construct with *wls* 3′UTR containing one *miR-8* predicted site was suppressed by *miR-8* in cell culture, and overexpression of *miR-8 in vivo* inhibited Wls protein expression and Wg secretion. This study also identified CG32767, a known positive regulator of Wg signaling (DasGupta et al., 2005), as a direct target of *miR-8*. Collectively, this data suggests that *miR-8* regulates the Wg pathway at multiple levels. Moreover, using a cell culture model of mouse mesenchymal stem cell differentiation, the authors demonstrated that this role of *miR-8* as a negative regulator of the Wg pathway is evolutionary conserved (Kennell et al., 2008).

miR-315 is another miRNA that was identified in a plasmid-based screen for miRNAs that affect the Wg pathway (Silver et al., 2007). This was a cell culture-based system employing a luciferase reporter controlled by a multimer of binding sites for T-cell-factor (TCF), which is the main sequences-specific transcription factor in the Wg pathway. The screen measured alterations in the luciferase expression by individual miRNAs in the presence or absence of exogenous Wg. *miR-315* was identified as a potent activator of luciferase. However, this activity was observed only in cells that were not stimulated with Wg, and was independent of the Wg receptor or coreceptor (Silver et al., 2007). *miR-315* was shown to activate Wg signaling in transgenic flies where misexpression of *miR-315* in wing imaginal discs was sufficient to respecify notum as wing and yielded four-winged flies, a phenotype that had been previously attributed to the misexpression of Wg (Ng et al., 1996). Axin and Notum were two critical negative regulators of the Wg pathway that were found to be targeted by *miR-315* via two conserved predicted sites in each of their 3′UTR. These targets were experimentally validated by coexpression of *miR-315* with *axin* or *notum* sensor constructs in S2 cells. Moreover, overexpression of *miR-315* inhibited ubiquitously expressed *axin* and *notum* GFP sensors in wing discs (Stark et al., 2003). Notably, ectopic *notum* or *axin* was able to partially rescue the mutant phenotypes induced by ectopic *miR-315*.

Conversely, gain-of-function of *miR-315* rendered flies extremely sensitive to heterozygosity for *axin* and *notum*, indicating that these are critical functional targets of *miR-315* in Wg signaling (Silver et al., 2007).

7.8. A miRNA in Notch signaling

miR-7 is an ancient and highly conserved miRNA (Lagos-Quintana et al., 2001; Prochnik et al., 2007). As described in Section 6 above, conserved regulatory motifs corresponding to the *miR-7* binding sites were identified in the 3′UTRs of many genes involved in the Notch signaling (Lai and Posakony, 1997, 1998; Leviten et al., 1997). These motifs (K boxes, Brd boxes, and GY boxes) were implicated in posttranscriptional regulation, but no function was assigned to the *miR-7* complementary GY boxes. However, based on the presence of the GY boxes, a later study predicted *miR-7* target sites in *hlhm3* and in *tom* (Lai, 2002). These predictions were extended to a much larger gene family and experimentally validated using transgenic flies coexpressing *miR-7* and sensor constructs (Lai et al., 2005; Stark et al., 2003). Moreover, ectopic expression of *miR-7* led to specific phenotypes that phenocopied a reduction in Notch signaling, indicating that these predicted genes were significant *in vivo* targets of *miR-7* (Lai and Posakony, 1998; Lai et al., 2005).

7.9. A miRNA that regulates nuclear receptor signaling

miR-14 is the first *Drosophila* miRNA to be studied functionally. It was initially identified in a screen for inhibitors of apoptotic cell death. Loss of *miR-14* enhanced Rpr-dependent cell death, whereas ectopic expression of *miR-14* suppressed cell death induced by expression of Rpr, Hid, Grim, and Dronc (Xu et al., 2003). *miR-14* loss-of-function mutants displayed semi-lethality, reduced lifespan, and increased sensitivity to stress and abnormal fat metabolism (Xu et al., 2003). In addition to these phenotypes, a later study reported a defect in anterior spiracle eversion during the larval-to-pupal transition of *miR-14* animals, suggesting a defect in metamorphosis (Varghese and Cohen, 2007). Potential *miR-14* binding sites were identified in the 3′UTRs of a number of apoptotic effectors including *drice*, *dcp-1*, *scythe*, *skpA*, and *grim*. Although none of these predicted binding sites were experimentally validated, elevated levels of Drice protein was detected in adult *miR-14* null flies and this increase in Drice protein was suppressed by two copies of *miR-14* (Xu et al., 2003).

In a later study, *miR-14* was shown to modulate a positive autoregulatory loop controlling steroid hormone signaling in flies (Varghese and Cohen, 2007). The steroid hormone Ecdysone and its receptor, EcR, play crucial roles during *Drosophila* development and metamorphosis.

Ecdysone signaling has been proposed to act in a positive autoregulatory loop to increase EcR levels and sensitize the animal to Ecdysone pulses (Koelle et al., 1991; Talbot et al., 1993). *miR-14* was shown to modulate this loop by targeting EcR, which in turn represses *miR-14* expression. Thus, *miR-14* participates in a mutual antagonistic feedback loop, wherein *miR-14* inhibits EcR positive autoregulation, and EcR reciprocally inhibits *miR-14* expression (Varghese and Cohen, 2007).

7.10. miRNAs that regulate Hox-cluster genes

Homeobox (*Hox*) genes are highly conserved homeodomain-containing transcription factors that specify the anterior–posterior axis and segment identity of organisms during embryonic development. The *hox* gene loci are clustered in a few genomic regions in insects and mammals. Some miRNAs encoded by the *hox* gene cluster include *miR-10* and *miR-iab-4/mir-196*, which derive from analogous positions in *hox* clusters in flies and vertebrates (Aravin et al., 2003; Lagos-Quintana et al., 2001; Yekta et al., 2004). These miRNAs have been shown to posttranscriptionally regulate Hox-protein-coding genes, thereby contributing to the extensive regulatory connections within *hox* clusters (Hornstein et al., 2005; Mansfield et al., 2004; Ronshaugen et al., 2005; Yekta et al., 2004). The *miR-iab-4* locus in flies encodes two miRNAs, *miR-iab-4-5p* and *miR-iab-4-3p*, that are generated from the two arms of the same hairpin (Aravin et al., 2003). *In vivo* analysis of *miR-iab-4-5p* showed that it inhibits the activity of one of its predicted targets Ultrabithorax (Ubx; Grun et al., 2005; Stark et al., 2003). Ubx protein is expressed throughout the haltere imaginal disc, where it imposes haltere identity by repressing the expression of genes that otherwise would direct wing development (Weatherbee et al., 1998). Moreover, ectopic expression of *miR-iab-4-5p* was shown to prevent accumulation of endogenous Ubx protein, and induced a classical homeotic mutant phenotype that transformed halteres to wings (Ronshaugen et al., 2005). A subsequent study showed that the *iab-4* locus in *Drosophila* produced a second miRNA from the opposite DNA strand (*miR-iab-4 AS*) that regulates neighboring *hox* genes via highly conserved sites (Stark et al., 2008). Computational analysis identified highly conserved binding sites for *miR-iab-4AS* in the 3′UTRs of several *Hox* genes that are proximal to the *iab-4* locus and are expressed in the neighboring more anterior embryonic segments, including *abdominal-A*, *ubx*, and *antennapedia* (Stark et al., 2007a). These targets were validated by luciferase reporters assays in S2 cells. Moreover, a clear homeotic transformation of haltere to wings was observed when *miR-iab-4AS* was misexpressed in the haltere imaginal disc (Stark et al., 2008). Notably, the haltere to wing transformation was much stronger than that reported for expression of *miR-iab-4* (Ronshaugen et al., 2005),

suggesting that the increased number of *miR-iab-4AS* target sites in *ubx* leads to stronger repression of *ubx* by *miR-iab-4AS* then *miR-iab-4*.

Recently, a combination of computational analysis as well as high-throughput sequencing efforts using small RNA libraries have predicted and confirmed the processing of miRNAs from more antisense transcripts (Ruby et al., 2007b; Stark et al., 2007a). These results suggest that sense/antisense miRNAs could be more generally employed in diverse contexts and in species as divergent as flies and mammals (Stark et al., 2008).

7.11. A miRNA that regulates circadian rhythm

Circadian clocks are crucial time-keeping mechanisms that allow organisms to anticipate daily changes in their environment. A recent study that addressed the roles of miRNAs in regulating circadian rhythms identified *bantam* as one of the miRNAs involved in the circadian clock (Kadener et al., 2009a). Ago1 immunoprecipitation from nervous system tissue followed by microarray analysis was used to identify mRNAs under miRNA control. This population of mRNAs included three of the core circadian mRNAs, *clock*(*clk*), *vrille*(*vri*), and *clockwork orange*(*cwo*). A parallel approach identified circadian miRNAs by using cell-specific inhibition of the miRNA biogenesis pathway followed by tiling arrays. These studies identified *bantam* along with nine other miRNAs as potential regulators of circadian rhythm (Kadener et al., 2009a). Interestingly, *bantam* had previously been predicted to regulate the *clk* 3′UTR (Grun et al., 2005). Cell culture experiments with *clk* sensor constructs confirmed that *bantam* regulates the translation via association with three binding sites located in the *clk* 3′UTR. Moreover, *clk* transgenes with mutations in the *bantam* binding sites were unable to provide full genetic rescue of the *clk* null mutant, thus confirming that *bantam* regulation of *clk* is important for *in vivo* circadian rhythmicity (Kadener et al., 2009a).

8. Concluding Remarks

A much clearer picture of the complex and diverse roles of miRNAs has emerged from the analysis of loss-of-function mutants in *Drosophila* miRNAs. These functional analyses suggest that the refinement of gene expression patterns and levels by miRNAs is critical in maintaining the fidelity and precision of cellular and developmental programs. Moreover, these studies also suggest that miRNAs employ multiple modes of action to regulate different target mRNAs. However, our current understanding of the endogenous functions of miRNAs is far from complete and additional

genetic studies are needed to understand whether miRNAs typically regulate only a handful of key targets or coordinately regulate many biologically relevant targets simultaneously.

One mode of miRNA activity that has not been explored enough is the role of miRNAs as reversible regulators in neurons. Local mRNA translation at dendritic spines is important for synaptic plasticity and long-term memory. In both *D. melanog*aster olfactory and mammalian hippocampal neurons, stimulation induces rapid proteolysis of the miRISC assembly factor Armitage, thus leading to local relief of miRNA-mediated repression of several proteins (e.g., CaMKII) involved in synaptic plasticity or memory formation (Ashraf et al., 2006; Banerjee et al., 2009). miRNA-mediated translational repression at synapses may allow for rapid activity-dependent changes in dendrites, which may be impossible to achieve by nuclear transcriptional regulation. Another key question that needs to be answered in this context is how specific miRNAs are transported to synapses.

Another area that has received only limited attention is the half-life and decay of miRNAs. Experimentation using RNA pol II inhibitors or depletion of miRNA processing enzymes have indicated that the half-lives of miRNAs may extend to many hours or even days (Krol et al., 2010a). However, several examples of accelerated or regulated miRNA turnover have been reported in other systems (Buck et al., 2010; Hwang et al., 2007; Krol et al., 2010a). Of particular interest is a study that reports that many miRNAs in neurons have very rapid turnover, and that this turnover is regulated by neuronal activity (Krol et al., 2010a). A study in *Drosophila* has indicated that a transient but profound turnover of miRNAs may occur in the developing eye during passage of the morphogenetic furrow (Lee et al., 2004b). Whether and how miRNA turnover and stability are regulated clearly represents an important issue for future investigation.

Another area of miRNA biology that have received only limited attention in the fruit fly model system is the posttranscriptional control of miRNAs. The identification of more interaction partners of individual miRNA precursors will further broaden the spectrum of control mechanisms. Future research in *Drosophila* will help answer some of these questions and continue to provide new insights into the regulation and functioning of miRNAs.

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CHAPTER TWO

New Insights into the Mechanism of Development of *Arabidopsis* Root Hairs and Trichomes

Rumi Tominaga-Wada,* Tetsuya Ishida,† *and* Takuji Wada‡

Contents

Abstract

Epidermis cell differentiation in *Arabidopsis thaliana* is a model system for understanding the mechanisms leading to the developmental end state of plant cells. Both root hairs and trichomes differentiate from epidermal cells and molecular genetic analyses using *Arabidopsis* mutants have demonstrated that the differentiation of root hairs and trichomes is regulated by similar molecular mechanisms. Molecular-genetic approaches have led to the identification of many genes that are involved in epidermal cell differentiation, most of which encode transcription factors that induce the expression of genes active in both root hair and trichome development. Control of cell growth after fate determination has also been studied using *Arabidopsis* mutants.

Key Words: Root hair, Trichome, Transcription factor, *Arabidopsis*, Cell differentiation.

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1. Introduction

The developmental specification and patterning of cell types is a crucial feature of multicellular organisms. Root hairs play a variety of roles in growth and metabolism, including anchorage, water absorption, and nutrient uptake. Some plant species have lost the ability to make root hairs, whereas in other species, every root epidermis cell forms a hair. The differentiation of epidermal cells in *Arabidopsis thaliana* has been used extensively as a relatively simple model for studying cell fate specification. Root epidermal cells are generated at the root apical meristem and differentiate into either of two cell types (hair cells or nonhair cells) in a cell position-dependent manner (Dolan et al., 1994) (Fig. 2.1A). Epidermal cells growing in contact with two flanking cortical cells differentiate into hair cells, whereas cells touching only one cortical cell develop into hairless cells (Fig. 2.1B). Wild-type *Arabidopsis* has eight hair cell files aligned longitudinally along the root (Dolan and Costa, 2001).

Trichomes differentiate from epidermal cells in the aerial parts of plants. These highly modified cells have a number of functions, including defense against herbivores, protection from UV irradiation, or reduction of transpiration (Myers and Bazely, 1991; Wagner et al., 2004). Trichomes can be unicellular or multicellular, glandular or nonglandular, depending on the plant species, suggesting that trichomes have multiple evolutionary origins (Glover and Martin, 2000; Ishida et al., 2008; Payne et al., 1999; Serna and Martin, 2006). Trichomes normally develop with regular spacing, which aids in their functionality. However, they are rarely formed adjacent to one

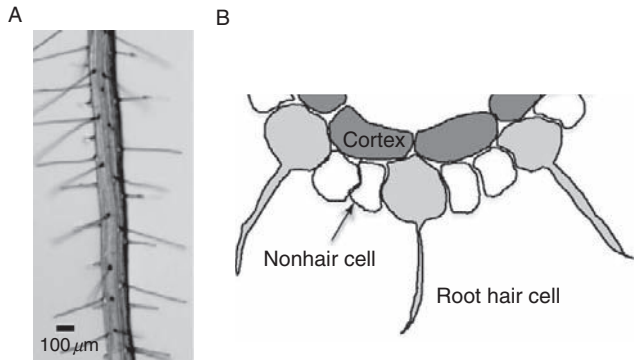

Figure 2.1 *Root hair phenotypes of Arabidopsis*. (A) Root hair pattern in a 5-day-old wild-type *Arabidopsis*. (B) Drawing of a cross-section of *Arabidopsis* root. Epidermal cells in contact with a single cortical cell are fated to become a nonhair cell. Epidermal cells in contact with two cortical cells differentiate into hair cells.

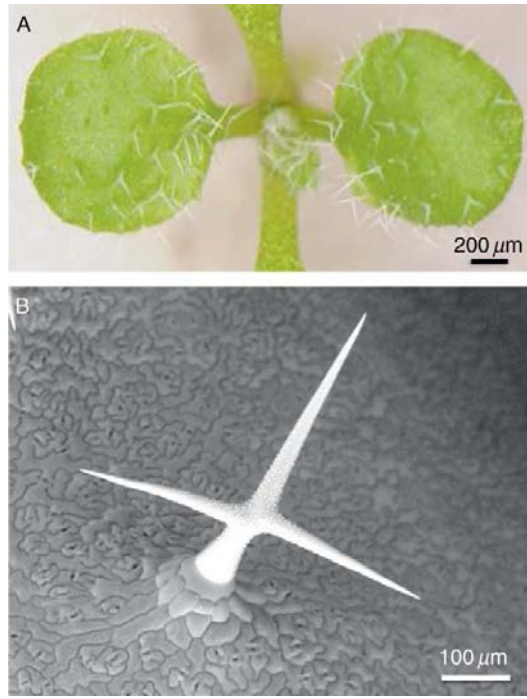

Figure 2.2 *Trichome phenotypes of Arabidopsis*. **(A) Trichome formation on a 5-day-old *Arabidopsis* first and second leaves. (B) Trichome phenotype of wild-type *Arabidopsis* leaf.**

another in wild-type *Arabidopsis*, suggesting that there is some developmental mechanism regulating trichome spacing (Hulskamp et al., 1994) (Fig. 2.2A). *Arabidopsis* trichomes have a characteristic three-branched phenotype (Fig. 2.2B), and several genes have been identified which play an important role in trichome cell development (Hulskamp et al., 1999).

2. Development of Root Hairs

2.1. Root hair initiation

2.1.1. Negative regulators of root hair cell fate

A number of genes involved in root hair cell fate determination have been identified. The *Arabidopsis* genes *GLABRA2* (*GL2*), *TRANSPARENT TESTA GLABRA1* (*TTG1*), and *WERWOLF* (*WER*) are involved in nonhair cell fate determination, as evidenced by the conversion of hairless cells to root hair cells in *ttg1*, *gl2*, and *wer* mutants (Galway et al., 1994;

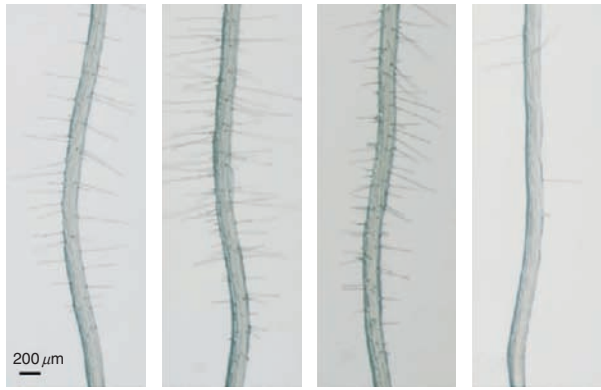

Figure 2.3 *Root hair pattern in 5-day-old Arabidopsis mutants*. **Hair and nonhair cell files are observed in wild-type root. A larger number of root hairs than wild type are formed, and all cell files produce root hairs in *glabra2* (*gl2*) and *werewolf* (*wer*) mutants. The *caprice* (*cpc*) mutant makes few root hairs.**

Lee and Schiefelbein, 1999; Masucci et al., 1996) (Fig. 2.3). *GL2* encodes a homeodomain-leucine zipper (HD-Zip) protein that is expressed preferentially in differentiating hairless cells (Di Cristina et al., 1996; Masucci et al., 1996; Rerie et al., 1994). *TTG1* had been thought to encode a basic helix-loop-helix (bHLH) protein, because the *ttg1* mutation is complemented by the ectopic expression of a maize gene, *R*, which encodes a protein with a bHLH domain (Galway et al., 1994; Lloyd et al., 1992). However, *TTG1* is now known to encode a protein with a WD40 motif (Walker et al., 1999). *WER* encodes an R2R3-type MYB protein that activates *GL2* expression (Lee and Schiefelbein, 1999). In addition to *GL2*, *TTG1*, and *WER*, *GLABRA 3* (*GL3*) and *ENHANCER OF GLABRA 3* (*EGL3*) also affect hairless cell fate determination in a redundant manner (Bernhardt et al., 2003). *GL3* and *EGL3* encode bHLH proteins (Bernhardt et al., 2003). There is a slight increase in the number of hair cells in both *gl3* and *egl3* single mutants, but in a *gl3 egl3* double mutant, most hairless cells are converted to root hair cells (Bernhardt et al., 2003). Like WER, these bHLH proteins also regulate *GL2* expression in nonhair cells (Bernhardt et al., 2003; Zhang et al., 2003). There are two other bHLH genes, *AtMYC1* (Urao et al., 1996) and *TRANSPARENT TESTA 8* (*TT8*) (Nesi et al., 2000), that are in the same subgroup as *GL3* and *EGL3* (Heim et al., 2003).

2.1.2. Positive regulators of root hair cell fate

In contrast to the negative regulation of root hair development provided by *GL2*, *TTG1*, *WER*, *GL3*, *EGL3*, *AtMYC1*, and *TT8*, specification of root hair forming cells is positively controlled by *CAPRICE* (*CPC*) (Wada et al.,

1997) (Fig. 2.3). The *cpc* mutant develops only a few root hairs (Wada et al., 2002). Although the typical plant MYB gene encodes an R2R3-type MYB region (Rosinski and Atchley, 1998), CPC encodes a small R3-type MYB of only 94 amino acids (Wada et al., 1997). Not long after isolation of *CPC*, *TRIPTYCHON* (*TRY*) was isolated as a *CPC*-homologous gene from a trichome-clustering mutant (Schellmann et al., 2002). More recently, *ENHANCER OF TRY AND CPC1 (ETC1)*, *ENHANCER OF TRY AND CPC2 (ETC2)*, and *ENHANCER OF TRY AND CPC3 (ETC3)/ CPC LIKE MYB3 (CPL3)* were isolated as *CPC*-homologs (Esch et al., 2004; Kirik et al., 2004a,b; Simon et al., 2007; Tominaga et al., 2008). The root hair phenotype of the *cpc-1* (WS background) (Wada et al., 1997) and *cpc-2* (Col-0 background) mutant lines is characterized by the formation of approximately one-fourth as many root hairs as the wild type (Tominaga et al., 2008), and the *etc3/cpl3* line has about 80% the number of root hairs as the wild type (Tominaga et al., 2008). The *cpc try* and *cpc etc1* double mutants have very few root hairs (Kirik et al., 2004b; Schellmann et al., 2002; Tominaga et al., 2008). The *cpc etc3/cpl3* double mutant has about 50% of the root hairs as the *cpc* single mutant (Tominaga et al., 2008). Plants transformed with a constitutive 35S::CPC construct grow ectopic root hairs (Wada et al., 1997), and 35S:: TRY, 35S::ETC1, 35S::ETC2, and 35S::ETC3/CPL3 also had more than the normal number of root hairs (Kirik et al., 2004a,b; Schellmann et al., 2002; Tominaga et al., 2008). Therefore, each of the CPC-like MYB homologs has a similar function for root hair formation when overexpressed under the control of the 35S promoter.

2.1.3. Transcriptional complex

Reporter gene expression studies indicate that WER, GL3, EGL3, and TTG1 act upstream of GL2 in the root epidermis transcriptional network (Bernhardt et al., 2003; Hung et al., 1998; Lee and Schiefelbein, 1999) (Fig. 2.4). Using the yeast two-hybrid system, GL3 and EGL3 were shown to interact with WER (Bernhardt et al., 2003) and with a WD40 protein TTG1 (Esch et al., 2003; Payne et al., 2000; Zhang et al., 2003), suggesting a transcriptional complex including MYB, bHLH, and WD40 protein. The transcription of GL2, which is thought to act farthest downstream in the root hair regulatory pathway (Bernhardt et al., 2005; Galway et al., 1994; Lee and Schiefelbein, 1999; Rerie et al., 1994; Wada et al., 1997), is controlled by a protein complex (Koshino-Kimura et al., 2005). WER directly binds to the *GL2* promoter region, indicating that a protein complex directly regulates GL2 transcription (Koshino-Kimura et al., 2005) (Fig. 2.4).

As presented in a trichome regulating model, TRY and GLABRA1 (GL1) compete for a GL3 protein-binding site to form different types of complexes that are involved in *Arabidopsis* trichome development (Marks and Esch, 2003). CPC protein has also been found to physically interact with both GL3 and EGL3 in yeast cells (Bernhardt et al., 2003), suggesting a

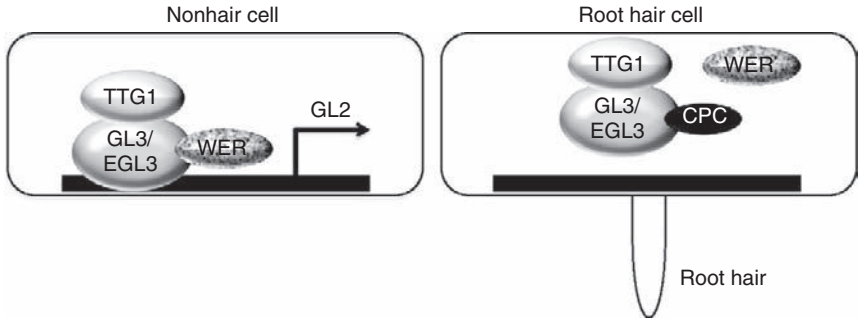

Figure 2.4 *Regulation model of root hair cell and nonhair cell fate by transcriptional complexes*. **The WEREWOLF (WER)–GLABRA3 (GL3)/ENHANCER OF GLABRA3 (EGL3)–TRANSPARENT TESTA GLABRA1 (TTG1) complex promotes *GL2* expression, which prevents root hair formation in nonhair cells. However, CPC protein competes with WER protein for binding to this transcriptional complex. The CPC–GL3/EGL3–TTG1 complex cannot promote *GL2* expression, resulting in root hair formation.**

competition model for CPC and WER (Bernhardt et al., 2003; Lee and Schiefelbein, 1999). CPC protein competes with WER protein for binding sites on GL3 or EGL3 (Tominaga et al., 2007). These results suggest that R3 Myb proteins inhibit root hairless cell differentiation by disturbing the formation of R2R3 Myb–bHLH–TTG1 complexes (Esch et al., 2003, 2004) (Fig. 2.4). An interesting point is that inhibitory effects differ among the R3 MYB proteins. Mutant phenotypes indicate that the effect of TRY is local, whereas that of CPC is more long ranging (Schellmann et al., 2002). TRY has a longer C terminus than CPC, which might be why the two proteins have different effects (Esch et al., 2004).

2.1.4. Cell-to-cell movement of transcriptional factors

Because the expression of CPC is required for developing root hair cells, it was expected that CPC would be expressed in the root hair cells, and more particularly at the root tip, because root epidermal cells elongate and begin to differentiate into hair cells after dividing from the epidermal initial cells located at the root meristem (Dolan et al., 1994; Galway et al., 1994). A GUS reporter gene under control of the CPC promoter indicated that the longitudinal pattern of CPC expression is consistent with the model predicting that CPC is involved in the development of hair cells (Wada et al., 2002). However, strong staining in transverse sections was observed in the nonhair cells (Wada et al., 2002). Unlike *GL2*, the expression pattern of *CPC* did not correlate with its site of action. *In situ* hybridization also showed that CPC is strongly expressed in nonhair cells (Wada et al., 2002), implying that CPC protein moves from nonhair cells to root hair cells (Fig. 2.5).

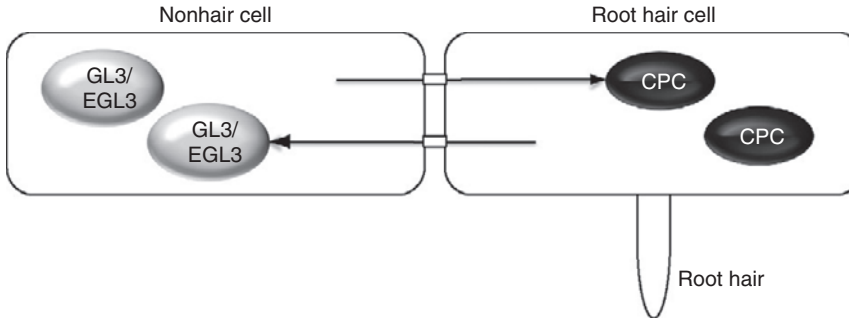

Figure 2.5 *Cell-to-cell movement of transcriptional factors*. **The CPC protein moves from nonhair cells to neighboring root hair cells, where it acts as a positive regulator of root hair formation. In contrast, the GL3/EGL3 protein moves from root hair cells to nonhair cells where it prevents root hair differentiation.**

The cell-to-cell movement of macromolecules is considered to be important in plant development (Zambryski, 2004), including the intercellular movement of plant transcription factors (Ruiz-Medrano et al., 2004). Several animal homeodomain proteins, such as Engrailed (En), Hoxa5, Hoxb4, Hoxc8, Emx1, Emx2, Otx2, and Pax6, can move from cell to cell in culture (Prochiantz and Joliot, 2003). Intercellular movement of En protein is driven by secretion and is regulated by phosphorylation of En itself (Joliot et al., 1998; Maizel et al., 1999, 2002). SHORT-ROOT (SHR), a member of the GRAS family transcription factors, moves from stele cells to the endodermis in *Arabidopsis* roots (Helariutta et al., 2000; Nakajima et al., 2001), but a SHR–GFP fusion protein did not move from phloem companion cells or epidermal cells when it was expressed under the control of tissue-specific promoters (Sena et al., 2004). Although SHR must be localized in the cytoplasm to move, the mere presence of SHR in the cytoplasm is not sufficient for movement (Gallagher et al., 2004). The maize homeobox protein KNOTTED1 (KN1) controls leaf formation and has been shown to move from inner cells to epidermal cells, possibly through plasmodesmata (Lucas et al., 1995). KN1 increases the size exclusion limit of plasmodesmata and induces the movement of the KN1 protein complex (Kragler et al., 2000; Lucas et al., 1995). KN1 protein can also move from the epidermal L1 layer to the inner cell layers in *Arabidopsis*. Thus, the movement of KN1 is regulated in a tissue-specific manner (Kim et al., 2002, 2003). However, not all intercellular regulatory proteins require export or import systems. For example, LEAFY (LFY), a floral identity transcription factor in *Arabidopsis*, moves by diffusion (Wu et al., 2003).

Using truncated versions of CPC fused to GFP, Kurata et al. (2005) identified a signal domain that is necessary and sufficient for CPC cell-to-cell movement in *Arabidopsis* root epidermal cells. Amino acid substitution

experiments indicated that W76 and M78 in the MYB domain are critical for targeted transport and that W76 is crucial for the nuclear accumulation of CPC protein (Kurata et al., 2005). In addition, CPC protein was able to move from root hair cells to nonhair cells, but could not exit from the stele, suggesting the involvement of tissue-specific regulatory factors in the intercellular movement of CPC (Kurata et al., 2005). Analyses with a secretion inhibitor, Brefeldin A, and with an *rhd3* mutant defective in the secretion process in root epidermis suggested that intercellular CPC movement is mediated through plasmodesmata (Kurata et al., 2005).

GL3 and *EGL3* are also thought to be cell-to-cell movement factors. Promoter-GUS and *in situ* hybridization analyses confirmed that *GL3* and *EGL3* are preferentially expressed in root hair cells (Bernhardt et al., 2005). GL3-YFP fusion protein accumulates in the nuclei of nonhair cells, indicating that GL3 (and presumably EGL3) moves from root hair cells to nonhair cells and regulates nonhair cell differentiation (Bernhardt et al., 2005) (Fig. 2.5). Mathematical simulations of root epidermal cell specification also support the mechanism of movement of CPC and GL3 proteins (Savage et al., 2008).

2.1.5. Positional cue

In *Arabidopsis* roots, root hair cells arise in a position-dependent manner and must respond developmentally in response to some signal, or positional cue, provided by their spatial context. Root hair cells lie over the junction of two cortical cells, whereas nonhair cells overlie one cortical cell only (Dolan et al., 1994; Galway et al., 1994) (Fig. 2.6). The positional relationship between cortical cells and epidermal cells was confirmed by the observation of small regions of two cell files (T-clones) that occasionally arise from a

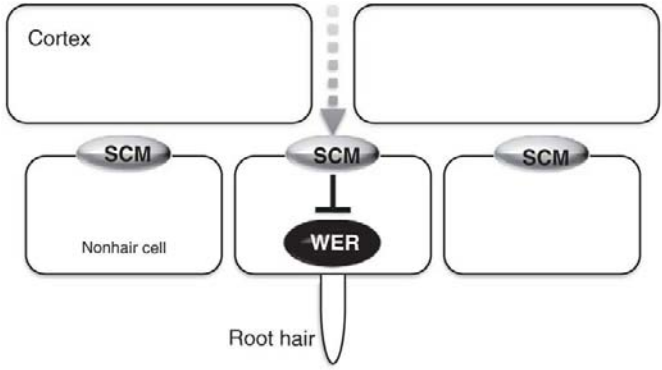

Figure 2.6 *Positional cue of root hair cell fate.* **A positional signal between two cortical cells activates SCRAMBLED (SCM) in epidermal cells located between two cortical cells. SCM represses *WER* expression in root hair cells, resulting in root hair formation.**

single hair cell file. One of the cell files stays in contact with only one cortical cell and does not form root hairs (Berger et al., 1998a).

The *SCRAMBLED* (*SCM*) gene was isolated as a candidate for mediating the positional cue (Kwak et al., 2005). *scm* mutants have disorganized *GL2::GUS*, *WER::GFP*, *CPC::GUS*, and *EGL3::GUS* backgrounds, suggesting that *SCM* acts upstream of these genes in cell fate determination (Kwak et al., 2005). *SCM* encodes a leucine-rich repeat receptor-like kinase (LRR-RLK), which is expressed throughout all developing tissue layers, including root epidermis (Kwak et al., 2005). Because *SCM* represses *WER*, the extracellular receptor domain of SCM on root hair cells may detect some unknown positional signal localized between cortical cells, and then an intracellular domain transmits its signal into the epidermal cell to establish the appropriate cell fate (i.e., hair cell or hairless cell) (Kwak and Schiefelbein, 2007) (Fig. 2.6). CPC moves into root hair cells and promotes the preferential accumulation of SCM in the root hair cell position, reinforcing root hair cell fate (Kwak and Schiefelbein, 2008). Interestingly, the intracellular kinase domain of SCM does not have kinase activity (Chevalier et al., 2005). The SCM ligand is a future target of cell fate determination research, as is the mechanism by which SCM transduces the correct signal to a complex of transcription factors.

Another leucine-rich receptor-like kinase, *BRASSINOSTERIOD INSENSITIVE 1* (*BRI1*) was also reported to convey positional information to root epidermal cells (Kuppusamy et al., 2009). BRI1 encodes brassinosteroid (BR), a class of steroid phytohormones known to promote growth in diverse plant species (Mussig, 2005). BR signaling is *via* a membrane-localized receptor (Li and Chory, 1997). Transcriptome analysis indicates that expression of *WER* is induced by BR treatment (Nemhauser et al., 2004). Although BRs are known to affect root elongation, Mussig et al. (2003) presented strong evidence that BRs are also required to maintain position-dependent fate specification in *Arabidopsis* roots (Kuppusamy et al., 2009).

2.1.6. Chromatin organization

Histone acetylation was reported to be involved in root hair or nonhair cell patterning of the root epidermis in *Arabidopsis* (Xu et al., 2005). Trichostatin A (TSA) is a specific inhibitor of histone deacetylase (HDAC) (Yoshida et al., 1990). TSA treatment of *Arabidopsis* seedlings alters the cellular pattern of the root epidermis to increase the number of root hair cells developing at ectopic positions (Xu et al., 2005). In *Arabidopsis* roots treated with TSA, histones H3 and H4 are hyperacetylated in *GL2*, *CPC*, and *WER* loci, resulting in altered expression levels and cell-specific expression of all three genes (Xu et al., 2005). However, both treatment of roots with TSA and mutation of the *HDA18* locus, which encodes HDAC, increase root hair density and cause the plant to form ectopic root hairs. This

inconsistency may indicate that an unknown mechanism mediates epidermal cell fate determination when histone acetylation occurs. *Arabidopsis* roots treated with TSA have much lower levels of HDAC activity, resulting in hyperacetylation of the core histones H3 and H4 in *GL2*, *CPC*, and *WER* loci. TSA also alters the expression levels and cell-specific expression of the patterning genes *CPC*, *GL2*, and *WER* (Xu et al., 2005). Analysis of HDAC-mutant cellular patterning further verified the participation of histone acetylation in cellular patterning and revealed that *HDA18* is a key component in the regulatory machinery of the *Arabidopsis* root epidermis (Xu et al., 2005). These results suggest that histone acetylation mediates positional cueing to direct expression of the patterning genes of *CPC*, *GL2*, and *WER* in root epidermal cells (Xu et al., 2005).

In roots, *GL2* is transcribed in nonhair cells but not in root hair cells in response to positional information (Berger et al., 1998a; Di Cristina et al., 1996; Hung et al., 1998; Masucci and Schiefelbein, 1996). Costa and Shaw (2006) demonstrated that the chromatin state around the *GL2* locus differs between nonhair cells and root hair cells. In a three-dimensional fluorescence *in situ* hybridization study on intact root epidermal tissue, a *GL2* bacterial artificial chromosome (BAC) probe hybridized with the genomic region of the *GL2* locus in nonhair cells but not in hair cells (Costa and Shaw, 2006). This suggests that the *GL2* locus of nonhair cells is in an open chromatin state and can be transcribed (Fig. 2.7). When an abnormal cell division made cell displacement from a root hair cell file into a nonhair cell file, it switched the cell's fate from root hair cell to nonhair cell (Berger et al., 1998b). The chromatin state at the *GL2* locus is reorganized in the

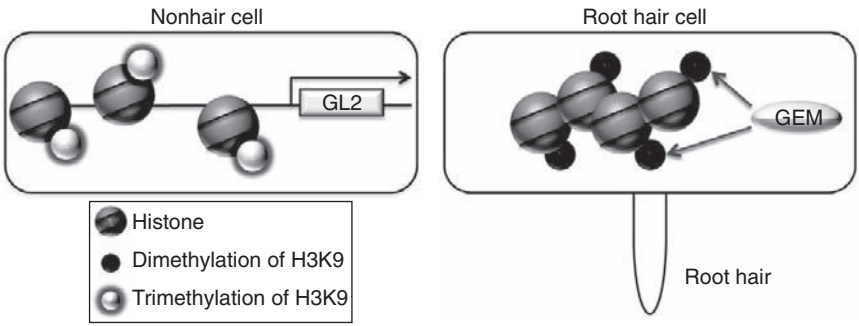

Figure 2.7 *Hypothetical chromatin structure around the GL2 locus*. **When histone H3 at the *GL2* locus is trimethylated, the *GL2* locus is open in nonhair cells. The transcription complex binds to the *GL2* promoter, induces *GL2* expression, and prevents root hair formation. In root hair cells, histone H3 at the *GL2* locus is dimethylated and the *GL2* locus is closed. Therefore, *GL2* is not expressed, resulting in root hair formation. GL2 EXPRESSION MODULATOR (GEM) mediates dimethylation of histone H3 in root hair cells.**

gap 1 (G1) phase of the cell cycle in response to positional information (Costa and Shaw, 2006). This ability to change chromatin organization may be involved in plasticity of plant cell fate determination. Therefore, alternative states of chromatin organization around the GL2 locus may be required to control position-dependent cell-type specification (Costa and Shaw, 2006).

Chromatin structure is altered by modification of core histones (Fuchs et al., 2006). Caro et al. (2007a) have identified a GL2-expression modulator, GEM, as an interactor with CDT1, a DNA replication protein. GEM also interacts with TTG1, a WD40-repeat protein involved in GL2-dependent cell fate decision, and modulates both cell division and GL2 expression (Caro et al., 2007a). In *Arabidopsis*, trimethylated K9 of histone H3 (H3K9me3) is a marker for the open chromatin state, and mono- and dimethylation with TTG1 suggest that GEM inhibits formation of R2R3 Myb–bHLH–TTG1 complexes. *GL2* and *CPC* promoters contain both H3K9me2 and H3K9me3 methylation markers. H3K9me3 increases and H3K9me2 decreases in these promoters in the *gem-1* background, but H3K9me3 decreases and H3K9me2 increases in *GEM* overexpressors. Acetylation of histone H3 in the *GL2* and *CPC* promoters increases in *gem-1* plants. These results suggest that *GEM* controls root epidermal cell fate through both interaction with TTG1, the regulator of *GL2* and *CPC* expression, and modification of histone H3 at the *GL2* and *CPC* loci (Caro et al., 2007a) (Fig. 2.7). GEM participates in the maintenance of the repressor histone H3K9 methylation status of root patterning genes, providing a link between cell division, fate, and differentiation during *Arabidopsis* root development (Caro et al., 2007b).

2.1.7. Phytohormone signaling

The phytohormones ethylene and auxin promote root hair formation. A number of studies have hinted at the involvement of ethylene in root epidermal development (Abeles et al., 1992). Root hair cell development in the root epidermis of *Arabidopsis* is positively regulated by ethylene (Tanimoto et al., 1995). Cormack (1935) induced a mass of root hairs on *Elodea* under conditions in which this aquatic plant normally formed no root hairs. Blocking perception or inhibiting ethylene synthesis by specific compounds reduces the number of root hairs, whereas treatment with the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) results in root hairs where hairless cells normally develop (ectopic root hairs) (Tanimoto et al., 1995).

Pharmacological and genetic studies have demonstrated that auxin also promotes root hair initiation (Masucci and Schiefelbein, 1994; Rahman et al., 2002). New knowledge about auxin signal–response pathways has come through the study of mutants in *Arabidopsis*. Mutations have been identified in a wide variety of auxin-response components, including auxin

transporters and transcriptional regulators. The auxin signal transduction pathway makes use of the Aux/IAA family of transcriptional regulators (Liscum and Reed, 2002). Stable dominant mutations in the *Aux/IAA* genes *AUXIN RESISTANT 3* (*AXR3*)/*IAA17*, *SOLITARYROOT* (*SLR*)/*IAA14*, or *AXR2/IAA7* reduce the number of root hairs compared with wild type, whereas a similar dominant mutation in *SHORT HYPOCOTYL 2* (*SHY2*)/*IAA3* shows early initiation of root hairs and prolonged hair elongation, suggesting that root hair formation stimulated by auxin is mediated by the *Aux/IAA* genes (Fukaki et al., 2002; Knox et al., 2003; Nagpal et al., 2000).

Ethylene and auxin also increase the rate of hair elongation (Pitts et al., 1998). To clarify the relationship between these hormones and cell fate determination factors *TTG1* and *GL2*, epistasis experiments and GUS reporter gene assays have been conducted. The nonhair cell fate-promoting genes *TTG1* and *GL2* are likely to have an effect during the early stages of ethylene and auxin activity, and *TTG1* and *GL2* negatively regulate the ethylene and auxin pathways. Studies of the developmental timing of these hormone effects indicate that ethylene and auxin pathways promote root hair formation after cell-type differentiation has been initiated. A genetic analysis of ethylene- and auxin-related mutants indicates that root hair formation is influenced by a network of hormone pathways, including through a partially redundant ethylene signaling pathway. A model has thus been proposed in which the patterning of root epidermal cells in *Arabidopsis* is regulated by the cell position-dependent action of the TTG1/GL2 pathway, and the ethylene and auxin hormone pathways promote root hair formation at a relatively late stage of differentiation (Masucci and Schiefelbein, 1996). These results clearly indicate that neither auxin nor ethylene regulates the *TTG1/GL2* pathway (Masucci and Schiefelbein, 1996). Mutations in *ROOT HAIR DEFECTIVE 6* (*RHD6*), a gene downstream of *GL2* that encodes a bHLH transcription factor, result in a reduction of root hair density and a shift in the site of root hair emergence (Masucci and Schiefelbein, 1994; Menand et al., 2007). These phenotypes are similar to those of the *axr2* mutant and the ethylene-resistant *ethylene response 1* (*etr1*) mutant and are rescued by application of auxin or ACC. In addition, wild-type roots treated with an inhibitor of ethylene biosynthesis phenocopy the *rhd6* mutant phenotype. These results suggest that *RHD6* promotes root hair formation through pathways that involve auxin and ethylene (Masucci and Schiefelbein, 1994).

A study in *Arabidopsis* focusing on ethylene signaling during root development highlights the importance of ethylene–auxin interactions during primary root cell elongation (Ruzicka et al., 2007; Stepanova et al., 2007; Swarup et al., 2007; Wu et al., 2007). Leblanc et al. (2008) report that N uptake and nitrate transporter *BnNrt2.1* transcript levels were markedly reduced in ACC-treated seedlings but were higher in AVG-treated

seedlings compared to the control in *Brassica napus*. Ethylene affects, in an integrated manner, the functioning of epidermal root hair cells as well as shoot epidermal stomatal guard cells (Beauclair et al., 2009). High ACC concentrations reduce exploratory root elongation and increase root hair length and number to allow water flux between the root and shoot (Beauclair et al., 2009).

2.2. Root hair growth

2.2.1. Ionic regulation

Root hair growth is a result of directed swelling of the cell by absorbed water, starting at the basal position of the hair cell (Fig. 2.8A). Polarity is established by auxin signaling and by the appropriate trafficking of the auxin influx carrier AUXIN RESISTANT 1 (AUX1) into the apical and basal ends of epidermal cells (Grebe et al., 2002; Masucci and Schiefelbein, 1996). In addition, the characteristic polar transport of auxin through nonhair cells sustains root hair development by accumulating at high concentrations at the root tip (Casimiro et al., 2001; Jones et al., 2009; Maes and Goossens, 2010; Sabatini et al., 1999; Sachs et al., 1991) (Fig. 2.8A). The application of auxin transport inhibitors (Maes and Goossens, 2010) changes root hair formation only at high concentrations and only after extended treatment

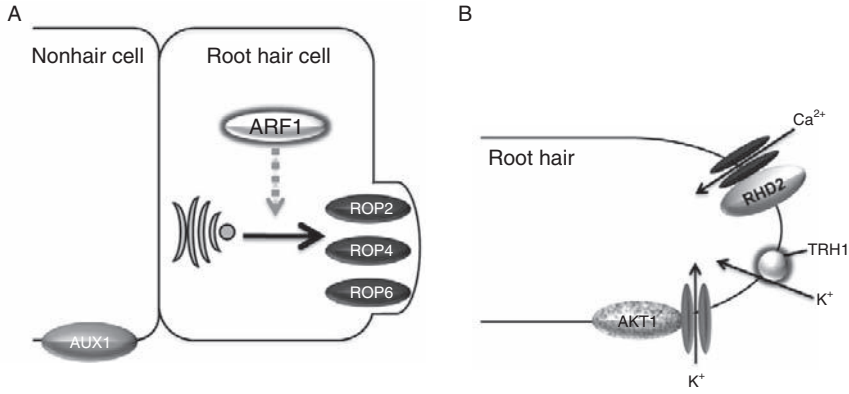

Figure 2.8 *Tip growth initiation of root hairs*. (A) Swelling occurs at the basal position of root hair cells. AUXIN RESISTANT 1 (AUX1) in nonhair cells contributes to the apical–basal hair cell polarity. Rho-related GTPase family members (ROP2, ROP4, and ROP6) localize to the initiation sight of root hair outgrowth. Their localization and activity are regulated by ADP ribosylation factor 1 (ARF1)-GTPase. (B) Ionic regulation of root hair tip growth. A calcium gradient is generated by calcium channel activity, which is activated by ROOT HAIR DEFECTIVE 2 (RHD2). Potassium acts to produce high turgor pressure in root hair tips. The activity of potassium channels is mediated by ARABIDOPSIS K TRANSPORTER 1 (AKT1). TINY ROOT HAIR 1 (TRH1), which encodes potassium transporter, also mediates potassium uptake.

times (Masucci and Schiefelbein, 1994; Sabatini et al., 1999). The presumptive auxin influx carrier *AUX1* (Bennett et al., 1996; Marchant et al., 1999) contributes to apical–basal hair cell polarity. The ROP GTPases ROP2, ROP4, and ROP6 mark the site where hair cell begins and are present throughout root hair outgrowth and development (Jones et al., 2002; Molendijk et al., 2001) (Fig. 2.8A). ROP GTPase localization and action are regulated by ADP ribosylation factor 1 (ARF1)-GTPase, a key component of vesicle transport, which is localized in Golgi and endocytic vesicles, and negatively regulated by Rho GTPase GDP dissociation inhibitor (RhoGDI1) (Yang, 2002), encoded by *SUPERCENTIPEDE1* (*SCN1*) (Carol et al., 2005; Xu and Scheres, 2005) (Fig. 2.5A). As an illustration of the importance of the correct positioning of ROP GTPases, in *scn1* mutants, ROP2 is mislocated, and supernumerary hair initiation sites are formed as a result (Carol et al., 2005). ROP2 regulates the activity of an NADPH oxidase encoded by *ROOT HAIR DEFECTIVE 2* (*RHD2*) that plays an important role in $Ca2^{+}$ uptake by root hair cells (Foreman et al., 2003; Jones et al., 2007; Wymer et al., 1997). $Ca2^{+}$ is indispensable for root hair tip growth (Schiefelbein, 1992). During root hair growth, the concentration of cytoplasmic $Ca2^{+}$ at the tip region can exceed 1 μM, which is considerably higher than the 100–200 nM found in the rest of the cell (Wymer et al., 1997). Blocking $Ca2^{+}$ channel activity causes the arrest of root hair growth, suggesting that $Ca2^{+}$ influx is required for higher concentrations at the tip region (Wymer et al., 1997). A set of mutants characterized by short root hairs and stunted roots, *rhd2*, are defective in $Ca2^{+}$ uptake and consequently cell expansion is compromised (Schiefelbein and Somerville, 1990; Wymer et al., 1997). RHD2/NADPH oxidase produces reactive oxygen species (ROS) (Torres et al., 1998), which accumulate at higher levels in wild type than in *rhd2*. An NADPH oxidase inhibitor, diphenylene iodonium (DPI), suppresses ROS production in wild type and phenocopies the *rhd2* mutant phenotype (Foreman et al., 2003). In addition, treatment with ROS causes the partial suppression of the *rhd2* phenotype and stimulates the activity of $Ca2^{+}$ channels, suggesting that ROS produced *via* an NADPH oxidase-dependent process activates $Ca2^{+}$ channels, which in turn regulates tip-focused calcium influx (Foreman et al., 2003) (Fig. 2.8B). Expression of the *Arabidopsis* gene *OXIDATIVE SIGNAL-INDUCIBLE 1 (OXI1)* encodes a serine/threonine kinase and is induced in response to ROS (Rentel et al., 2004). OXI1 kinase activity is itself also induced by ROS (Rentel et al., 2004). *oxi1* null mutants have shorter root hairs than wild type (Rentel et al., 2004), likely because OXI1 is required for full activation of the *Arabidopsis* mitogen-activated protein kinases (MAPKs) AtMPK3 and AtMPK6 when stimulated by treatment with ROS (Rentel et al., 2004). Because AtMPK6 is an ortholog of SIMK (stress-induced MAPK), which is involved in actin organization (described below), the regulatory target of AtMPK6 may be the cytoskeleton.

The role of potassium in root hair tip growth has also been investigated. *Arabidopsis* plants homozygous for a complete loss-of-function *tiny root hair 1* (*trh1*) mutation have short root hairs and frequently initiate more than one root hair per root hair cell (Rigas et al., 2001). *TRH1* encodes an *Arabidopsis* K^+ transporter protein. *TRH1* mediates K^+ transport in *Arabidopsis* roots and is responsible for specific K^+ translocation, which is essential for root hair elongation (Fig. 2.8B). *AKT1* encodes an inward-rectifying potassium channel (Gaymard et al., 1998; Sentenac et al., 1992) expressed in the root epidermis, endodermis, and cortex of *Arabidopsis* (Desbrosses et al., 2003) (Fig. 2.8B). Plants homozygous for the *akt1-1* mutation develop longer root hairs than wild type when grown in the absence of external potassium, but develop shorter hairs than wild type when grown in high concentrations of potassium (Desbrosses et al., 2003). In addition to genetic approach with transporter mutants *trh1* and *akt1*, experiments with a biochemical blocker of K^+ transport indicated that K^+ transporters are required for root hair tip growth and for the determination of initiation sites (Desbrosses et al., 2003; Rigas et al., 2001).

2.2.2. Cytoskeleton

Root hair growth starts at the base of root hair cells, and the arrangement of the cortical ER changes as the root hair grows (Ridge et al., 1999). Expanding root hairs, however, have extensive perforated sheets of cortical ER, which transform quite abruptly into a loose reticulum at the basipetal end of the elongation zone. The reticulum compacts in the root hair beginning at sites where root hairs are about to emerge. The compacted form is maintained throughout the hair until growth ceases, at which time the open reticulate ER reforms (Ridge et al., 1999). As the root hair bulge expands, actin cytoskeleton also accumulates and takes over maintenance of root hair tip growth (Baluska et al., 2000). Using GFP-talin to visualize actin structures, a dense mesh or cap can be seen in the dome region of the root tip, and less-dense actin filaments are observed at the basal region of the root hair (Baluska et al., 2000) (Fig. 2.9A). The tip-growing root hair apices are equipped with dense F-actin meshworks, which are assembled during outgrowth. Moreover, Profilin (PFN), along with its mRNA, accumulates within outgrowing bulges and growing hair tips (Baluska et al., 2000). Latrunculin B, an inhibitor of actin polymerization, arrests the tip growth of *Arabidopsis* and maize root hairs (Baluska et al., 2000; Bibikova et al., 1999). Depletion of F-actin by the G-actin sequestering agent Latrunculin B blocked root hair formation after the bulge formation stage (Baluska et al., 2000). This biochemical result was confirmed by genetic studies. Mutations in *ACTIN2* (*ACT2*), encoding one of eight actin isovariants in *Arabidopsis*, cause a hairless or short-hair phenotype with a swollen shape (Gilliland et al., 2002; Nishimura et al., 2003; Ringli et al., 2002). *ACT2* is essential for bulge site selection and tip growth during root hair development (Ringli et al., 2002). Both vegetative and reproductive actin isovariants

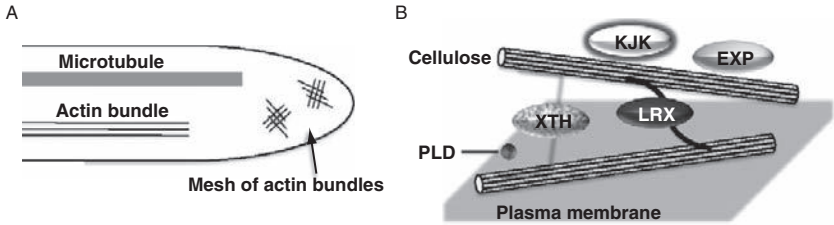

Figure 2.9 *Cytoskeleton and cell wall structure in root hairs*. (A) A mesh of actin bundles caps the tip region, and actin bundles are localized in the basal region. Microtubules (MTs) are axially organized and oriented in the direction of tip growth. (B) In the cell wall of swelling region, GLABRA2 (GL2)-regulating gene product, phospholipase D (PLD), hemicellulose reconstructing enzyme, xyloglucan endotransglycosylases (XTH), cellulose synthase-like protein, KOJAK (KJK), cell wall structural protein, leucine-rich repeat/extensin protein (LRX), and cell wall-loosening protein expansin (EXP) are localized.

complement the stunted root hair phenotype of the *act2-1* mutation (Gilliland et al., 2002). Direct observation of actin in the dominant-negative *act2-2D* mutants indicates that actin filament bundles in root epidermal cells of *act2-2D* are shorter than in wild type (Nishimura et al., 2003).

At the crossroads of signal transduction and the actin cytoskeleton, PFN is an important regulator of actin dynamics (Sohn and Goldschmidt-Clermont, 1994). PFN's role *in vivo* progressed from that of a simple actin-binding protein, which inhibits actin polymerization, to one which promotes actin polymerization under the appropriate circumstances (Sohn and Goldschmidt-Clermont, 1994). *PFNs* are encoded by a multigene family in *Arabidopsis*. Transgenic *Arabidopsis* plants harboring a *PFN1-GUS* reporter gene fusion showed expression in roots and root hairs and in a ring of cells at the elongating zone of the root tip (Ramachandran et al., 2000). *PFN1*-overexpressing lines have longer root hairs than wild type (Ramachandran et al., 2000). Actin-interacting protein 1 (AIP1) can cap F-actin and enhances the activity of actin depolymerizing factor (ADF) *in vitro* (Allwood et al., 2002; Ono, 2003). AIP1 enhances the depolymerization of F-actin in pollen grains (Allwood et al., 2002), and ADF enhances turnover of actin filaments by severing and depolymerizing filaments (Ono, 2003). Inducible expression of AIP1 RNAi in knockdown lines of *Arabidopsis* results in thicker actin bundles, abnormal actin organization, and shorter root hairs than wild type (Ketelaar et al., 2004).

Involvement of the stress-activated alfalfa mitogen-activated protein kinase (SIMK) has also been implicated in root hair tip growth regulation (Samaj et al., 2002). During root hair formation, SIMK is activated and redistributed from the nucleus into growing tips of root hairs possessing dense F-actin meshworks (Samaj et al., 2002). SIMK colocalized with actin filaments, suggesting that SIMK activity was affected by organization of the

actin cytoskeleton (Samaj et al., 2002). ACTIN-RELATED PROTEINS 2 and 3 form the subunits of the ARP2/3 complex, which is a regulator of actin organization (Mathur et al., 2003a). Two *Arabidopsis* genes *WURM* and *DISTORTED1* encode the plant ARP2 and ARP3 orthologs, respectively (Mathur et al., 2003a). Mutations in these genes result in sinuous root hairs (Mathur et al., 2003a). *Arabidopsis CROOKED* also encodes a subunit of the Arp2/3 complex, and the mutant *crooked* locus has wavy root hairs under rapid tip-growing conditions (Mathur et al., 2003b).

Microtubules (MTs) are also essential for the growth polarity of root hairs. Reorganization and *in vivo* dynamics of MTs during *Arabidopsis* root hair development have been described (Van Bruaene et al., 2004). In actively growing *Arabidopsis* root hairs, the nucleus moves into the bulge and locates at a fixed distance from the hair tip. Biochemical inhibitor studies show that MTs are not involved in the positioning of the nucleus, but that subapical actin between the nucleus and the root hair tip is required to maintain nuclear position with respect to the growing root hair tip (Baluska et al., 2000; Ketelaar et al., 2002). During *Arabidopsis* root hair development, cortical MTs, which form helical arrays in undifferentiated root epidermal cells, reorganize into a longitudinal position (Van Bruaene et al., 2004). The MTs reorient after apparent contact with other MTs and support a model for MT alignment based on repeated reorientation during dynamic MT growth (Van Bruaene et al., 2004). Endoplasmic MTs primarily associate with the nucleus and become longitudinally oriented along the growth axis of the root hair (Baluska et al., 2000; Bibikova et al., 1999; Van Bruaene et al., 2004) (Fig. 2.9A). Depolymerizing or stabilizing the MT cytoskeleton of apically growing root hairs with oryzalin and taxol, respectively, led to a loss of growth directionality and the formation of multiple, independent growth points in a single root hair (Bibikova et al., 1999). Furthermore, reduced expression of α-tubulin genes (*TUA6*) in *Arabidopsis* affects root hair development (Bao et al., 2001). Transgenic plants in which *TUA6* is suppressed produce ectopic root hairs (Bao et al., 2001). As described above, the development of root hair and nonhair cells is dependent on positional information supplied by spatial context within a cell file. This pattern of cell differentiation and the expression of molecular markers of cell fate are altered in the *ectopic root hair 3* (*erh3*) mutant root hair epidermis (Webb et al., 2002). *ECTOPIC ROOT HAIRS 3*, which encodes KATANIN-p60, severs MTs (Webb et al., 2002). These results suggest that MTs are required for not only establishment of root hair growth polarity but also cell fate determination.

2.2.3. Phospholipid signaling

Phospholipid-based signaling, including lipid kinase and phospholipase activities, is a novel second messenger system in plant cells (Meijer and Munnik, 2003). Phospholipase D (PLD) is reported to be crucial for root hair growth and development (Samaj et al., 2004). The *Arabidopsis phospholipase Dζ 1*

(*AtPLDζ 1*) gene was identified as a direct target of GL2 in root hair pattern formation (Ohashi et al., 2003). Inducible expression of *AtPLDζ 1* promoted ectopic root hair initiation (Ohashi et al., 2003). Ohashi et al. (2003) concluded that GL2 regulates root hair development by modulating phospholipid signaling (Ohashi et al., 2003). PLD produces the second messenger phosphatidic acid (PA), which is required for tobacco pollen tube growth (Potocky et al., 2003). The lipid signaling pathway in plants is downstream of PA and involves the *Arabidopsis* 3-phophoinositide-dependent kinase 1 (AtPDK1). AGC2-1, which is an AtPDK1-interacting protein kinase, is also activated by PA in an AtPDK1-dependent manner (Anthony et al., 2004). A *agc2-1* knockout mutation results in shorter root hair length than wild type, suggesting a role for AGC2-1 in root hair growth and development (Anthony et al., 2004). These studies indicate that suppressed *GL2* expression in root hair cells promotes *AtPLDζ 1* expression, induces PA accumulation, and thus results in the activation of AtPDK1 and AGC2-1. This regulatory cascade maintains the continuous growth of root hairs (Fig. 2.9B). *AGC2-1* encodes the same gene as *OXI1* (encoding a serine/threonine kinase mentioned above) (Samaj et al., 2004), indicating that PA and ROS cooperatively regulate root hair tip growth. Phosphatidylinositol (PtdIns) transfer proteins (PITPs) regulate signaling interfaces between lipid metabolism and membrane trafficking and also play a role in the growth of *Arabidopsis* root hairs (Bohme et al., 2004; Vincent et al., 2005). PITPs transfer PtdIns or phophatidylcholine (PtdCho) monomers (Allen-Baume et al., 2002; Cleves et al., 1991). Mutations in *CAN OF WORMS 1* (*COW1*)/*AtSFH1*, which encodes a phosphatidylinositol transfer protein, reduce root hair length compared with wild type because of a defect in a protein containing a Sec14p-nodulin domain (Bohme et al., 2004; Grierson et al., 1997; Vincent et al., 2005). COW1/AtSFH1 localizes in discrete plasma membrane domains and in the root hair tip cytoplasm (Bohme et al., 2004; Vincent et al., 2005). Vincent et al. (2005) further suggest that COW1/AtSFH1 regulates the polar phosphoinositide localization that involves membrane trafficking, Ca^{2+} signaling, and thus cytoskeleton organization in root hair growth.

2.2.4. Cell wall

The synthesis and rearrangement of cell wall materials are critical processes for root hair tip growth. The cell wall is an important determinant of plant cell shape. The primary cell wall is composed of a network of cellulose microfibrils and hemicelluloses, like xyloglucan polymers, which are embedded in a matrix of pectins (Carpita and Gibeaut, 1993). Root hairs are particularly suitable for studying cell expansion and morphogenesis because cell wall rearrangements occur during a defined developmental time period, they are easily observed independent of surrounding cells, and there are a number of mutant lines available that affect root hair growth (Tanimoto et al., 1995). Root hair cells locally "loosen" the cell wall and

undergo highly localized expansion at their outer surface to form a bulge in the cell wall (Leavitt, 1904). Once initiated, cell wall deposition is confined to the expanding tip of the growing hair, leading to the elongated hair-like outgrowth (Schnepf, 1986).

Xyloglucan endotransglycosylases (XTHs) are enzymes that cleave or catalyze transfer of xyloglucan chains, which are cell wall polysaccharides (Nishitani and Tominaga, 1992; Thompson and Fry, 2001). Root hair initiation is primarily coupled to a highly localized increase in XTH action at the basal initiation site of hair cells, followed by general distribution over the surface of the growing root hair (Vissenberg et al., 2001). XTH action takes place at the site of future bulge formation, where the root hair cell locally loosens its cell wall structure (Vissenberg et al., 2001). This suggests an important role for XTH in the beginning of root hair tip growth (Fig. 2.9B).

An *Arabidopsis* mutant homozygous for the *KOJAK* (*KJK*) gene causes the root hair to rupture soon after initiation. *KJK* encodes a cellulose synthase-like (subfamily D) protein, AtCSLD3, which is preferentially expressed in root hair cells (Favery et al., 2001; Wang et al., 2001). *KJK* is the first member of this subfamily of proteins to be shown to have a function in cell growth. KJK is located on the endoplasmic reticulum, suggesting that it is required for the synthesis of a noncellulosic wall polysaccharide. Consistent with the *kjk* mutant phenotype, KJK is mainly expressed in root hair cells in *Arabidopsis* root epidermis (Favery et al., 2001). The *kjk* mutant phenotype and KJK expression pattern suggest the early involvement of KJK in biosynthesis of the polysaccharides required during root hair growth (Favery et al., 2001).

Other evidence supports the involvement of cell wall components in root hair development. The *root hair deficient 1* (*rhd1*) mutant possesses short root hairs and bulge (Schiefelbein and Somerville, 1990; Seifert et al., 2002). The *RHD1* gene product appears to be necessary for proper initiation of root hairs (Schiefelbein and Somerville, 1990). *RHD1* is one of the five widely expressed family genes that encode UDP-D-glucose 4-epimerase (UGE4). UGE4 is an enzyme which acts in the formation of UDP-D-galactose and may catalyze interconversion between UDP-D-glucose and UDP-D-galactose (Seifert et al., 2002). RHD1 is specifically required for the galactosylation of xyloglucan and arabinogalactan but is not involved either in D-galactose detoxification or in galactolipid biosynthesis (Seifert et al., 2002). Immunocytological analyses revealed that *rhd1* root epidermal cell walls lack arabinosylated (1→6)-β-D-galactan and galactosylated xyloglucan (Seifert et al., 2002), leading to the assumption that these galactose-related cell wall polymers are required for root hair tip growth. In addition, genetic studies suggest that ethylene and auxin together participate in the flux control of UDP-D-galactose into cell wall polymers (Seifert et al., 2004). Ethylene rescues the deficiency of galactose-containing xyloglucan and arabinosylated galactan cell wall polysaccharides in the *rhd1* mutant (Seifert et al., 2004).

Structural proteins also contribute to cell wall maintenance. There are many structural proteins in the plant cell wall, falling essentially into three main classes: proline-rich proteins (PRPs), glycine-rich proteins (GRPs), and hydroxyproline-rich glycoproteins (HRGPs), also called extensins (Cassab, 1998; Keller, 1993; Showalter, 1993). Extensins might be involved in plant development as regulators of cell wall expansion (Carpita and Gibeaut, 1993) or as chains connecting the cell wall with the plasma membrane (Knox, 1995). LRX1, a chimeric leucine-rich repeat/extensin protein, is specifically localized in the cell walls of root hair cells in *Arabidopsis* (Baumberger et al., 2001). *LRX1* is expressed in root hair cells and LRX1 is localized in root hair cell walls (Baumberger et al., 2001). *lrx1* mutants are characterized by aborted initiation, swelling, and/or branching root hairs (Baumberger et al., 2001). *LRX2*, a paralog of *LRX1*, is also expressed mainly in roots. *LRX1* and *LRX2* act synergistically in cell wall formation during root hair growth (Baumberger et al., 2003). Thus, LRX1 and LRX2 regulate tip growth of root hair cells *via* cell wall synthesis or assembly (Baumberger et al., 2001, 2003) (Fig. 2.9B).

Root hair growth starts within the basal region of root hair cells with a localized lowering of pH in the cell wall and is associated with localized expansion in the lateral root hair cell wall (Bibikova et al., 1998). Acidification starts with root hair initiation and continues until tip growth begins (Bibikova et al., 1998). Inhibition of cell wall acidification with buffers prevents root hair initiation, but root hair growth restarts when the cell wall is returned to an acidic pH (Bibikova et al., 1998).

Cell wall-loosening protein expansins (EXP) also accumulate within root hair bulges when tip growth is initiated (Baluska et al., 2000). Under partial low-pH cell wall conditions, expansins may become active (Baluska et al., 2000), which would allow cell wall extension under acidic conditions without hydrolytic breakage of cell wall structural components (Cosgrove, 2000; Lee et al., 2001; McQueen-Mason et al., 1992). Two EXP genes, *EXP7* and *EXP18*, are expressed specifically in *Arabidopsis* root hair cells (Cho and Cosgrove, 2002). These two genes are involved in root hair initiation and subsequent elongation (Cho and Cosgrove, 2002) (Fig. 2.9B).

3. Development of Trichomes

3.1. Trichome initiation and growth

3.1.1. Trichome initiation

The *Arabidopsis* trichome is a large single cell with three branches (Fig. 2.2), which plays a role in protecting the plant as a parrying barrier on the leaf surface (Glover and Martin, 2000). *Arabidopsis* trichomes normally develop singly and initiate in the epidermis of developing *Arabidopsis* leaf primordia

(Hulskamp et al., 1994; Larkin et al., 1996; Schwab et al., 2000). Trichome spatial distribution is not random, but spacing does not appear to be due to any pattern-generating mechanism (Glover and Martin, 2000). However, the *triptychon* (*try*) mutant develops trichomes in clusters which are not clonally related (Schnittger et al., 1999).

Trichome development in *Arabidopsis* is controlled by more than 40 genes (Hulskamp et al., 1999). Positive regulators of *Arabidopsis* trichome formation include the R2R3 type Myb transcription factors GLABRA1 (GL1) (Oppenheimer et al., 1991) and MYB23 (Kirik et al., 2005), the basic helix-loop-helix (bHLH) transcription factors GLABRA3 (GL3) and ENHANCER OF GLABRA3 (EGL3) (Payne et al., 2000; Zhang et al., 2003), WD repeat protein TRANSPARENT TESTA GLABRA1 (TTG1) (Galway et al., 1994; Walker et al., 1999), and the homeodomain protein GLABRA2 (GL2) (Masucci et al., 1996; Rerie et al., 1994). No trichome formation was observed in *gl1*, *gl2*, *gl3*, and *ttg1* mutant leaves (Fig. 2.10). The involvement of these genes provides a direct genetic link between root hair and trichome formation. Negative regulators include the R3-type MYB transcription factors CAPRICE (CPC) (Wada et al., 1997, 2002), TRY (Schellmann et al., 2002; Schnittger et al., 1999), ETC1 (Esch et al., 2004; Kirik et al., 2004a), ETC2 (Kirik et al., 2004b), and ETC3/CPL3 (Simon et al., 2007; Tominaga et al., 2008; Wang et al., 2008). A number of genetic studies have provided solid evidence for the connection between gene expression and trichome spatial determination. For example, *cpc try* double mutants produce trichome clusters (Fig. 2.10). Epidermal cells on the adaxal surface of *cpc try etc1 etc3* quadruple mutant leaves are entirely converted to trichomes (Tominaga et al., 2008) (Fig. 2.10). A ternary complex composed of R2R3-type MYB transcription factors GL1 or MYB23, bHLH transcription factors GL3 or EGL3, and WD repeat-containing protein TTG1 promote trichome development. The MYB–bHLH–WD40 complex activates the expression of downstream genes *GL2* and *TRY*, and GL2 directs the development of trichomes (Ishida et al., 2007; Schellmann et al., 2002; Szymanski et al., 1998; Zhao et al., 2008) (Fig. 2.11). TRY protein is thought to migrate to neighboring non-trichome cells, where it inhibits trichome initiation by disturbing the formation of GL1–GL3/EGL3–TTG1 complexes (Esch et al., 2003, 2004) (Fig. 2.11). The current trichome patterning model involves a local autonomous circuit of multiple transcription factors acting at leaf primodia (Bouyer et al., 2008; Pesch and Hulskamp, 2004; Yoshida et al., 2009).

3.1.2. Cell cycle regulation

Protodermal cells committed to a trichome fate exit the mitotic cycle and enter an endoreduplication cycle while the surrounding epidermal cells continue to divide (Hulskamp et al., 1994). During *Arabidopsis* leaf development, each trichome executes an average of four endoreduplication

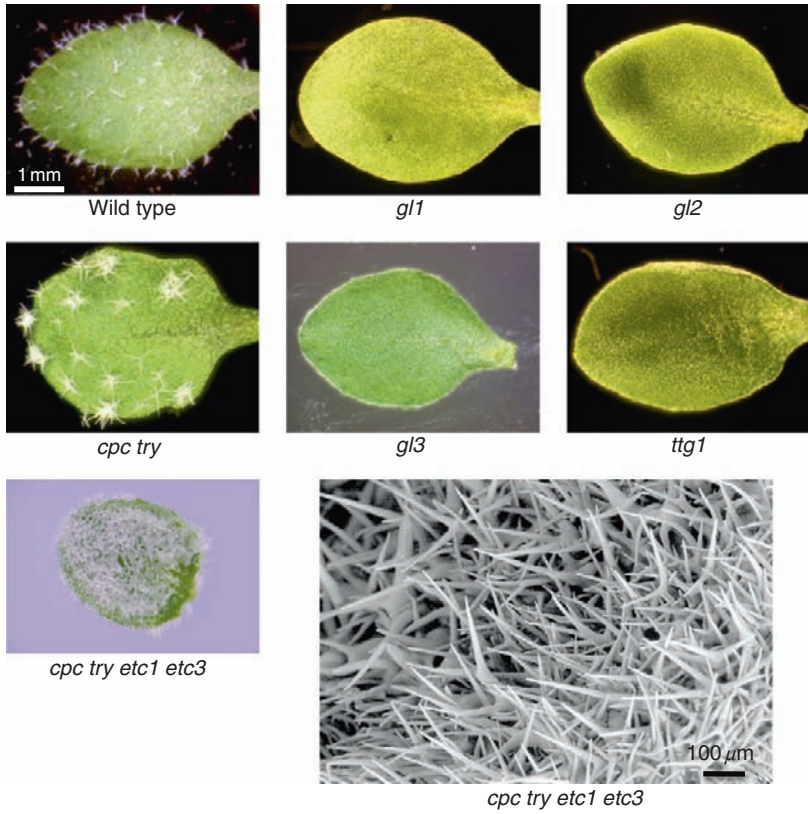

Figure 2.10 *Trichome phenotype on the 2-week-old third leaves in Arabidopsis mutants*. No trichome formation was observed in *gl1*, *gl2*, *gl3*, and *ttg1* mutant leaves. Trichome clusters were observed in *cpc try* double mutant leaves. Adaxal site of *cpc try etc1 etc3* quadruple mutant leaf was entirely covered by trichomes.

cycles, achieving an average nuclear DNA content of 32C (C equals haploid DNA content per nucleus) (Hulskamp et al., 1994; Schnittger and Hulskamp, 2002). Trichomes develop from protodermal precursor cells entering the first endoreduplication cycle, which is likely to be completed prior to outgrowth (Schnittger and Hulskamp, 2002). The second endoreduplication cycle starts with the first branching, and the third endoreduplication cycle begins with the second branching. The last endoreduplication cycle results in the mature three-branched *Arabidopsis* trichome (Schnittger and Hulskamp, 2002). Several characterized mutations affect either the change from mitosis to endoreduplication or the number of endoreduplication cycles. The *SIAMESE* (*SIM*) gene controls the endoreduplication cycle in *Arabidopsis* trichomes by suppressing mitotic cycling (Walker et al., 2000)

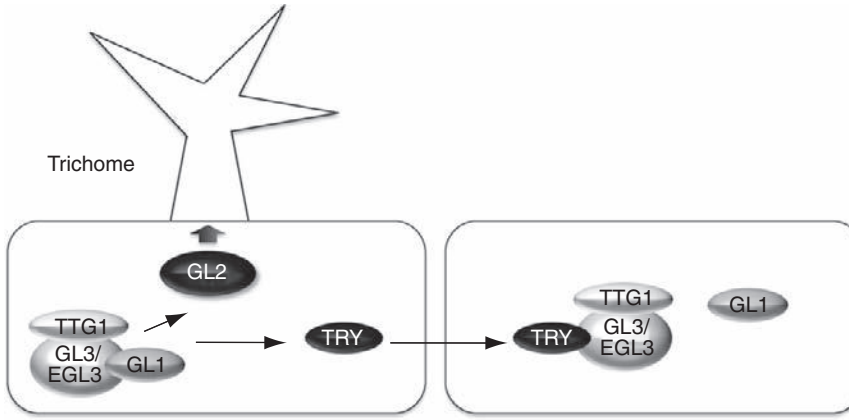

Figure 2.11 *Regulatory model of trichome formation on Arabidopsis leaf epidermis*. **The GLABRA1 (GL1)–GLABRA3 (GL3)/ENHANCER OF GLABRA3 (EGL3)–TRANSPARENT TESTA GLABRA1 (TTG1) complex activates *GLABRA2* (*GL2*) and *TRIPTYCHON* (*TRY*) expression. GL2 induces trichome differentiation. TRY protein moves to neighboring cells and competes with GL1 for the binding site of the complex. TRY–GL3/EGL3–TTG1 complex promotes neither GL2 expression nor trichome induction.**

(Fig. 2.12). A recessive mutation in the *sim* locus results in multicellular trichomes, but their gross morphology is normal (Walker et al., 2000). The *SIM* gene encodes a nuclear protein containing a cyclin-binding motif and motif similar to one found in INHIBITOR/INTERACTOR OF CYCLIN-DEPENDENT KINASE/KIP-RELATED PROTEINs (ICK/KRP), which are inhibitors of cyclin-dependent kinases (CDKs), which interacts with D-type cyclins (CYCDs) and CDKA (Churchman et al., 2006). CYCD–CDKA complexes are usually associated with control of the gap1/synthesis (G1/S) checkpoint. However, ectopic expression of CYCD in trichomes promotes not only DNA replication, but relaxation of the G1/S checkpoint also induces multicellular trichomes (Schnittger et al., 2002a). Expression of the CDK inhibitors ICK1/KRP1 can rescue the *sim* mutant phenotype, and variation in endoreplication levels correlates with *ICK1/RKP1* transcription levels (Weinl et al., 2005). These results suggest that SIM inhibits CDK function by interacting with CYCD–CDKA complexes (Fig. 2.12). Ectopic expression of B-type cyclin (CYCB) in trichomes using the *GL2* promoter induces mitotic divisions, resulting in multicellular trichomes (Schnittger et al., 2002b). CYCB regulates the gap2/mitosis (G2/M) transition. Therefore, its ectopic expression may induce a change from endoreduplication to mitosis. No *CYCB* expression is detected in wild-type *Arabidopsis*. RNA *in situ* hybridizations and *CYCB* promoter *GUS* reporter analyses suggest that *CYCB* is expressed in *sim* trichomes

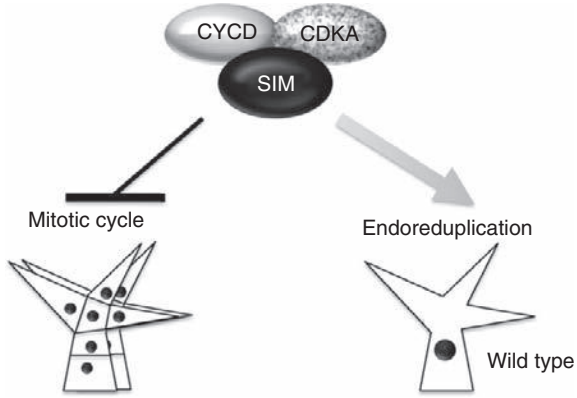

Figure 2.12 *Regulation of endoreduplication cell cycle in Arabidopsis trichomes*. **Endoreduplication cycling starts when the mitotic cycle stops. SIAMESE (SIM) interacts with D-type cyclin–CYCLIN-DEPENDENT KINASE A (CYCD–CDKA) complex and inhibits the mitotic cycle, resulting in a switch to endoreduplication cycles. Mitotic cycle progression induces multicellular trichomes in contrast to the unicellular trichomes produced by wild-type plants.**

(Schnittger et al., 2002b), suggesting that *SIM* inhibits *CYCB* expression (Schnittger et al., 2002b). The involvement of several different molecular pathways regulating endoreduplication cycles has been proposed based on DNA replication (Buhler et al., 1998; Hartung et al., 2002; Sugimoto-Shirasu et al., 2002), programmed cell death (Bowling et al., 1997; Schnittger et al., 2003), ubiquitination (Downes et al., 2003; El Refy et al., 2003), and GA signaling (Perazza et al., 1998, 1999) studies. Some mutants have a trichome phenotype with supernumerary or reduced numbers of branches because of perturbation of endoreduplication. The *GL1* gene is required for trichome initiation, but *GL1* overexpression reduces trichome number because of its effect on endoreduplication cycles (Szymanski and Marks, 1998). *TRY* and *GL3* also regulate not only trichome initiation but also endoreduplication cycles. The *try* mutant trichomes have an average DNA content of 64C with increased branches, suggesting that one additional endoreduplication cycle occurs (Szymanski and Marks, 1998). The *gl3-1* mutant has trichomes with an average DNA content of 16C and reduced branching, suggesting reduced endoreduplication cycles (Hulskamp et al., 1994). Two dwarf mutants, *root hairless2* (*rhl2*) and *hypocotyl6* (*hyp6*), have reduced endoreduplication cycles and a decreased number of trichome branches compared to wild type (Hartung et al., 2002; Sugimoto-Shirasu et al., 2002). The *Arabidopsis* genome has three homologs of the archaeal DNA topoisomerase VI (topoVI) subunit A, including *RHL2*, which encodes *AtSPO11-3*. It also contains the *HYP6* gene which encodes *AtTOP6B*, a homolog of subunit B. TopoVI from the

hyperthermophilic archaeon *Sulfolobus shibatae* is required to relax and decatenate chromosomes during DNA replication (Buhler et al., 1998). Cells of *rhl2* and *hyp6* mutants proceed through only the first two rounds of endoreduplication, achieving 8C (Hartung et al., 2002; Sugimoto-Shirasu et al., 2002). These results suggest that the TopoVI complex is required for *endocycles* successive endocycle rounds beyond the 8C level (Hartung et al., 2002; Sugimoto-Shirasu et al., 2002). The *CONSTITUTIVE PATHOGEN RESPONSE 5* (*CPR5*) gene mutant in *Arabidopsis* mimics programmed cell death in the absence of pathogens (Bowling et al., 1997). The *cpr5* mutant has trichomes with a reduced number of branches and small size compared with wild type, and these mutant alleles have been used to demonstrate that endoreduplication cycles stop after the second cycle is finished (Kirik et al., 2001). A correlation between cell cycle regulation and cell death has also been observed (Schnittger et al., 2003). Transgenic plants misexpressing CDK inhibitor *ICK1/KRP1* in trichomes under control of the *GL2* promoter possess trichomes with reduced endoreduplication cycles and small cell size and ultimately undergo cell death (Schnittger et al., 2003). These observations imply the presence of a pathway controlling both cell cycle regulation and programmed cell death. *KAKTUS* (*KAK*) gene mutants in *Arabidopsis* produce trichomes with a large nucleus and an average DNA content of 64C by increased endoreduplication cycles (Perazza et al., 1999). *KAK* encodes a protein with an amino acid sequence similar to the E6AP C terminus (HECT) ubiquitin ligase domain, indicating that ubiquitin-regulated protein degradation represses endoreduplication cycles in *Arabidopsis* trichomes (Downes et al., 2003; El Refy et al., 2003).

3.1.3. Regulation by phytohormones

Phytohormones also regulate trichome initiation and growth (Maes and Goossens, 2010). However, little is known about the effects of phytohormone signaling on the induction of trichomes in *Arabidopsis*. Gibberellin (GA) controls both initiation and morphogenesis of *Arabidopsis* trichomes (Chien and Sussex, 1996; Perazza et al., 1998). GA treatment increases trichome number on *Arabidopsis* leaves. Inhibitors of GA biosynthesis, including paclobutrazol and uniconazol, reduce trichome number and branching (Chien and Sussex, 1996; Perazza et al., 1998). The *SPINDLY* (*SPY*) gene encodes a repressor of GA signaling. SPY contains a tetratricopeptide domain (Jacobsen et al., 1996) and an O-linked *N*-acetylglucosamine transferase-related domain (Robertson et al., 1998). The *spindly-5* mutant produces more trichomes and branching than wild type, and these trichomes contain 64C DNA (Payne et al., 2000). Conversely, GA-deficient mutant *ga1-3* (Barendse et al., 1986; Perazza et al., 1998) has few trichomes, and the rare *ga1-3* trichomes have reduced branching (Perazza et al., 1998). GA controls trichome formation via upregulation of *GLABROUS1* (*GL1*) or its homologs, which in turn induces endoreduplication (Perazza et al., 1998). The glabrous

phenotype of *gl1-1* is epistatic to *spy-5*, which is demonstrated by the increased trichome phenotype in the *gl1-1 spy-5* double mutant, suggesting that the GA signaling pathway is upstream of GL1 activity (Perazza et al., 1998).

Jasmonic and salicylic acid are also involved in trichome formation in *Arabidopsis* (Traw and Bergelson, 2003). Jasmonic acid promotes trichome induction on leaves (Traw and Bergelson, 2003), whereas salicylic acid has a negative effect on induction and reduces the effect of jasmonic acid (Traw and Bergelson, 2003). These results suggest that negative crosstalk between jasmonic and salicylic acid take place during trichome development (Traw and Bergelson, 2003).

Cytokinins also increase trichome formation (Greenboim-Wainberg et al., 2005). The GA response inhibitor SPY enhances the cytokinin signaling pathway in *Arabidopsis*, suggesting crosstalk between cytokinin and GA (Greenboim-Wainberg et al., 2005). GA and cytokinin signaling pathways are integrated by the *Arabidopsis* transcription factors GLABROUS INFLORESCENCE STEMS (GIS), ZINC FINGER PROTEIN 8 (ZFP8), and GLABROUS INFLORESCENCE STEMS 2 (GIS2) in the regulation of trichome cell fate, and they control *GL1* gene expression (Gan et al., 2006, 2007). Three phytohormone classes, the GAs, jasmonates, and cytokinins, all promote *Arabidopsis* trichome formation but cause various morphogenic effects via species- and trichome-specific mechanisms (Maes and Goossens, 2010).

3.2. Trichome branching

Many genes that are involved in trichome endoreduplication cycles also regulate trichome branching. Some of these genes control MT organization (Mathur and Chua, 2000). During trichome branching, cortical MTs reorient from a transverse to a longitudinal direction with respect to the branch growth axis (Mathur and Chua, 2000). Treatment with the MT-stabilizing drug paclitaxel induces a change in growth directionality and branch initiation of trichomes in the *Arabidopsis* mutant *stichel* (*sti*), which possesses unbranched trichomes, but MT-disrupting drugs such as oryzalin and propyzamide does not (Mathur and Chua, 2000). Taxol also induces trichome branch points in the *Arabidopsis zwichel* (*zwi*) mutant, which has less branched trichomes (Mathur and Chua, 2000). These results suggest that trichome cell branching in *Arabidopsis* is mediated by the function of MTs, which reorient to new growth directions along with the trichome branch. Trichome branching is severely affected by genes involved in the formation of α/β-tubulin heterodimers. *Arabidopsis lefty1* and *lefty2* are dominant-negative mutants in α-tubulin 4 and α-tubulin 6, respectively, and trichome branching of both mutants was highly reduced (Abe et al., 2004). However, the expression of modified α-tubulins (TUA6 D251A/E254A) makes MTs stable and increases trichome branching (Abe and Hashimoto, 2005).

Mutations in *KIESEL* (*KIS*), *PORCINO* (*POR*), and *KATANIN-p60* genes reduce trichome branching compared with wild-type *Arabidopsis* (Burk et al., 2001; Kirik et al., 2002a,b). The *Arabidopsis* gene *KIS* encodes similar sequences to the tubulin-folding cofactor A (*TFC A*) gene and functions mainly as a buffer protecting the cell from an unbalanced α/β-tubulin ratio to maintain the balance between the α/β-tubulin monomers (Kirik et al., 2002a). *POR* encodes tubulin-folding cofactor C (*TFC C*) and also has an essential role in MT function *in vivo* (Kirik et al., 2002b). The *KATANIN* gene product severs MT proteins in an ATP-dependent manner and is required for reorientation of cortical MT arrays (Burk et al., 2001). These mutant analyses suggest that control of MT synthesis and MT severing is important for *Arabidopsis* trichome branching (Burk et al., 2001; Kirik et al., 2002a). As mentioned above, *zwi* mutants show a reduced number of branching phenotype compared with wild-type trichomes. *ZWI* encodes a member of the kinesin superfamily of MT motor proteins (Oppenheimer et al., 1997). ZWI binds to MTs and moves directionally along the MTs, suggesting an essential role for kinesin-like protein in trichome branching (Oppenheimer et al., 1997).

DNA contents and branching are increased in *kak* mutant trichomes (Perazza et al., 1999). *KAK* encodes a member of the HECT ubiquitin ligase family, suggesting that ubiquitin-regulated protein degradation inhibits trichome branching (Downes et al., 2003; El Refy et al., 2003).

3.3. Trichome expansion

After branching, trichome expansion occurs over the length of the trichome rather than tip growth (Schwab et al., 2003). Actin filaments play an important role during trichome expansion, presumably because the direction of trichome expansion depends on the actin cytoskeleton (Szymanski et al., 1999). After early stages of expansion in which the actin cytoskeleton is diffuse, filamentous actin becomes reorganized from fine filaments to thick bundles longitudinally aligned to the growth axis (Mathur et al., 1999; Szymanski et al., 1999). Application of the actin-disrupting drug cytochalasin D affects the maintenance and coordination of the normal pattern of trichome cell growth (Szymanski et al., 1999). Furthermore, cytochalasin D treatment mimics the phenotype of the *distorted* class of mutations that cause distorted trichome morphology (Mathur et al., 1999; Szymanski et al., 1999). The *DISTORTED* group genes encode subunits of the actin-related protein (Arp)2/3 and Wiskott–Aldrich syndrome protein family verprolin homologous protein (WAVE) complexes, which function in actin polymerization and regulate a cell morphogenesis pathway (Szymanski, 2005). A motile system of actin-directed tracks was observed in plant organelles such as the Golgi apparatus and peroxisomes (Boevink et al., 1998; Mathur et al., 2002; Nebenfuhr et al., 1999). The *Arabidopsis*

CROOKED gene encodes the smallest subunit of the ARP2/3 complex, and a *crooked* mutant produces abnormal cell shapes because of misdirected expansion (Mathur et al., 2003b). Nonexpanded areas of trichomes in the *crooked* mutant occur where the actin filaments are densely accumulated and Golgi and peroxisome motility is reduced (Mathur et al., 2003b). However, Golgi and peroxisomes in the expanded areas of trichomes in the *crooked* mutant can move as in the wild type (Mathur et al., 2003b). ARP2/3 is known as an important regulator of actin organization and is involved in cell shape development (Mathur et al., 2003a). At the subcellular level, cell shape changes are affected by actin filament aggregation and compromised vacuole fusion, suggesting that actin filaments affect membrane fusion (Mathur et al., 2003a). *WRTM* and *DISTORTED1* encode for *ARP2* and *ARP3*, respectively (Mathur et al., 2003a). Trichomes of both *wrm* and *dis1* mutants have many small vacuoles near a large vacuole and display defects in vacuole morphology, indicating that membrane fusion is also controlled by actin (Mathur et al., 2003a). Actin dynamics play an essential role in cell morphology, cell motility, and cell shape changes, and these processes are controlled by members of the Rho family of small GTPases and the Arp2/3 complex (Bompard and Caron, 2004). Rho GTPases are activated by CDM family proteins (named for *Caenorhabditis elegans* CED-5, human DOCK180, and *Drosophila melanogaster* myoblast city, which has been hypothesized to mediate cytoskeletal reorganization in response to diverse extracellular signals), which function as a guanine nucleotide exchange factor (Bompard and Caron, 2004; Ishida et al., 2008; Kiyokawa et al., 1998; Ohashi et al., 2003). Rho GTPase binds to a WAVE complex and activates the whole complex or releases active subcomplex, either of which interacts with and activates the Arp2/3 complex in nonplant cells (Bompard and Caron, 2004). The *Arabidopsis* genome encodes 11 Rho-related GTPases (ROPs) and CDM-homologous gene *SPIKE1* (*SPK1*) (Qiu et al., 2002; Yang, 2002). Future research will need to clarify the WAVE-Arp2/3 pathway, because contradictory results have been observed in the interactions between ROP and WAVE complex subunits (Basu et al., 2004; Uhrig et al., 2007).

4. Concluding Remarks

Numerous studies have revealed a number of the details of root hair and trichome development in *Arabidopsis* epidermis. WEREWOLF (WER), GLABRA3 (GL3)/ENHANCER OF GLABRA 3 (EGL3), and TRANSPARENT TESTA GLABRA 1 (TTG1) make a transcriptional complex and promote *GLABRA 2* (*GL2*) expression, which negatively regulates root hair cell formation. In contrast, *CAPRICE* (*CPC*) and its homologous gene products (TRY, ETC1, ETC2, and ETC3/CPL3)

interfere with the formation of WER–GL3/ETL3–TTG1 transcriptional complexes, inhibit *GL2* expression, resulting in root hair formation. The regulation of cell-to-cell movement of the CPC protein from nonhair cells to root hair cells and GL3/EGL3 proteins from root hair cells to nonhair cells is involved in preferential accumulation of CPC protein in root hair cells and GL3/EGL3 proteins in nonhair cells. Root hair cells arise in a position-dependent manner. However, the factors that determine positional cues in root hair formation are still unknown. Chromatin organization is also involved in root hair or nonhair cell patterning. The chromatin structure of *GL2* is affected by modification of histone H3, resulting in a clear difference in the expression state of *GL2* between root hair cells and nonhair cells. The phytohormones ethylene and auxin promote root hair formation but have their effects after cell fate determination by *GL2* expression. During root hair growth, several cellular factors and components such as ionic regulation, actin bundles, MTs, phospholipid signaling, and cell wall materials are involved.

GLABRA1 (GL1), GL3/EGL3, and TTG1 form a transcriptional complex and promote *GLABRA 2* (*GL2*) expression, which induces trichome formation. In contrast, *CPC* and its homologous gene products (TRY, ETC1, ETC2, and ETC3/CPL3) interfere with the formation of GL1–GL3/ETL3–TTG1 transcriptional complexes, inhibit *GL2* expression, resulting in inhibition of trichome formation. Trichome initiation is also affected by GA signaling *via* GL1–GL3/ETL3–TTG1 transcriptional complex activity. Trichome development is accompanied by cell cycle regulation, which coordinates a progression of endoreduplication cycles by several molecular pathways. Trichome branching is controlled by MT reorientation and MT-dependent vesicle transport and organelle delivery. Directional trichome expansion is affected by actin organization, which is regulated by actin-related protein 2/3 (Arp2/3) and Wiskott–Aldrich syndrome protein family verprolin homologous protein (WAVE) complex.

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CHAPTER THREE

Metastasis Suppressor Genes: At the Interface Between the Environment and Tumor Cell Growth

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Abstract

The molecular mechanisms and genetic programs required for cancer metastasis are sometimes overlapping, but components are clearly distinct from those promoting growth of a primary tumor. Every sequential, rate-limiting step in the sequence of events leading to metastasis requires coordinated expression of multiple genes, necessary signaling events, and favorable environmental conditions or the ability to escape negative selection pressures. Metastasis suppressors are molecules that inhibit the process of metastasis without preventing growth of the primary tumor. The cellular processes regulated by metastasis suppressors are diverse and function at every step in the metastatic cascade. As we gain knowledge into the molecular mechanisms of metastasis suppressors and cofactors with which they interact, we learn more about the process, including appreciation that some are potential targets for therapy of metastasis, the most lethal aspect of cancer. Until now, metastasis suppressors have been described largely by their function. With greater appreciation of their biochemical mechanisms of action, the importance of context is increasingly recognized especially since tumor cells exist in myriad microenvironments. In this chapter, we assemble the evidence that selected molecules are indeed suppressors of metastasis, collate the data defining the biochemical mechanisms of action, and glean insights regarding how metastasis suppressors regulate tumor cell communication to–from microenvironments.

Key Words: Metastasis suppressor, Angiogenesis, Intravasation, Extravasation, Proliferation, Apoptosis, Cell migration.

1. Introduction

Cancer metastasis is an arduous pathological process that is the major contributor to the morbidity and mortality of cancer patients (Eccles and Welch, 2007; Jemal et al., 2010). Upon diagnosis, a patient may feel that cancer has suddenly struck and their life has immediately changed. In reality, however, diagnosis follows a culmination of years—possibly decades—of alterations occurring at the genetic, molecular, cellular, tissue, and organismal levels. Fortunately, the processes of tumor formation and, particularly, metastasis are extremely inefficient and only small fractions of cells from a tumor mass actually overcome the many hurdles to grow at a distant site (Eccles and Welch, 2007; Fidler, 1973a; Weiss, 1990). To metastasize, expression of particular genetic programs is required by a tumor cell to enable the appropriate interactions with changing microenvironments to promote continued survival and proliferation at secondary sites. Understanding these genetic programs and how they affect cellular interactions and signaling cascades is key to understanding the complex process of metastasis.

The existence of tumor suppressors and oncogenes is now accepted as dogma and is well supported by experimental and clinical data. Genes involved in the promotion of metastasis at distinct stages of the disease are also well accepted. However, the hypothesis for the existence of molecules that inhibit the process of metastasis without preventing primary tumor growth was initially met with much skepticism as demonstrated by the three-time rejection of the manuscript reporting the first metastasis suppressor gene *NM23* (Steeg, 2004b). Since that time, multiple labs, using many different model systems, have demonstrated the existence of a multitude of protein coding and noncoding genes that significantly reduce metastasis without preventing primary tumor formation. It is now understood that metastasis, the ultimate step in tumor progression, involves many pathological processes, and, just as there are several hallmarks of primary tumor formation (Hanahan and Weinberg, 2000), there also exist hallmarks of metastatic cells (Fig. 3.1). Inhibition of a single step in the metastatic cascade leads to suppression of metastasis (Bruns et al., 2000; Eccles and Welch, 2007; Fidler and Radinsky, 1996). In this chapter, the process of metastasis and the functionality of metastasis suppressing molecules are discussed with the objective that this information can be utilized to identify potential antimetastatic therapeutic strategies. Before discussing metastasis suppressors, it is first necessary to establish the context in which they function.

1.1. Genesis of cancer and neoplastic progression

The evolution of a normal cell into a neoplastic cell with progression to a potentially lethal macroscopic metastatic mass is referred to as neoplastic progression or, in the vernacular, tumor progression (Foulds, 1954; Welch and Tomasovic, 1985). There have been several distinct models to depict the cellular mechanisms for this progression including linear and parallel progression models, mutation–selection theory, cancer stem cells, and derivatives of each (Brabletz et al., 2005; Fidler, 2003; Fidler et al., 2007; Klein, 2009; Talmadge and Fidler, 2010; Welch, 1989; Welch and Tomasovic, 1985; Wellner et al., 2009). One of the primary difficulties in constructing generalized model systems for the study of cancer has been the fact that cancer is a heterogeneous disease. As the disease progresses, heterogeneity also increases (Heppner, 1984; Nowell, 1976, 1986). In fact, metastatic cells are behaviorally distinct from cells remaining at the site of primary tumor origin (Steeg and Theodorescu, 2007). These behavioral differences arise at multiple levels including intrinsic cellular changes (genetic and epigenetic heterogeneity), from characteristics of the physical environment (positional heterogeneity; e.g., O_2, pH, growth factors, cytokines, chemokines, etc.) and/or from transient events (temporal heterogeneity; e.g., stage of cell cycle, manipulation of the tumor; Nicolson, 1984; Rubin, 1990; Welch, 1989; Welch and Tomasovic, 1985). The intrinsic molecular mechanisms

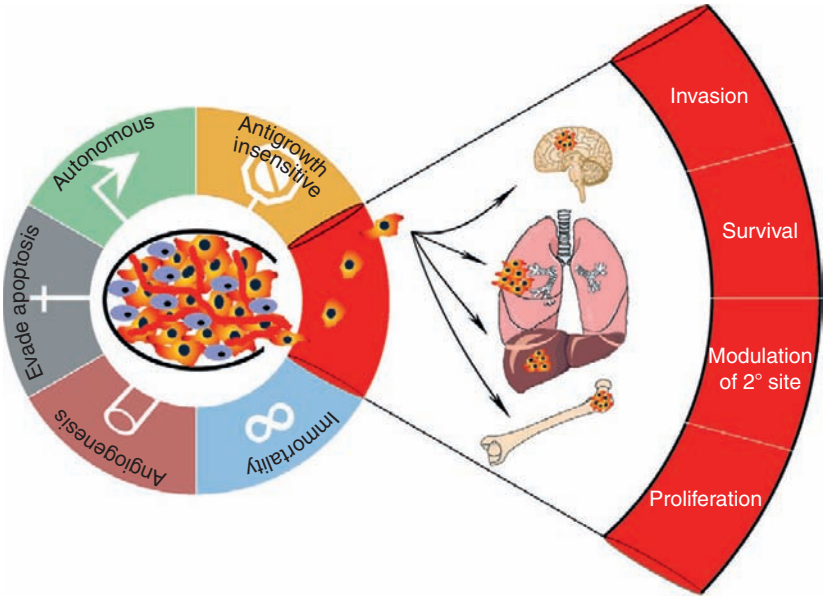

Figure 3.1 Hallmarks of metastasis. The necessary traits to form a neoplasm are illustrated for metastasis as an adaptation of the hallmarks of cancer proposed by Hanahan and Weinberg (2000). Only subsets of neoplastic cells successfully invade and metastasize. To invade, cells must alter cell:cell and cell:matrix adhesion, reorganize the extracellular matrix, and become motile. Upon detachment from the primary tumor mass, cells must survive sheer stress and avoid detachment-induced cell death (anoikis). Then, following arrest or adhesion at secondary sites, disseminated cells modify the local microenvironment in order to enable continued growth. Environmental alterations can be accomplished directly by tumor cells or through surrogates, such as inflammatory cells or mobilized bone marrow stem cell populations. While millions of cells initiate the process of dissemination, only a small fraction completes the process by proliferating to form a macroscopic metastatic mass.

underlying phenotypic differences that characterize a metastatic cell are still being elucidated. However, appreciation for the interrelationships between the surrounding microenvironment and cancer cell-associated genes is increasing (Albini et al., 2007; Ben-Baruch, 2003; Bodenstine and Welch, 2008; Finger and Giaccia, 2010; Joyce and Pollard, 2009; Lin et al., 2009; Pietras and Ostman, 2010; Witz and Levy-Nissenbaum, 2006). Selective regulation of gene transcription also occurs through chemical modifications of DNA and chromatin. Epigenetic modifications are modulated, in part, by how cells interact with the microenvironment(s) in which they find themselves (Lin et al., 2009; Marusyk and Polyak, 2010).

Heterogeneity, for the most part, does not result from multicellular transformation. Data from isoenzyme patterns, karyotypes, and protein

production all indicate that the vast majority of tumors are derived from a single cell (Frumkin et al., 2008; Heppner and Miller, 1998; Welch and Tomasovic, 1985). Likewise, analogous methods have been used to show that >90% of metastases are also the result of single-cell outgrowth (i.e., clonal origin) rather than emboli seeding various tissues (Jones et al., 2005, 2008; Talmadge et al., 1982; Wang et al., 2009a; Yamamoto et al., 2003).

Genetic instability may be the chief driver of heterogeneity during tumor progression by random (i.e., not sequentially acquired) generation of variants as described by the mutation–selection theory (Balmain, 2001; Boveri, 1914). However, there are others who advocate that metastatic ability may be a trait acquired early, or commensurate with, tumorigenesis (Bernards and Weinberg, 2002). Regardless, neoplastic cells are significantly more genetically unstable than normal counterparts as shown by fluctuation analyses for multiple genes and loci (Cifone and Fidler, 1981; Otto et al., 1989; Tlsty, 1990; Tlsty et al., 1989). As a result, progression is most often believed to occur as a result of mutation and coupled selection. Subpopulations of cells that have acquired the ability to migrate, invade, and colonize ectopic sites may have a selective advantage since these tumor cells "acquired" the ability to respond, adapt, and/or survive changing environments. Ultimately, with continued selection and variant generation, subpopulations of cells may acquire the ability to penetrate a basement membrane (i.e., invade). Invasion is the unequivocal hallmark that defines malignancy. It should be emphasized that tumor stage is typically measured in terms of the tumor mass and location, rather than individual cells within the mass. Grade is typically defined by the most malignant cells identified within a tumor. Even if the majority of individual cells within a neoplasm are indolent, the term malignant is applied even if a single cell has penetrated a basement membrane. Microdissection of tumor cells has identified chromosomal and genetic changes between subpopulations within a tumor mass (Frost et al., 2001; Steeg and Theodorescu, 2007). This information has been useful for the prediction of genetic underpinnings controlling tumorigenesis, invasiveness, and metastasis. However, it is important to note that adjacent, apparently normal cells also have evidence of genetic instability (Hida and Klagsbrun, 2005).

The complexity of tumor progression leading to a metastatic cell, as described above, shows—not surprisingly, given the numerous steps required to complete the process of metastasis—that a single genetic change is insufficient to accurately predict the likelihood of a lesion progressing to a metastatic phenotype. In fact, defined subsets of genes can be used as prognostic tools (Jorissen et al., 2009; Liu et al., 2007). While multiple genes are required for the progression from primary tumor formation to metastasis, expression of even a single gene that disrupts any of these events would have the ability to suppress metastasis. Although cofactors may be necessary for suppressor function, identification of metastasis suppressors is,

overall, usually less technically challenging and easier to interpret than the identification of metastasis-promoting genes.

1.2. Distinctions between tumorigenicity, metastasis, and steps in metastasis

As alluded to, the cellular and molecular events along the progression of a tumor cell into a fully metastatic macroscopic lesion can be broken down into discrete steps. These steps are often discussed interchangeably, therefore, incorrectly. Thus, it is first critical to define metastasis. Doing so is necessary for two reasons. First, *metastasis* is both a verb and a noun. The process of metastasis (the verb) was defined above. And, the product of the process is a metastasis, the noun. Therefore, it is important to recognize the context in which the discussion of metastasis occurs. Second, the definitions provide the framework to understand the mechanisms involved and develop therapeutic strategies.

In recent years, five misconceptions regarding metastasis have crept into the scientific and medical literature (Welch, 2006, 2007). (1) Metastasis is an inherent property of cancer cells. (2) Metastasis and invasion are equivalent phenotypes. (3) Metastases arise *only* from cells disseminated via the blood or lymphatics. (4) Tumor cells at secondary sites are metastases. (5) Extravasated cells are metastases. By looking at the definition of metastasis and the mechanisms underlying the process of metastasis, we hope to dispel these misconceptions.

1.2.1. Tumorigenicity and metastasis

Usually, when a primary mass is apparent to the individual or the diagnosing physician, it often comprises at least 10^{10} cells based on the fact that a cubic centimeter of tissue contains $\sim 10^9$ cells (Tannock, 1983). Although histological analysis reveals these cells to be pleiomorphic and single-cell clones isolated from a tumor vary dramatically in terms of biological behavior, not all cells in a neoplasm are capable of completing the required steps for metastasis.

In their outstanding review, Hanahan and Weinberg described six hallmarks of cancer cells (Hanahan and Weinberg, 2000). Besides immortality (apparently limitless replicative potential), abnormal growth regulation (i.e., failure to respond to growth-inhibitory signals or hyperresponsiveness to progrowth signals), self-sufficient growth, evasion of apoptosis and sustained angiogenesis, invasion and metastasis were listed as distinguishing characteristics. Unfortunately, some have interpreted the list as meaning that all tumors are invasive and/or metastatic, which is certainly not true. Some tumors are highly aggressive and metastatic (e.g., small cell carcinoma of the lung, melanoma, pancreatic carcinoma), while others rarely metastasize despite being locally invasive (e.g., basal cell carcinomas of the skin,

glioblastoma multiforme). Therefore, metastasis is not an inherent property of all neoplastic cells (Welch, 2007).

In fact, the process of metastasis begins before cells migrate from a primary tumor mass. Several groups have discovered that the presence of a tumor elicits mobilization of hematopoietic (Erier et al., 2009; Kaplan et al., 2005) and mesenchymal (Hurst and Welch, 2007; Karnoub et al., 2007; Kitamura et al., 2007; Ojalvo et al., 2010; Patsialou et al., 2009; Wyckoff et al., 2007; Yan et al., 2010) stem cells. Both cell types can facilitate tumor cell migration and invasion (Barkan et al., 2010; Ojalvo et al., 2010; Patsialou et al., 2009; Wyckoff et al., 2007) and reorganize tissues in order to manipulate a "niche" into which tumor cells migrate and/or proliferate (Psaila and Lyden, 2009).

1.2.2. Invasion, motility, and metastasis

In most textbooks, metastasis is described in terms of blood-borne (i.e., hematogenous) dissemination. However, secondary tumors can arise because tumor cells have migrated via lymphatics (i.e., lymph node metastases are extremely common in many carcinomas; Eccles et al., 2007; Nathanson, 2003); traversing body cavities (e.g., ovarian carcinoma cells most frequently establish secondary tumors by dissemination in the peritoneum while rarely forming metastases via hematogenous spread; Lengyel, 2010); along capillaries (i.e., many melanomas migrate along already-existing vessels; Lugassy et al., 2002, 2004, 2006; Shields et al., 2007); or along nerves (i.e., pancreatic and prostate carcinomas often exhibit perineural spread; Liebig et al., 2009). So, the route of dissemination is not inherent to a definition of metastasis (Eccles and Welch, 2007; Welch, 2006, 2007). Rather, development of a metastasis needs only incorporate spread of tumor cells to secondary sites.

Although proteolysis-dependent invasion is not an inherent requirement for all tumors to metastasize, it is required for the majority of cancers since physical barriers usually surround a tumor. Understanding the complexity of invasion is necessary to appreciate the mechanisms of many metastasis suppressor genes. Invasive cells have often acquired other traits necessary to metastasize; however, if an invasive cell cannot complete any other step in the metastatic cascade, it will not form a metastasis.

Invasion requires substantial changes of cell morphology and phenotype in addition to modifications of the surrounding environment. During invasion, three important processes are dynamically regulated, including adhesion, ECM reorganization, and motility (Liotta, 1992; Wolf and Friedl, 2006). Normally, epithelial cells form polarized sheets that are maintained by tight intercellular junctions and are anchored to basement membranes by hemidesmosomes, associated intermediate filaments, and integrins. Invading cells have altered cell–cell and cell–matrix adhesion that must be balanced. If a cell is too strongly adherent, it cannot move, and, like a person trying to walk or drive on ice, if to lose an adhesion, cells do not have the traction

to move. The structural and functional proteins that regulate cell adhesion and migration are key downstream targets of oncogenes and tumor suppressor-controlled signaling pathways and provide insights into how oncogenic transformation results in progression to an invasive phenotype. Many of the proteins involved in tumor invasion also affect cell survival, growth, apoptosis and angiogenesis, and hallmarks of malignancy. This highlights the intricate network of interrelated pathways modulating cancer cell behavior.

Many dramatic changes in tumor cell morphology during invasion are reminiscent of a normal process that occurs during embryonic development (Hay, 2005; Thiery, 2002), known as epithelial-to-mesenchymal transition (EMT). The EMT describes conversion from an epithelial morphology to a nonpolarized, motile, spindle-shaped cell resembling a fibroblast (Polyak and Weinberg, 2009; Thiery, 2002; Thompson and Newgreen, 2005). EMT is associated with the loss of epithelial-specific E-cadherin from the adherens junctions, and a switch from the expression of keratins as the major intermediate filament to the mesenchymal intermediate filament, vimentin. EMT is influenced by the tumor microenvironment and has been observed primarily at the tumor stromal interface (Polyak and Weinberg, 2009; Thompson and Newgreen, 2005), but a role for EMT in cancer invasion is not universally observed (Cardiff, 2005, 2010; Tarin, 2005). A key regulator of EMT is transforming growth factor beta (TGF-β) signaling (Bierie and Moses, 2006a,b; Creighton et al., 2010; Heldin et al., 2009; Huber et al., 2005; Oft et al., 1998; Pardali and Moustakas, 2007) but other mediators include hepatocyte growth factor/scatter factor (HGF/SF; Yang et al., 2009), PI3 kinase signaling pathway (Pon et al., 2008), MAP kinases (Bakin et al., 2002; Janda et al., 2002), Sprouty4 (Tennis et al., 2010), and the transcriptional factors ZEB1 (Wellner et al., 2009), Twist and Snail (Moreno-Bueno et al., 2008; Onder et al., 2008). Other signaling pathways implicated in stem cell maintenance that are linked to EMT are Wnt (Debies et al., 2008; ten Berge et al., 2008), Notch (Sahlgren et al., 2008), and Hedgehog (Bailey et al., 2007). Tumor cells may also reverse the process and undergo a mesenchymal-to-epithelial transition (MET) in the absence of EMT-inducing signals (Chaffer et al., 2006; Hugo et al., 2007). This transient nature of EMT helps explain why metastatic cells morphologically resemble primary tumor cells despite the fact that they by necessity accomplished all the steps of the metastatic cascade.

Cells induced to undergo EMT not only exhibit enhanced motility but are resistant to apoptosis (especially anoikis), another key requirement for successful metastasis. However, some cancer cells use EMT-independent modes of migration, including collective and amoeboid (Yilmaz and Christofori, 2010). For example in a clever study, Tsuji and colleagues isolated two populations from a single tumor (Tsuji et al., 2008). One population, herein designated Cell-I, exhibited properties of EMT and was able to enter the vasculature (i.e., intravasate). Cell-I was, however,

unable to form metastases if injected directly into the vascular compartment. The second population, herein designated Cell-II, displayed an epithelial morphology and was not able to enter the blood stream or metastasize when injected orthotopically. However, Cell-II would colonize tissues when directly injected into the vasculature. If Cell-I and Cell-II were coinjected orthotopically, both were found in metastases. Critical to this review, however, cells undergoing EMT were not themselves successful for metastasis. The authors demonstrated that cellular cooperation existed within the primary tumor and was critical to form metastatic lesions. This would suggest that tumor heterogeneity not only exists but may also be essential for tumor progression.

The extracellular matrix (ECM) provides scaffolding for cells and spatial cues that dictate cellular behavior (Barkan et al., 2010). Matrices comprise proteins, primarily triple-helical collagens, glycoproteins such as laminin and fibronectin, and proteoglycans (Catchpole, 1982; Engbring and Kleinman, 2003; Iozzo et al., 2009; Liotta, 1986; Timpl, 1993; Timpl and Aumailley, 1989). Basement membranes are specialized ECM that form barriers separating polarized epithelial, endothelial, and muscle cells from the underlying tissue. Interstitial matrices provide structural characteristics to connective tissues (Erler and Weaver, 2009). The molecular composition of ECM varies between tissues and organs, and provides important contextual information to cellular constituents (Egeblad et al., 2010). In addition, the ECM interacts with many secreted molecules to serve as a repository for regulatory proteins and growth factors (Rozario and DeSimone, 2010). Thus, cell:matrix interactions dictate survival, growth, differentiation, and migration. Correspondingly, selective proteolysis of ECM components leads to release of fragments collectively known as matrikines (Arroyo and Iruela-Arispe, 2010; Duca et al., 2004; Tran et al., 2004), which further regulate protein function and may be involved in cell signaling.

Adhesion of cells to matrix occurs primarily through a family of transmembrane glycoproteins known as integrins, which are heterodimers assembled as specific combinations of 18 alpha and 8 beta subunits (Desgrosellier and Cheresh, 2010; Shattil et al., 2010). Each heterodimer binds distinct, but sometimes overlapping, ECM components. Integrin–ECM binding may be either tumor-promoting or -inhibitory. During tumor progression, cancer cells tend to downregulate the integrins that mediate adhesion and induce maintenance of a quiescent, differentiated state while simultaneously upregulating integrins that promote survival, migration, and proliferation. Although there is a cell-type dependency on integrin function, generally integrins $\alpha_2\beta_1$ and $\alpha_3\beta_1$ are viewed as suppressors of tumor progression, while $\alpha_v\beta_3$, $\alpha_v\beta_6$, and $\alpha_6\beta_4$ promote cellular proliferation and migration (Desgrosellier and Cheresh, 2010).

Integrins bidirectionally mediate signals so that changes in intracellular signaling pathways can modulate cellular adhesion (i.e., inside-out

signaling) and changes in cellular adhesion can alter cellular phenotype (i.e., outside-in signaling). Integrin–ECM interactions often modulate cell function by cooperative signaling with different growth factor receptors (Askari et al., 2010; Desgrosellier and Cheresh, 2010). Many cellular responses induced by activation of receptor tyrosine kinases are dependent upon proper cellular adhesion to ECM substrates in an integrin-dependent manner. Signaling in response to ECM interaction usually activates focal adhesion kinase (FAK) and nonreceptor tyrosine kinases of the Src-family.

The ECM can be remodeled by degradative enzymes that are produced by the tumor cells themselves and surrounding stromal cells (Bhowmick et al., 2004). These enzymes contribute to matrix degradation and facilitate tumor cell invasion. Proteolytic enzymes, representing virtually every class of proteases, have been implicated in tumor cell invasion (Boyd, 1996; Gabbert, 1985; Khokha and Denhardt, 1989; Liotta and Stetler-Stevenson, 1991; Nakajima and Chop, 1991; Nicolson, 1982b; Pauli et al., 1983; Roycik et al., 2009; Stracke et al., 1994). Tumor progression-associated proteases include, but are not limited to, serine proteinases (plasmin, plasminogen activator, seprase, hepsin, and several kallikreins), cysteine proteinases (e.g., cathepsin B), aspartyl proteinases (e.g., cathepsin D), and metal-dependent proteinases (e.g., matrix metalloproteinases—MMP and a disintegrin and metalloproteinases—ADAM families). Other matrix-degrading enzymes such as heparanase, an endoglycosidase that cleaves heparin sulfate proteoglycans, and hyaluronidase that cleaves hyaluronic acid, have also been causally associated with tumor progression and invasion (Nakajima et al., 1983; Sanderson et al., 2004; Vlodavsky et al., 1990, 2002).

Liotta and colleagues observed that metastatic potential correlates with the degradation of type IV collagen found predominantly in the basement membrane and focused attention on the metal-dependent type IV collagenases or gelatinases that are now recognized as MMP-2 and MMP-9 (Thorgeirsson et al., 1985; Turpeenniemi-Hujanen et al., 1985). Subsequently, many of the 23 members of the MMP family of matrix-degrading metalloproteinases have been associated with tumor progression (Nelson et al., 2000). Elevated MMP levels correlate with invasion, metastasis, and poor prognosis in many cancer types. Animal models provide evidence for a causal role for MMP activity in cancer progression (Coussens et al., 2001; McCawley and Matrisian, 2000; Sternlicht and Werb, 2001; Sternlicht et al., 1999). Additionally, the plasminogen activator/plasmin system has been causally implicated in cancer invasion, and urokinase plasminogen activator (uPA) and plasminogen activator inhibitor-1 (PAI-1) are validated prognostic and predictive markers for breast cancer (Andreasen et al., 1997; Carlsen et al., 1984; DeClerck et al., 1997; Hildenbrand et al., 2009).

Regulation of matrix proteolysis occurs at multiple levels. In addition to the expression of proteases themselves, many cells also produce endogenous

inhibitors including the tissue inhibitors of metalloproteinases (TIMPs; Chirco et al., 2006), serine proteinase inhibitors (SERPINs Bailey et al., 2006), and cysteine protease inhibitors (CYSTATINs; Cox, 2009). Some inhibitors accumulate in high concentrations within the ECM and paradoxically exhibit tumor-promoting functions, including protease activation (Jiang et al., 2002). Conversion of pro-MMP-2 to active MMP-2 requires the activity of MT1-MMP (MMP-14), a transmembrane MMP that is activated intracellularly by the propeptidase family member furin, and TIMP-2 (Hernandez-Barrantes et al., 2000). The stoichiometry of each of these molecules is critical for proper function and regulation. Other proteolytic cascades are important for regulating protease activity during the degradation of ECM, including cathepsin(s) → uPA → plasmin → MMP (Affara et al., 2009). Each protease in this cascade can cleave ECM components; therefore, attribution of function requires detailed and systematic evaluation of each component in the cascade.

The original view that proteolytic enzymes function predominantly to remove physical ECM barriers has been expanded with the realization that proteolysis regulates multiple steps of tumor progression. For example, MMP substrates in the matrix or on the cell surface that modulate cellular growth, differentiation, apoptosis, angiogenesis, chemotaxis, and migration have been identified (Kessenbrock et al., 2010). The abundant evidence for a role of MMPs in tumor progression led to the design and testing of synthetic MMP inhibitors for cancer therapy. These inhibitors proved to be disappointingly ineffective in clinical trials (Coussens et al., 2002), results that have been explained by problems with inhibitor or clinical trial design, as well as a lack of understanding of the broad range of MMP activities resulting in both cancer-promoting and cancer-inhibitory effects (Krüger et al., 2010; Lopez-Otin and Matrisian, 2007).

In addition to ECM remodeling, cell locomotion occurs via coordinated polymerization and depolymerization of the actin cytoskeleton to extend pseudopodia at the leading edge of the cell, known as invadopodia (Buccione et al., 2009; Weaver, 2006), followed by contraction associated with disassembly of cell:matrix adhesive contacts at the trailing edge (Wolf and Friedl, 2006). Adhesion molecules, including several β1 integrins and CD44, and proteases, including MMP and ADAM, are an intricate part of the invadopodia. Inside the plasma membrane, invadopodia contain actin and actin assembly molecules as well as multiple signaling molecules, including FAK, Rac1, and synaptojanin 2; src associated proteins such as p130Cas and Tks5/FISH; and the small GTPases cdc42, Arf1 and Arf6 (Chuang et al., 2004; Guarino, 2010; Muralidharan-Chari et al., 2009; Seals et al., 2005; Tannock, 1983; Yamaguchi et al., 2005). Actin cytoskeletal reorganization involves the Arp2/3 complex and its regulators, WASP, cortactin, and the GTPase Rac (TenKlooster et al., 2006). Actin contractility is regulated by myosin light chain kinase and upstream small GTPases, in

particular, Rho and its effector ROCK (Kosako et al., 2000; Olson and Sahai, 2009). Many of these molecules have been targeted since invadopodia are implicated as key cellular structures that coordinate and regulate the process of invasion (Buccione et al., 2009; Poincloux et al., 2009; Weaver, 2006).

As alluded to above in the discussion of EMT, single cells migrate either with a spindle-shaped morphology, referred to as mesenchymal migration, or with the less adhesive ellipsoid shape used by leukocytes and *Dictyostelium* termed amoeboid migration (Wyckoff et al., 2006). Collective migration can occur when the cells retain cell:cell junctions and clusters of cells move in single file through a tissue (Sahai, 2005; Yilmaz and Christofori, 2010). It is noted, however, that the ability of cells to utilize amoeboid migration has been called into question since methods used for reconstitution of matrix resulted in inferior barriers and protein:protein interactions (Sabeh et al., 2009). Another mechanism by which cells traverse cellular barriers is termed entosis (Overholtzer et al., 2007). Briefly, tumor cells transit *through* other cells and emerge on the other side. Amazingly, many times neither cell is harmed during the process. Based upon some *in vitro* estimates, entosis can sometimes be quite common. However, the frequency *in vivo* has not been well studied.

Each type of motility is governed by a variety of cellular factors. Cellular motility is triggered by autocrine inducers of random movement (Jiang et al., 2006; Silletti et al., 1994). Tumor cells produce lysophospholipase D (autotaxin) which stimulates motility, as does lysophosphatidic acid (LPA; Liu et al., 2009; Stracke et al., 1992). LPA can be produced by autotaxin activity on lysophosphatidylcholine. Likewise, HGF/SF interacts with its receptor, c-met, to induce chemokinetic activity of epithelial cells, resulting in an invasive phenotype (Klominek et al., 1998). In fact, disruption of the HGF axis is currently the target of drug development against metastasis (Cecchi et al., 2010; Eder et al., 2009). Directional motility is a chemotactic (following a soluble concentration gradient) or haptotactic (following an insoluble concentration gradient) effect in response to a gradient of soluble or localized factors, respectively. Chemotaxis is often the result of growth factors such as insulin-like growth factor (IGF) and chemokines of the CCR and CXC families (Mantovani et al., 2010). Among the best studied CCR/CXC interactions in metastasis is cellular response to SDF1 (CXCL-12; stromal derived factor-1) as a ligand for the CXCR4 receptor (Gladson and Welch, 2008; Muller et al., 2001; Teicher and Fricker, 2010). SDF-1 levels are often high in tissues commonly colonized by tumor cells (e.g., lung, bone) that express abundant CXCR4. As with the HGF axis, inhibitors of CXCR4 are being studied in preclinical models and are showing efficacy in multiple tumor types (Kim et al., 2008; Richert et al., 2009). Haptotaxis is characterized as a response to gradients of ECM components such as laminin-5 and fibronectin and can be modulated positively or negatively by proteolysis (McCarthy et al., 1985).

Even cells that have been selected for invasive and metastatic capacity exhibit low efficiency for developing metastasis, seldom exceeding 0.1%. Entry of cells into the blood stream (termed *intravasation*) is apparently not uncommon. In fact, more than a million cells per gram of tumor can be shed daily (Butler and Gullino, 1975). Tarin and colleagues illustrated metastatic inefficiency of hematogenous metastases using peritovenous (Levine) shunts to palliate ascites burden for patients suffering from various cancer types (Tarin et al., 1984). Although millions of tumor cells were directly deposited into the vena cava daily, the patients did not develop secondary blood-borne tumors with higher frequency.

The fate of already intravasated tumor cells is uncertain because of apparently contradictory experimental evidence. Using radiolabeled cells, Fidler et al. found that most do not survive (Fidler, 1970, 1973b; Fidler and Nicolson, 1977) because of hemodynamic sheer (Weiss, 1989, 1990; Weiss and Schmid-Schonbein, 1989; Weiss et al., 1985), anoikis (Kim et al., 1999; Phadke et al., 2008; Wong et al., 2001), or immune selection (Fidler, 1974; Gorelik et al., 1980; Hanna, 1985; North and Nicolson, 1985; Van Netten et al., 1993; Young and Newby, 1986). In contrast, using a fluorescent tag Naumov et al. (1999, 2002) showed that a majority of cells not only survived but also extravasated. Muschel et al. used intravital microscopy in lung and brain metastasis models to show that the majority of cells remained intravascular and began to proliferate (Carbonell et al., 2009; Wong et al., 2001). Their data illustrate how extravasation is not essential for successful establishment of a secondary mass. Plausible explanations for these dichotomous results include different cell monitoring methods (i.e., radiolabeling vs. fluorescent tagging), analysis of tumor cell behavior in two different tissues (i.e., lung vs. liver), and whether the studies were done completely *in vivo* versus *ex vivo*.

Critically, all of these observations highlight the importance of tumor–stromal interactions in the metastatic process and clearly demonstrate that a "one-size-fits-all" description of the metastatic process does not exist. For a cell to accomplish all these "steps" involved in invasion, specific genetic programs must be expressed and functional. Once again, it is stressed that inhibition of any of these requirements would render a cell less metastatic.

1.3. Organotropism of metastasis

Secondary tumors can arise because tumor cells have migrated via lymphatics (i.e., lymph node metastases are extremely common in many carcinomas), the blood vasculature, or across body cavities (e.g., ovarian carcinoma cells most frequently establish secondary tumors by dissemination in the peritoneum while rarely forming metastases via blood-borne routes). Lugassy and colleagues recently documented dissemination of melanoma cells along the space between endothelium and basement membrane (Lugassy et al., 2002, 2004, 2007). That is, the cells do not appear to enter

the vascular lumen *per se*. The latter route of dissemination is reminiscent of perineural spread, which is common in pancreatic and prostatic carcinomas in which tumor cells migrate along nerve sheaths (Liebig et al., 2009). Thus, the route of dissemination is not inherent to a definition of metastasis. Nonetheless, the varying pathways to metastasis illustrate different barriers which tumor cells must surmount.

English surgeon Stephen Paget asked, "What is it that decides what organs shall suffer in a case of disseminated cancer?" (Paget, 1889). Upon reviewing autopsy records from 735 women with breast cancer, he recognized discrepancies between the blood supply going to specific organs and the frequency of metastasis to those organs. For example, despite abundant blood circulation to the heart, spleen, and kidney, breast cancers (indeed most cancers) infrequently colonize these tissues. Paget concluded that unequal distribution of metastases could not be exclusively explained by passive embolus arrest in the first capillaries encountered, as supported by the famed pathologists, Rudolph Virchow (Talmadge and Fidler, 2010; Virchow, 1858), Leonard Weiss (Bross and Blumenson, 1976; Weiss, 1979, 1992; Weiss and Ward, 1982), and James Ewing (Ewing, 1919). Autopsy results for patients succumbing to multiple types of cancer indeed show that most metastases are found in the first lymph node or capillary beds encountered by intravasated tumor cells (Gershenwald and Fidler, 2002; Hess et al., 2006; Park et al., 2009). However, there are several well-known examples of metastatic colonization patterns that simply cannot be explained (Table 3.1).

Throughout the latter half of the twentieth century, numerous studies supported a blending of the seed and soil and the mechanical hypotheses. As alluded above, many tumor cells can seed lots of tissues, most commonly at the first lymph node or capillary bed encountered. However, the capacity of cells to proliferate and complete the metastatic process is determined by

Table 3.1 Patterns of clinical metastases that cannot be explained by circulatory patterns or mechanical lodgment of blood-borne tumor cells

Cancer type	Common sites of metastasis
Bladder carcinoma	Bone, liver, brain
Breast adenocarcinoma	Bone, brain, adrenal gland
GI, Kruckenberg adenocarcinoma	Ovary, liver
Kidney, clear cell carcinoma	Bone, liver, thyroid
Lung, small cell carcinoma	Brain, liver, bone
Melanoma, cutaneous	Brain, liver, bowel
Melanoma, uveal	Liver
Neuroblastoma	Liver, adrenal gland
Prostate adenocarcinoma	Bone
Testicular carcinoma	Liver
Thyroid, follicular adenocarcinoma	Bone

the ability of tumor cells to respond to growth promoting while avoiding growth inhibitory signals.

2. Genetic Regulation of Metastasis

The field of metastasis genetics and the very existence of genes that control specifically metastasis have been called into question (Steeg, 2004a). Inarguably, functional data with the metastasis suppressor genes specifically control metastasis, not tumorigenicity. Some array data were interpreted to suggest that metastatic potential is inherent in tumor cells (Bernards and Weinberg, 2002), but the metastasis suppressor data argue against this interpretation (Eccles and Welch, 2007). Furthermore, recent deep sequencing studies in human pancreatic carcinomas and metastases revealed selective genetic changes consistent with the existence of specific metastasis-regulatory genes (Campbell et al., 2010; Yachida et al., 2010). These new data further show that presumably asynchronous metastases share some, but not all, of the same genetic changes, suggesting multiple pathways in which a cell could succeed in its quest to metastasize. In general, metastasis-regulatory genes can be grouped into promoting and inhibiting classes. Recent findings have added a third group that can be thought of as the underlying background upon which the promoting and suppressing genes operate. Since the promoting and suppressing genes operate upon this background, we will begin the discussion of metastasis genes with them.

2.1. Quantitative trait loci (QTL)

Complex phenotypes or traits—like metastasis—logically involve contributions from numerous genes, both positive and negative for a phenotype (Cookson et al., 2009; Winter and Hunter, 2008). Analyses to ascribe involvement is challenging because the contribution of each gene is individually relatively small, making linkage challenging. Ultimately, even if each step in metastasis was governed by one gene, more than a dozen genetic changes would be implicated. In reality, as illustrated for adhesion, migration, and invasion, there are scores of genes involved for each.

Thus, in somewhat overly simplistic terms, each of the genes contributes to the quantity of metastases rather than qualitative determination of metastasis development. Kent Hunter and colleagues have tackled this challenging problem and collected some very important and revolutionary data that support the existence of metastasis genes using breeding strategies in mice. Using a transgene-induced mouse mammary tumor model (MMTV-PyMT, in which the polyoma middle T oncogene is driven by the murine mammary tumor virus promoter), mice were crossed with mice of varying genetic backgrounds. Significant differences in metastasis were found in the F_1 progeny despite failure to alter tumor initiation or growth kinetics in some

strains (Lifsted et al., 1998). Since all the mouse tumors were initiated by the same oncogenic event, differences in metastasis and gene expression were most readily explained by genetic background. His data reinforced a notion introduced earlier—gene context is an important parameter in determining metastatic potential. Although this review is focused upon metastasis suppressors, appreciation of QTL and metastasis-promoting molecules is essential to understand structure–function relationships of the metastasis suppressors.

2.2. Pro-metastatic genes

It is clear that subsets of tumor cells are endowed with capabilities not present in their nonmetastatic counterparts. It follows, then, that metastatic cells turn on genes that promote metastasis. However, it is difficult to identify prometastatic genes because the ability to metastasize requires a cell to accomplish numerous tasks in multiple different microenvironments. Therefore, experimental studies are prone to false-negative studies for metastasis-promoting genes. More accurately, metastasis-promoting genes should probably be designated metastasis *efficiency-enhancing* genes.

Despite these caveats, mutated *ras* expressed in NIH-3T3 cells can confer tumorigenicity and metastatic capacity (Bondy et al., 1985; Chambers et al., 1990). Likewise, introduction of mutant MEK mutants—which mimic constitutively activated MEK—also render NIH-3T3 cells tumorigenic and metastatic (Welch et al., 2000). While this is true in experimental models using fibroblasts, the ability of Ras or MEK to transform and induce progression in all cell types remains to be determined. Together, these studies implicate signaling through the Ras-Raf-Mek-Erk pathway in metastasis. However, cross-talk to and from this signaling cascade affects numerous downstream mediators, thereby providing a plausible mechanism for coordinated expression of the multiple molecules necessary for metastasis. Similarly, Kang et al. (2003) and Minn et al. (2005) in the laboratory of Joan Massague have studied gene expression patterns that begin to explain organotropism of metastasis. Both found that coordinated expression of multiple genes is required for bone and lung metastasis, respectively. Interestingly, many of the genes implicated in both metastatic sites are downstream of TGF-β, a well-known promoter of tumor invasion and EMT as discussed above.

As discussed above with studies identifying QTL, context is critical. This concept is readily apparent when considering the role(s) of TGF-β in the development of multiple carcinomas. In normal breast, TGF-β is generally growth inhibitory (Nam et al., 2008; Wakefield and Stuelten, 2007); however, sometime during tumor progression, there is a paradoxical switch in which malignant behavior is promoted (Welch et al., 1990). Although numerous studies have focused on this phenomenon, the precise molecular mechanisms remain elusive.

2.3. Metastasis suppressor genes and methods to identify metastasis suppressors

Any single gene that disrupts a necessary biological process involved in the metastatic cascade could suppress metastasis. Since metastases develop only from neoplastic cells, tumor suppressors will also, by definition, suppress metastasis. However, we distinguish metastasis suppressors by their ability to inhibit metastasis without preventing primary tumor formation. (Note: some metastasis suppressors can delay tumor growth, but do not prevent tumor growth.) Metastasis suppressors have been found in virtually all cellular compartments and have a wide range of functions including cell adhesion, cell–cell communication, signaling, cell invasion, transcriptional regulation, etc. Below is a summary of metastasis suppressors grouped in broad functional categories. Table 3.2 provides a quick summary of the key points and Fig. 3.2 depicts key pathways involved.

Metastasis suppressor genes have mostly been identified by first comparing loss of heterozygosity (LOH) and karyotypic abnormalities in different stage human cancers. Then, microcell-mediated chromosomal transfer (MMCT) was used to introduce individual chromosomes thought to encode one or more metastasis suppressors. This method has been the most lucrative and proved successful for the discovery of metastasis suppressors on chromosomes 1, 2, 7, 8, 10–13, 16, 17, and 20. Individual genes have also been successfully identified by subtractive hybridization, differential display, comparative genomic hybridization (CGH), microdissection, real-time RT-PCR, microarray, and proteomic approaches (Rinker-Schaeffer et al., 2006; Vaidya and Welch, 2007). Details for the discovery of individual genes are discussed in Section 3.

More recently discovered metastasis suppressors are the direct result of improved techniques to identify functions associated with metastasis. Genome-wide shRNA screens were used to identify Growth Arrest-Specific 1 (GAS1) and Krüppel-Like transcriptional Factor 17 (KLF17) as melanoma and breast cancer metastasis suppressors, respectively (Gobeil et al., 2008; Gumireddy et al., 2009). In their study, Gobeil et al. discovered 22 genes in which shRNA knockdown resulted in an increase in metastasis using the highly metastatic B16–F10 murine melanoma cell line. They focused on GAS1 since it was substantially downregulated in the B16-F10 cells. It is presently unclear why the identified genes in their screen did not overlap with already known metastasis suppressor genes.

In the study by Gumireddy et al. (2009), an shRNA library and *in vivo* screen in which the nonmetastatic 168FARN breast cancer cells that metastasized were selected. RNAi for the KLF17 gene was identified and subsequently chosen for more detailed studies. KLF17 was found to bind to the promoter region of inhibitor of differentiation 1 (Id1) leading to inhibition of invasion and EMT. Improved techniques such as these

Table 3.2 Metastasis suppressors and proposed mechanisms

Metastasis suppressor	Chromosomal location	Proposed mechanism(s) of action	Cellular localization[a]	Step(s) in metastasis inhibited
BRMS1	11q13.1–q13.2	Transcriptional regulation via interaction with SIN3:HDAC complexes; downregulates PtdIns(4,5)P_2	N, some C	Multiple; colonization
Caspase 8	2q33–q45	Induction of apoptosis if cells bind to unliganded integrins	C	Transport
E-cadherin	16q22	Cell:cell interactions	M	EMT; invasion
N-cadherin	8q11.2	Cell:cell interactions	M	EMT; invasion
Cadherin-11	16q22.1	Cell:cell, cell:matrix interactions	M	EMT; invasion
CD44	11p13	Hyaluronic acid receptor; osteopontin receptor stem cell marker (selected)	M	Migration
DCC	18q21.3	Regulates cytoskeletal organization; regulates MAPK signaling	C	Transport; migration
DLC1	8p22–p21.3	RhoGTPase activating protein; regulates cytoskeletal structure	C	Motility; migration; invasion
DRG1	8q24.3	Unknown	C, some N	Angiogenesis; colonization (?); intravasation (?)
GAS1	9q21.3–q22	Inhibit cell cycle	N, some C	Unknown
Gelsolin	9q33	Regulates cytoskeletal structure; reduces motility	C	Motility; migration
HUNK	21q22.1	Protein kinase	C	Migration; invasion
KAI1	11p11.2	Interacts with endothelial DARC to induce apoptosis	M	Intravasation; transport
KISS1 (kisspeptins)	1q32	Maintains dormancy at secondary sites	S	Colonization

KISS1R	19p13.3	G-protein coupled receptor	M	Colonizaton
KLF17	1p34.1	Transcription	N	Invasion; EMT
LSD1	1p36.12	Chromatin remodeling	N	Invasion
MKK4	17p11.2	Stress-activated MAPK signaling	C	Colonization; migration
MKK7	19p13.3–p13.2	Stress-activated MAPK signaling	C	Colonization; migration
p38	6p21.3–p21.2	Stress-activated MAPK signaling	C	Colonization; migration
Nm23	17q22	Phosphorylates KSR to prevent downstream activation of MAPK pathways	C, some N	Migration; colonization
OGR1	14q31	GPCR signaling	M	Migration
RhoGDI2	12p12.3	Regulates Rho; negatively alters endothelin 1 and neuromedin U expression	C	Migration; colonization
RKIP	12q24.23	Competitive inhibitor of RAF1–MEK interactions	C	Migration; invasion
RRM1	11p15.5	Increases PTEN expression; decreases FAK phosphorylation	C	Motility; invasion
SSeCKS	6q24–q25.1	Scaffold protein for PKA and PKC; inhibits osteopontin, VEGF expression; up regulates vasostatin	C	Angiogenesis; migration
TIMPs	Multiple	Inhibit metalloproteinases; signaling	C, S, M	Angiogenesis; migration; invasion; transport

[a] Nuclear (N), cytoplasmic (C), membrane (M), secreted (S).

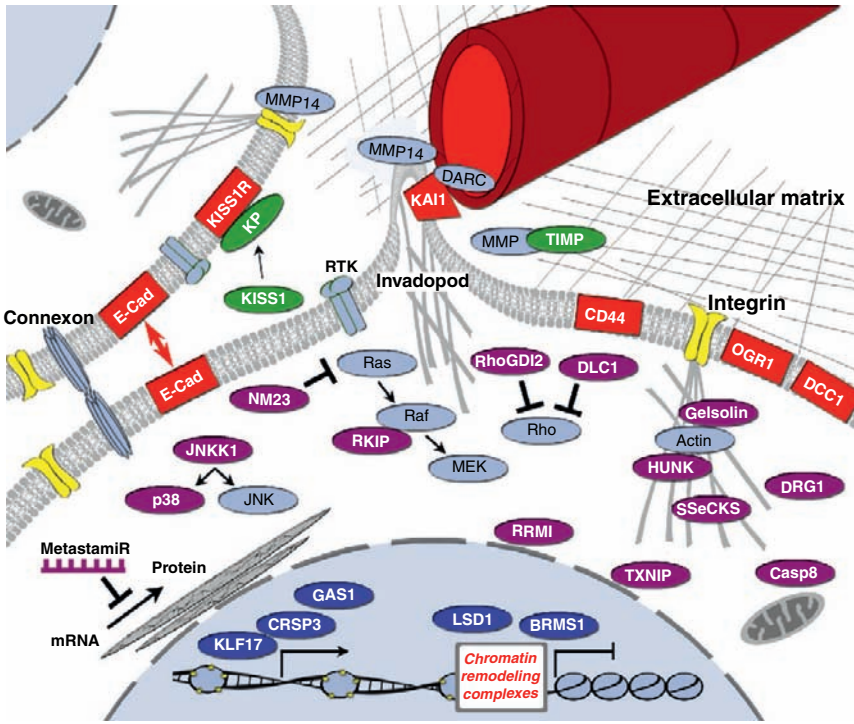

Figure 3.2 Metastasis suppressors exist in every cellular compartment and in the extracellular milieu. Predominantly nuclear (blue), cytoplasmic/signaling (purple), membrane (red), or extracellular (green) molecules are shown, some with key interacting molecules/complexes (gray). Based upon current knowledge regarding location and function, inferences regarding mechanism of action are described in the text.

shRNA screens should increase our discovery of metastasis suppressor genes. Questions regarding specific cell line or model systems may become crucial to our understanding of how context-dependent factors play a major role in suppressor function.

3. Functionally Validated Metastasis Suppressor Genes

3.1. Transcriptional regulators

3.1.1. BRMS1

Because metastasis requires the coordinated expression of particular genes at multiple steps, a key regulatory molecule would be one that functions by regulating metastasis-associated gene transcription. Breast cancer metastasis

suppressor-1 (BRMS1) alters the expression of multiple metastasis-associated genes including osteopontin (OPN; Hedley et al., 2008; Samant et al., 2007; Shevde et al., 2006), uPA (Cicek et al., 2005, 2009), fascin (Zhang et al., 2006), epidermal growth factor receptor (EGFR; Hurst et al., 2008; Vaidya et al., 2008), CXCR4 (Yang et al., 2008), as well as coordinately regulating many metastamiR (Edmonds et al., 2009a,b; Hurst et al., 2009a). These genes are associated with metastasis at many different steps. Likewise, BRMS1 affects multiple phenotypes implicated in cancer metastasis (Phadke et al., 2008), including restoration of homotypic (Saunders et al., 2001; Shevde et al., 2002) and heterotypic (Kapoor et al., 2004) gap junctional intercellular communication, inhibition of migration and invasion, promotion of anoikis, and differential modulation of growth factor signaling. Additionally, the selective downregulation of phosphoinositide phosphatidylinositol (4,5) bisphosphate (PtdIns(4,5)P_2) has been demonstrated (Champine et al., 2007; DeWald et al., 2005; Vaidya et al., 2008) that may have dramatic signaling effects in response to the microenvironment. *In vivo* experiments have demonstrated that BRMS1 inhibits several steps of metastasis, including the ultimate step, colonization at the secondary site (Phadke et al., 2008).

BRMS1 was originally identified by analysis of differentially expressed genes in the metastatic breast carcinoma cell line MDA-MB-435 (Chambers, 2009; Grijalva et al., 2003; Hollestelle and Schutte, 2009; Montel et al., 2009) following MMCT of neomycin-tagged chromosome 11 (Seraj et al., 2000). Differential display was used to compare chromosome 11-containing with parental cells, which led to the identification and cloning of BRMS1, which was subsequently mapped to 11q13.1–q13.2. It was then directly transfected into metastatic breast cancer cell lines that express no detectable levels of BRMS1 transcript and using xenograft (Seraj et al., 2000) and syngeneic (Samant et al., 2002, 2006) mammary tumor models was found to significantly suppress metastasis. Since that time, multiple labs using several different model systems have found that BRMS1 suppresses metastasis of melanoma (Shevde et al., 2002), ovarian (Zhang et al., 2006) and nonsmall cell lung carcinomas (Smith et al., 2009) in addition to breast carcinoma.

Determining the mechanism of action for BRMS1 has occurred in a somewhat circuitous manner. Protein sequence homology provided few clues regarding possible mechanisms. So, protein:protein interaction studies using yeast two-hybrid genetic screens and coprecipitations were undertaken. Almost simultaneously, both approaches identified a direct interacting partner for BRMS1, Rb-binding protein-1 (RBBP1), which is now known as AT rich interacting domain 4A (ARID4A) (Hurst et al., 2008; Meehan et al., 2004). Also, directly binding to BRMS1 is suppressor of defective silencing-3, SUDS3 (a.k.a. mSDS3 or SAP45; Hurst et al., 2008; Meehan et al., 2004; Silveira et al., 2009). Both ARID4A and SUDS3 are

components of the SIN3 histone deacetylase chromatin remodeling complexes. Other groups studying SIN3 complexes and associated proteins have identified BRMS1 by mass spectrometry, affinity purification, and coimmunoprecipitation (Doyon et al., 2006; Le Guezennec et al., 2006; Nikolaev et al., 2004; Shiio et al., 2006; Smith et al., 2010).

When BRMS1 is present in complexes with the Gal4 promoter–luciferase reporter, transcriptional repression is observed (Hurst et al., 2008; Meehan et al., 2004; Silveira et al., 2009). However, mRNA expression arrays reveal a complex pattern of >500 nonrandom expression changes (Champine et al., 2007; Cicek et al., 2005). Cicek et al. were the first to demonstrate selective differential expression of proteins in BRMS1-expressing breast cancer cells using 2D gel-electrophoresis (Cicek et al., 2004), and recently Rivera et al. used a similar approach in melanoma cells to identify differentially expressed proteins (Rivera et al., 2007). Some differentially expressed proteins were identified in both studies, for example, annexins and glutathione-*S*-transferases, but overlap was not predominant probably because BRMS1 regulation may be cell-type dependent. By both proteomic and genomic discovery approaches, BRMS1 regulates genes involved in lipid metabolism and transport, secretion, and cellular architecture.

To date, however, whether gene regulation effects are direct versus indirect has not been clearly demonstrated. Jones et al. (Liu et al., 2006; Smith et al., 2009) identified interaction of BRMS1 with the p65 subunit of the NFκB transcription factor. Presumably, recruitment of SIN3::HDAC complexes reduces NFκB activity, a finding that has been observed using reporter assays. Inhibition of NFκB activity through recruitment of HDAC1 has been observed by different laboratories (Cicek et al., 2005, 2009; Samant et al., 2007). Although it is likely that BRMS1 will interact with other transcription factors, their identities, if any, have not yet been determined.

Mutational analysis of BRMS1 has determined that direct ARID4A and SUDS3 interactions are not essential for metastasis suppression (Hurst et al., 2008; Silveira et al., 2009). Different domains of the BRMS1 protein bind each molecule; however, BRMS1 remained associated with SIN3 and HDAC1/2. Interestingly, disruption of each direct BRMS1 interaction alters the gene expression profiles of cells reexpressing the BRMS1 mutants (Hurst et al., 2008; Silveira et al., 2009). These findings reveal that the mix-and-match nature of the SIN3::HDAC::BRMS1 complexes determines the expression of individual genes. In fact, Smith et al. showed that HDAC inhibitors could differentially cause modification to the SIN::HDAC complex composition (Smith et al., 2010).

Clinical studies with BRMS1 have been relatively inconsistent with regard to patient survival and metastasis correlations. The inconsistencies are thought to be primarily because most clinical studies measured mRNA expression, but BRMS1 mRNA and protein do not always correlate (Hurst et al., 2009c). Also, BRMS1 protein is sensitive to proteasome degradation

and is stabilized by the heat shock protein HSP90 (Hurst et al., 2006), highlighting the importance of measuring protein levels. Ultimately, simply measuring protein levels for a protein that functions differentially depending upon its interaction partners may be moot. Nonetheless, BRMS1 protein expression using IHC is predictive for survival and metastasis development in subsets of breast (Frolova et al., 2009; Hicks et al., 2006) and nonsmall cell lung carcinomas (Smith et al., 2009).

3.1.2. CRSP3 and TXNIP

Two additional transcriptional regulators, CRSP3 (a.k.a. cofactor required for SP1 activity; DRIP130, Vitamin D regulatory interacting protein 130) and TXNIP (a.k.a. thioredoxin interacting protein; TBP2, thioredoxin binding protein 2; VDUP, vitamin-D3 upregulated protein) have been identified as metastasis suppressors (Goldberg et al., 2003). Both molecules have been studied with regard to their redox regulation and/or signaling in addition to their apparent association with the vitamin D transcription complex. CRSP3, which maps to chromosome 6q23.2, upregulates TXNIP, which maps to chromosome 1q, which, in turn, regulates the KISS1 metastasis suppressor (see Section 3.3.3).

3.1.3. LSD1

Lysine-Specific Demethylase 1 (LSD1) is an amine oxidase catalyzing the demethylation of histone proteins and has been shown to be a component of many chromatin remodeling complexes including CoREST (Lee et al., 2005; Shi et al., 2005), CtBP (Wang et al., 2007), and other HDAC containing complexes (You et al., 2001). More recently, it was found to be an integral component of the Nucleosome Remodeling and Deacetylase (NuRD) protein complex to inhibit invasion and suppress metastasis in breast cancer model systems (Wang et al., 2009b).

LSD1 was found to be downregulated in breast carcinomas and expression was inversely correlated with TGFβ1. Interestingly, the NuRD complexes have also been implicated in promoting metastasis as several studies show important functions associated with histone deacetylation for the metastasis-associated proteins (MTA; Bagheri-Yarmand et al., 2004; Nicolson et al., 2003; Ohshiro et al., 2010; Toh et al., 1994, 2004). Analogous to the complexes formed by BRMS1, the paradigm supported by LSD1 is that the function of chromatin remodeling complexes is clearly dependent on the specific composition of each complex.

3.2. Posttranscriptional regulators

It is clear that metastasis is regulated by the expression of genes necessary for phenotypes required for each step in the cascade and transcriptional regulation of metastasis-associated genes is one key mechanism to inhibit or

promote metastasis. The majority of proteins in a cell are also regulated posttranscriptionally and this serves as yet another level for controlling metastasis. It has recently been shown that several microRNA (miRNA) genes significantly influence several steps in the metastatic cascade that have now been given the term metastamiR. Other molecules that regulate signaling in response to the microenvironment or affect adhesion to the microenvironment may dramatically inhibit metastasis.

3.2.1. MetastamiR and noncoding RNA

With the initial discovery of miRNA in the control of the timing of *Caenorhabditis elegans* larval development (Lee et al., 1993), they were identified in plant (Park et al., 2002) and mammalian cells less than a decade later (Wightman et al., 1993). These small RNA genes are typically transcribed by RNA polymerase II to the pri-miRNA that adopts a characteristic hairpin loop structure. They are further processed to pre-miRNA by the RNAse 3 Drosha and exported to the cytoplasm by Exportin 5 where the enzyme Dicer processes the hairpin to a mature 18–26 nucleotide miRNA that associates with the RNA-induced silencing complex (RISC). Both Drosha and Dicer form complexes with proteins containing dsRNA-binding domains. The Drosha partner is DiGeorge syndrome critical region gene 8 (DGCR8) and the Dicer partner is TAR RNA binding protein (TRBP).

The latest release of the miRBase database has catalogued >1000 miRNA in humans. Each mature miRNA (19–24 nt) complements the 3′-UTR of mRNA. Moreover, microRNA can regulate the translation of hundreds of genes through sequence-specific binding to mRNA depending on sequence complementarity will result in the inhibition of translation and/or degradation of target mRNAs (Stefani and Slack, 2008). However, some microRNA upregulate some genes by direct and indirect mechanisms. As a result of such promiscuity, it is perhaps not surprising that a single metastamiR might regulate metastasis similarly to a transcription factor that exerts its effect on multiple mRNA or proteins.

Altered regulation of miRNA expression exerts profound effects on cell phenotypes. Soon after their discovery in mammalian cells, miRNA were reported to play key roles in cancer, recurrence, development of metastases, and/or survival (Edmonds et al., 2009b; Hurst et al., 2009b; Nicoloso et al., 2009). At least, a dozen miRNA have been shown to promote or inhibit metastasis in experimental models and that number will likely grow even further because >20 more have been shown to impact critical steps in the metastatic cascade, such as EMT, apoptosis, and angiogenesis. Typically, metastamiR were discovered using *in vitro* screens for individual steps in metastasis including proliferation, EMT, adhesion, migration, invasion, apoptosis, and/or angiogenesis. As mentioned previously, a critical

point to validate a miRNA as a *bona fide* metastasis suppressor is to perform *in vivo* assays.

The first suppressing metastamiR was identified by Tavazoie et al., who compared miRNA expression in metastatic variants derived from the human breast carcinoma cell line, MDA-MB-231 (Tavazoie et al., 2008). They identified six miRNAs with low relative expression in the metastatic cells. Three of these, miR-335, -126, and -206, suppressed metastasis *in vivo*; however, miR-126 also inhibited cell proliferation and tumorigenesis, removing it from the metastasis suppressor category, by definition. Both miR-335 and -206 inhibited invasion and migration *in vitro*. miR-335 targets SOX4 (SRY-box containing transcription factor), PTPRN2 (receptor type tyrosine protein phosphatase), MERTK (c-Mer tyrosine kinase), and possibly TNC (tenascin C). Additionally, inhibition of SOX4 or TNC by shRNA inhibited invasion *in vitro* and metastasis *in vivo*. Their findings elegantly demonstrate how a single miRNA could impact several downstream pathways by arborizing signaling pathway components. There was also a clinical association of miR-335 expression with metastasis-free survival in a set of 20 primary breast tumor samples.

Several groups had shown roles for miR-146 in inflammation through regulation of NFκB (O'Connell et al., 2010). Although the miR-146a and -146b genes are encoded on different chromosomes, their mature sequence differs by only two nucleotides at the 3′ region. So their mRNA targets are predicted to overlap significantly. Indeed, both miR-146a and -146b inhibit invasion and migration of breast cancer cells by downregulating NFκB by targeting IRAK1 and TRAF6 (Bhaumik et al., 2008). These studies were extended *in vivo* by demonstrating miR-146a and -146b suppressed metastasis that may involve targeting of EGFR (Hurst et al., 2009a) or ROCK1 (Nicoloso et al., 2009), both of which are involved in promoting invasion and metastasis. In clinical samples, miR-146a expression is inversely correlated with prostate cancer progression, further supporting a metastasis suppressor function for this metastamiR (Lin et al., 2008).

While inhibition of any step in the metastasis cascade precludes metastasis, a single metastamiR could result in more robust inhibition of the metastatic process by targeting multiple steps. Evidence to support this conclusion comes from studies with miR-31, which inhibits invasion, anoikis, and colonization leading to a 95% reduction in lung metastasis in an orthotopic model of breast cancer (Valastyan et al., 2009, 2010). Additionally, miR-31 levels were lower in a pilot study of breast cancer patients with metastasis.

MetastamiR are not limited to suppressors of metastasis. miR-10b was the first metastamiR discovered by Ma et al. (2007a). They hypothesized that certain miRNA could regulate specific stages of tumor progression and found that miR-10b was highly expressed only in metastatic breast cancer cell lines compared to primary human mammary epithelial or spontaneously

immortalized cells. After showing that miR-10b enhanced migration and invasion *in vitro* and metastasis *in vivo,* they identified a pathway where the prometastatic gene TWIST1 upregulates miR-10b that targets HOXD10 leading to an increase in RHOC. Additionally, RTQ with 23 primary breast tumors was used to show a general increase in miR-10b expression in patients with metastasis.

Interestingly, the BRMS1 metastasis suppressor that regulates miR-146a/b also regulates TWIST, miR-10b, and RhoC expression (Edmonds et al., 2009a). Whether the regulation of these genes by BRMS1 is direct or indirect is still not known. Regardless, the data all point to common pathways impacted by these metastasis-regulatory molecules (Fig. 3.3).

Huang and colleagues transduced nonmetastatic MCF7 human breast cancer cells with an miRNA expression library and screened the

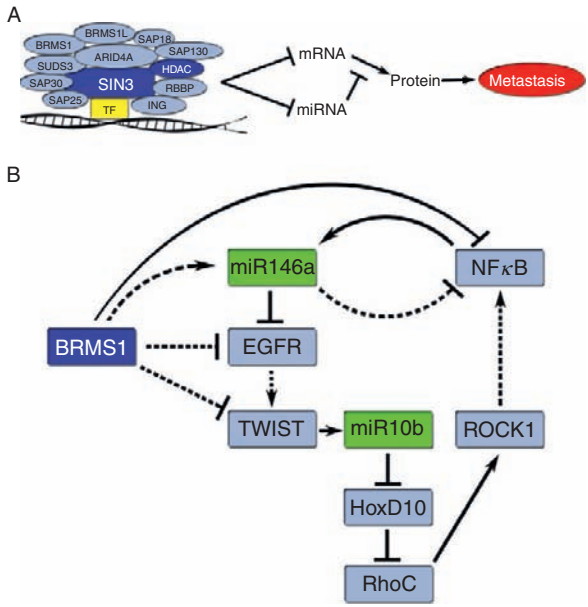

Figure 3.3 Metastasis suppressor networks. Metastasis suppressors may function together to inhibit metastasis-associated networks at multiple levels. (A) BRMS1 is part of a chromatin remodeling complex that represses transcription. This leads to direct repression of gene transcription and indirect repression through downregulation of upstream repressors. Additionally, noncoding genes including miRNA are repressed leading to altered translation of mRNA. (B) BRMS1 regulates the expression of EGFR by downregulation of transcription and by increased expression of miR-146a that targets EGFR. This leads to decreased levels of TWIST1 and the metastasis-promoting metastamiR, miR-10b. Translation of the transcription factor, HoxD10, is then increased with a subsequent decrease in RhoC and ROCK1. This pathway feeds back through NFκB. Dotted lines indicate indirect mechanisms or multiple steps not shown and solid lines indicate direct regulation.

transductants using a transwell migration assay (Huang et al., 2008). Both miR-373 and -520c promoted migration and were subsequently found to increase *in vivo* metastasis, at least in part, by targeting the hyaluronate receptor and stem cell marker, CD44. Clinically, miR-373 expression was higher in lymph-node metastasis compared with the primary tumors from 11 pairs of matched samples.

Invasion and migration are increased while apoptosis is decreased by miR-21 expression in breast, colon, and glioma (Asangani et al., 2008; Gabriely et al., 2008; Zhu et al., 2008). miR-21 targets TPM1 (tropomyosin 1), PDCD4 (programmed cell death 4), and regulators of MMP. miR-143 and miR-182 promoted hepatocellular carcinoma and melanoma metastasis, respectively (Segura et al., 2009; Zhang et al., 2009b). miR-143 is upregulated by NFκB and decreases adhesion. miR-182's effects can be reversed by reexpression of MITF (microphthalmia-associated transcription factor M) or FOXO3. miRNA-182 is part of a cluster (miR-183-96-182). Many miRNA are encoded as genetically linked clusters (perhaps operons?) and are expressed as a single pri-miRNA. As a result, it is not always possible to distinguish biological effects that are the result of a single miRNA or the collective actions of multiple miRNA. Since many experimental studies manipulate single members of a cluster, interactions or feedback mechanisms may be missed if the cluster expression is not taken fully into account.

3.2.2. MetastamiR pathways, concepts, and future directions

While metastamiR have only been recognized for slightly more than 3 years, the rapid discovery of this important family of molecules is impressive. MetastamiR are components of complex pathways and are often expressed downstream of pro- or antimetastatic signals, including pathways regulated by NFκB, EGFR, TWIST1, BRMS1, ZEB1/2, and HIF1α. Unfortunately, understanding the mechanisms by which miRNA are regulated is still in its infancy.

Interestingly, positive and negative feedback loops have been found whereby the upstream effectors are themselves targets of the miRNA that they regulate (Aguda et al., 2008; Bracken et al., 2008; Castellano et al., 2009; Cheloufi et al., 2010; Taganov et al., 2006; Wellner et al., 2009). This implies an important role for metastamiR in modulating key signaling pathways involved in tumorigenicity and metastasis. Because of their position as nodes within signaling pathways and their promiscuity with regard to downstream targets, each metastamiR can (and probably does) amplify pro- and antimetastatic signaling events. It is likely that metastamiR regulation of these signaling events is context dependent, relying on microenvironmental cues in both directions. We predict that yet-to-be-discovered cofactors will lead to specificity of miRNA effects on selected pathways; however, their existence is speculation at this time. We find ourselves in the midst of a revolution with regard to the biochemical and molecular regulation of

cancer metastasis. Old notions of equating tumorigenicity with metastasis have to be discarded. There are clear distinctions between the phenotypes; biologically, biochemically, and genetically. Understanding the interrelationships between regulatory genes and gene products and how these are modulated by the microenvironmental context is beginning to unravel the complex tapestry that is cancer metastasis.

During the course of assembling references for this chapter, the expanding complexity of miRNA and more specifically, metastamiR, exploded. As targets are assigned to individual miRNA, one must now take into account previously ignored pseudogenes. Poliseno et al. showed that the pseudogene, PTENP1, was biologically active by virtue of regulating cellular levels of the tumor suppressor PTEN (Poliseno et al., 2010). Although PTENP1 is not translated into protein due to a missense mutation of the initiator methionine codon, it still possesses a 3′ UTR with high homology to PTEN. As a result, increased expression of PTENP1 serves as a "decoy" for PTEN targeting miRNA and leads to increased translation and protein levels of biologically functional PTEN. Therefore, pseudogenes are now going to have to be considered when analyzing various functions of miRNA.

Future studies regarding noncoding RNA involved in metastasis will not be limited to miRNA. A recent report describes the large intergenic noncoding RNA (lincRNA) HOTAIR that promotes metastasis (Gupta et al., 2010). HOTAIR associates with the chromatin remodeling complexes Polycomb Repressive Complex 2 (PRC2) and LSD1 and alters the methylation pattern on histone lysine residues, specifically methylation of H3K27 and demethylation of H3K4 (Gupta et al., 2010; Tsai et al., 2010). This leads to epigenetic changes in gene expression that are favorable for metastasis. Another recent report identified p53 as a mediator of many lincRNA including lincRNA-p21 (Huarte et al., 2010) demonstrating the likelihood of identifying many more metastasis-associated lincRNA.

3.3. Regulators of cellular communication

In retrospect, it seems obvious that the cell surface would be a key site for critical molecules involved in cancer metastasis since tumor cells encounter numerous different microenvironments during their journey. Three lines of evidence have been used to support the involvement of cell-surface molecules in the process of metastasis. The first is that enzymatic modification of cell-surface components can alter adhesion, survival in the circulation, and arrest at secondary sites (Hagmar and Norrby, 1973; Welch, 1997; Welch et al., 1994a). The second is involved in biosynthetic modification of surface glycoproteins and glycolipids (Gasic and Gasic, 1962; Irimura et al., 1981; Shaikh et al., 2008). The third has involved transfer of cell-surface molecules from metastatic to nonmetastatic cells with a corresponding

enhancement of metastatic efficiency (Legrue, 1982; Poste and Nicolson, 1980; Poste et al., 1980). There are abundant more examples for each of these experimental strategies. Readers are referred to several excellent reviews for additional details (Geiger and Peeper, 2009; Lu and Kang, 2009; Nicolson, 1982a, 1988a,b). The examples listed below focus exclusively on metastasis suppressor genes that are found on the cell-surface or cell–cell junctions.

3.3.1. Cell-surface receptors and junctions

3.3.1.1. CD44 CD44 is a transmembrane glycoprotein that binds ECM components such as hyaluronic acid and the prometastatic factor, osteopontin (Underhill, 1992). CD44 is proposed to modulate adhesion, lymphocyte homing, and activation (Kallakury et al., 1996) and maps to 11p13 (Rudy et al., 1993). In clinical samples, there is a correlative loss of CD44 expression in high-grade tumors and metastases (Kallakury et al., 1996). Depending on the type of cancer, cell line used, and the model being evaluated, CD44 expression can increase tumorigenicity and metastatic potential or function as a metastasis suppressor (Kallakury et al., 1996; Rudy et al., 1993). In recent years, many cancer researchers have become enamored by the cancer stem cell theory or the cancer progenitor cell theory (Brabletz et al., 2005). Briefly, the theory proposes that migrating cells with properties similar to stem cells—capacity to self renew for extended times, ability to regenerate a mixed population of cells with both specialized and unspecialized properties. Since the majority of metastases are clonal in origin yet heterogeneous by the time overt, macroscopic metastases are diagnosed, there are abundant similarities. Several laboratories have indicated that CD44 surface expression is a marker for cancer stem cells (Bauerschmitz et al., 2008; Sackstein et al., 2008; Tang et al., 2007), further raising questions regarding the role(s) of CD44 in metastatic behavior.

Ambiguity regarding metastasis-promoting or -suppressing effects by CD44 probably rests in the high degree of posttranscriptional and splicing variation that occur in different cell types. As a result, some of the splice variants may have different functions from others. Until reagents are developed and the cell-specific changes are categorized, the issue cannot be resolved.

3.3.1.2. E-cadherin Epithelial cell–cell interactions are mediated primarily by cadherins, transmembrane glycoproteins that form Ca^{+2}-dependent homotypic complexes (Harris and Tepass, 2010). For many tumor types, loss of E-cadherin occurs during EMT and correlates with increased invasion and metastasis. Reexpression in experimental models can block invasion. Taken together, these observations suggest that loss of E-cadherin is causative for invasion. E-cadherin loss occurs because of transcriptional repression and proteolytic degradation (Jeanes et al., 2008; Li et al., 2004; Onder et al.,

2008; Van Roy and Berx, 2008). The zinc finger transcriptional repressors Snail and Slug, in particular, have been implicated in regulating EMT by virtue of their ability to repress E-cadherin transcription. Cadherins are regulated by catenins (α, β, γ, and p120 catenins), cytoplasmic proteins that functionally link the cadherin complex to the actin cytoskeleton. β-catenin is both a cell adhesion protein and a transcription factor. In addition to its role in adherens junctions, β-catenin participates in canonical Wnt signaling (Behrens, 1999; Giles et al., 2003), a signaling pathway implicated in development and cancer. E-cadherin levels and function are also disrupted by loss of p120 catenin, which may also contribute to metastasis.

E-cadherin is not the only cell:cell adhesion molecule associated with invasion and metastasis. Another member of the immunoglobulin cell adhesion molecule (Ig-CAM) family, NCAM, is downregulated in several tumor types. NCAM loss increases the ability of tumor cells to disseminate in some tumor types (Crnic et al., 2004). Still other Ig-CAMs, such as DCC, CEACAM1, and Mel-CAM, have reduced expression in some cancers. Please note: not all cell:cell adhesion molecules can be viewed as potential invasion suppressors. Several adhesion molecules, such as L1, CEA, and ALCAM (Cavallaro and Christofori, 2004), are overexpressed in advanced cancers. Additionally, N-cadherin promotes cell motility (Hazan et al., 2000; Nieman et al., 1999). This complexity may be explained by (in)direct signaling functions for these molecules that are distinct from their roles in cell:cell adhesion (Behrens, 1999; Jeanes et al., 2008; Van Roy and Berx, 2008). Because of the interrelatedness of proliferation and invasion, adhesion and growth effects and the complexity of tumor tissue (including complexity that still exists in well-defined experimental models), it is not always possible to distinguish the myriad functions of so-called adhesion molecules.

Another cadherin implicated as a metastasis suppressor is N-cadherin, which when overexpressed in the LM8 osteosarcoma line, inhibited pulmonary metastasis (Kashima et al., 2003). However, there are contradictory data showing that N-cadherin can increase aggressiveness and metastasis in breast and melanoma cell lines (Hazan et al., 2000; Li et al., 2001). Clearly, more work will be required to understand how cadherins play a role in metastasis suppression. Nonetheless, it is clear that different cadherins will play distinct roles in different tissues. This highlights an emerging theme in the metastasis suppressor field—context is critical.

3.3.1.3. KAI1 Kang-Ai1 (Chinese for anticancer; a.k.a. CD82/C33/TIP30) was first identified in AT3.1 and AT6.1 rat Dunning prostate cancer cells (Dong et al., 1995). Similar to the story for BRMS1 discovery, human chromosome 11 was introduced by MMCT because previous work had implicated chromosomal aberrations in late-stage prostate carcinoma. Chromosome 11 hybrids significantly blocked metastasis without preventing

primary tumor formation, indicating the presence of one or more metastasis suppressors. Positional cloning mapped KAI1 to 11p11.2 (Dong et al., 1995; Ichikawa et al., 1992). Subsequent experiments demonstrated that KAI1 inhibits metastasis of breast and melanoma cell lines (Phillips et al., 1998; Wei et al., 1996; Yang et al., 1997). Consistent with its role as a metastasis suppressor, KAI1 expression is frequently downregulated during prostate (Dong et al., 1995), breast (Phillips et al., 1998; Yang et al., 2000), colorectal (Lombardi et al., 1999), ovarian (Liu et al., 2000), cervical (Liu et al., 2000, 2001), oral (Farhadieh et al., 2004), and nonsmall cell lung (Goncharuk et al., 2004) cancers.

Regulation of KAI1 is complex and is still being elucidated. KAI1 expression is positively regulated by p53, junB, and AP2 (Marreiros et al., 2003) is induced following etoposide treatment through p53 and c-Jun pathways (Mashimo et al., 2000); appears to be dependent upon Tip60 and β-catenin–reptin complexes (Kim et al., 2005a); and can be induced by protein kinase C (PKC); Akita et al., 2000). As with many of the other metastasis suppressor genes, *KAI1* is associated with abundant methylation of CpG islands in the promoter; however, treatment of cells with 5-aza-2-deoxycytidine (which prevents cytosine methylation) or trichostatin-A (which inhibits histone deacetylation) failed to upregulate KAI1 (Sekita et al., 2001), suggesting other regulatory controls.

Since KAI1 is a member of the tetraspanin superfamily; much of the regulation of, and mechanistic insights regarding, *KAI1* are inferred from studying the role of tetraspanin family members in T-cells. Consistent with a role in metastasis, KAI1 and other tetraspanins are thought to stabilize molecular networks regulating motility, invasion, and other cellular processes (Hemler, 2005; Longo et al., 2001; Sridhar and Miranti, 2006; Sugiura and Berditchevski, 1999; Zhou et al., 2004a), many of which are already associated with metastasis.

Tetraspanins, including KAI1, interact with a multitude of other signaling molecules. KAI1 interacts with other tetraspanins, immunoglobulins, integrins, and histocompatibility molecules (Angelisova et al., 1994; Delaguillaumie et al., 2002, 2004; Horvath et al., 1998; Lee et al., 2004; Mannion et al., 1996; Mashimo et al., 2000; Shibagaki et al., 1999; Szollosi et al., 1996; Zhang et al., 2003). Palmitoylated KAI1 can directly interact with EGF receptor (Odintsova et al., 2003), which instigates EGFR endocytosis and migration signals. When palmitoylation was disrupted, motility and invasion were disrupted, suggesting that KAI1 might suppress metastasis by controlling cellular responses to external signals.

The most convincing studies suggesting a mechanism of action for KAI1 involve discovery that KAI1 directly interacts with a cell-surface molecule, DARC (Duffy antigen receptor for chemokines/gp-FY) on vascular endothelial cell surfaces. Watabe and colleagues, in a series of systematic and elegant studies, obtained data supporting a model in which KAI1–DARC

interaction induces tumor cell senescence via induction of the cyclin kinase inhibitor $p21^{WAF1}$ (Bandyopadhyay et al., 2006). Curiously, senescence occurred even in the absence of KAI1-dependent activation of DARC signaling (Horuk et al., 1993). Collectively, their model proposes that KAI1-expressing cancer cells grow and invade locally, but upon intravasation and interaction with DARC-expressing endothelial cells, the tumor cells cannot complete subsequent steps in the metastatic cascade.

3.3.1.4. KISS1R Although discussed in more detail below in the context of Section 3.3.3, the KISS1 receptor (KISS1R, a.k.a. GPR54, AXOR12, hOT7T175) appears to be critical for metastasis suppression in some tumor cells. Briefly, KISS1R is a G-protein-coupled receptor that is expressed almost ubiquitously at low levels, but is abundantly present in specialized neurons located within the hypothalamus, pituitary, and arcuate nucleus, where it is responsible for regulating pubertal development in the hypothalamic-pituitary-gonadal access (Beck and Welch, 2010; Colledge, 2009; Hameed and Dhillo, 2010; Oakley et al., 2009). Three laboratories had identified an orphan GPCR (Kotani et al., 2001; Muir et al., 2001; Ohtaki et al., 2001), which was subsequently shown to bind internal fragments derived from the KISS1 metastasis suppressor protein. Ohtaki et al. showed that overexpression of KISS1R in B16 melanoma cells diminished metastasis when mice were treated with KISS1-derived polypeptides (Ohtaki et al., 2001). However, an autocrine loop has not been formally established in any cell line that has not been transfected with the receptor (Beck and Welch, 2010).

In studies attempting to characterize whether KISS1- or KISS1-derived peptide(a.k.a. kisspeptins) secretion was required for metastasis suppression, a surprising finding was that none of the cell lines that were suppressed for metastasis following transfection and reexpression of KISS1 possessed detectable levels of the receptor, arguing that an autocrine loop was not responsible in the majority of cases. Thus, we speculated that paracrine signaling to surrounding stroma might be responsible for the metastasis suppressing effects of KISS1 (Beck and Welch, 2010; Nash and Welch, 2006). Beck and colleagues recently demonstrated that primary cultures derived from skin and lung differentially expressed KISS1R. Moreover, the primary cultures from lung secrete growth inhibitory signals more abundantly than the skin primary cultures (Beck, B.H. and Welch, D.R., unpublished observations). These findings illustrate how melanoma cells expressing KISS1 might be able to grow in the skin, but fail to grow after they have already disseminated (Nash et al., 2007).

3.3.1.5. OGR1 Another GPCR, ovarian cancer G-protein coupled receptor (OGR1, a.k.a. GPR58), when overexpressed in PC3 prostate cancer cells did not inhibit tumor cell proliferation and significantly

inhibited metastasis to multiple organs (Singh et al., 2007). To date, only preliminary mechanisms have been proposed regarding OGR1 function and these have been based upon the roles of related family members in mediating the functions of lysophospholipids (Xu, 2002).

Functionally, OGR1 regulates endothelial barrier function, proliferation, and tube formation as well as T-cell migration, glucocorticoid-induced thymocyte apoptosis, and globoid cell formations (Im et al., 2001; Kim et al., 2005b; Qiao et al., 2006; Radu et al., 2005; Tosa et al., 2003). Interestingly, OGR1 and other family members also exhibit proton sensing properties. Overexpression of OGR1 increased the levels of $G\alpha_{i1}$ transcription and translocation to the cell membrane concomitant with secretion of a hydrophobic factor which appears to be important for OGR1 antimetastatic actions (Singh et al., 2007).

3.3.1.6. DCC1 Deleted in colon cancer (a.k.a. UNC-40 or Frazzled) was first described as a tumor suppressor in colorectal cancer (Fearon et al., 1990). However, expression loss in late-stage cancers led to studies regarding its potential role in metastasis (Iino et al., 1994; Itoh et al., 1993; Kikuchi-Yanoshita et al., 1992; Ookawa et al., 1993). In esophageal (Miyake et al., 1994), bladder (Brewster et al., 1994), neuroblastoma (Ikeami et al., 1985; Reale et al., 1996), and glioma (Reyes-Mugica et al., 1997), DCC expression is lower in lymph-node metastases, invading, and disseminated cells. And further correlation has been described in an experimental model using Madin-Darby canine kidney cells, transfection with DCC resulted in decreased lymph-node and lung metastasis without impairing growth at the site of injection (Rodrigues et al., 2007). However, the results are not entirely clear because a tumor cell marker (i.e., luciferase) was significantly reduced despite overall tumor size appearing to be identical. This leaves open the possibility that a tumor suppressing effect was somehow mast by other cells that had been recruited to the site of tumor cell injection.

The mechanism of action for DCC is largely unclear because it has been implicated in so many diverse functions, such as axon guidance. Among the mechanisms by which DCC is thought to direct cellular movement is by induction of the apoptosome (Forcet et al., 2001). However, induction of apoptosis in metastatic cells has not been measured to the best of our knowledge.

3.3.2. Intracellular signaling molecules

Once cells have received a signal from the milieu, they must interpret and transmit appropriate signals to alter tumor cell function. The majority of metastasis suppressors identified to date are involved in signal transduction.

3.3.2.1. RKIP Raf Kinase Inhibitor Protein was discovered as a metastasis suppressor gene in prostate cells (Fu et al., 2003; Keller et al., 2004). Clinical data only shows a correlative relationship of RKIP as a metastasis suppressor

gene in breast cancer, that is, expressed in primary tumors, but absent in matched lymph-node metastases (Hagan et al., 2005).

RKIP binds directly to Raf, inhibiting MEK1 activation with a concomitant decrease of downstream signaling. Since MEK and RKIP compete to bind to RAF, the presumed mechanism of action is the regulation of ERK signaling. Interestingly, RKIP appears to selectively regulate Raf1, but not Braf (Trakul et al., 2005). This latter observation suggests that RKIP exerts antimetastatic effects only in certain cell types, but this has not been formally tested. Recently, in immune cells, RKIP has been implicated in NFκB and Snail signaling (Wu and Bonavida, 2009), which suggests that it plays roles in EMT and associated phenotypes.

Dangi-Garimella and colleagues recently showed that RKIP suppresses a metastasis signaling cascades involving the microRNA LIN28 and let-7 (Dangi-Garimella et al., 2009). They showed that inhibition of invasion by RKIP involves reduced MAPK signaling and decreased transcription of LIN28 by Myc. Correspondingly, reductions in LIN28 lead to enhanced let-7 processing and inhibition of the chromatin remodeling protein HMG2A, which is involved in regulating prometastatic genes such as Snail.

3.3.2.2. Nm23 Nm23 (nonmetastatic clone #23) was the first metastasis suppressor discovered. It was isolated from a differential colony hybridization screen using murine K1735 melanoma cell lines (Steeg et al., 1988). Since that time, seven other Nm23 family members have been identified, but only Nm23-H1 and Nm23-H2 have been demonstrated to suppress metastasis in experimental models (Lacombe et al., 2000). Metastasis suppression has been observed in multiple tumor types (Freije et al., 1998; Marshall et al., 2010).

Since it was the first discovered, Nm23 has been studied much more extensively than other metastasis suppressors in clinical samples. Generally, with the exception of neuroblastoma, Nm23 expression is inversely correlated with poor survival and tumor grade for breast, gastric, ovarian, non-small cell lung, hepatocellular, oral squamous cell carcinomas, and melanoma (Guan-Zhen et al., 2007; Hartsough and Steeg, 2000; Katakura et al., 2002; Mao et al., 2001; Niu et al., 2002; Terasaki-Fukuzawa et al., 2002; Wang et al., 2004). It is important to acknowledge that not all studies evaluating Nm23 in clinical specimens have revealed prognostic value. While this can be explained away because of tumor heterogeneity, technical issues have also contributed to muddying the literature. Antibody specificity is called into question for virtually all commercially available antibodies/antisera (Bordeaux et al., 2010), so readers are cautioned to be skeptical unless data validating antibody specificity is provided.

Transfection of Nm23 reduces motility in response to multiple growth factors *in vitro* (Kantor et al., 1993; Leone et al., 1993; Suzuki et al., 2004). Horak et al. showed that Nm23 downregulates the LPA receptor, EDG2,

and the HGF, c-Met (Horak et al., 2007a,b). EDG2 reexpression in Nm23-expressing cells completely restored motility in Nm23-H1-expressing cells while c-Met reexpression only partially restored motility, indicating that EDG2 regulation is closely associated with Nm23-induced metastasis suppression.

Using coimmunoprecipitation and yeast two-hybrid genetic analyses, Nm23 has been found to interact with Tiam1 (Kuppers et al., 2005), Rad (Tseng et al., 2001; Zhu et al., 1999), glyceraldehyde 3-phosphate dehydrogenase (Engel et al., 1998), vimentin (Pinon et al., 1999), various G-proteins (Kimura and Shimada, 1990), casein kinase 2 (CK2) (Biondi et al., 1996), and numerous other proteins (Salerno et al., 2003). Together, these findings implicate Nm23 in an extensive array of cytoskeletal organizing and signaling pathways. However, the physiological relevance of many interactors remains speculative because Nm23 is "sticky." Nm23 definitely directly interacts with and phosphorylates kinase suppressor of Ras (KSR) at Ser^{392}, possibly altering KSR binding to other proteins and preventing downstream activation of the MAPK pathway. This hypothesis is strengthened by the observation that Nm23-H1 transfectants show reduced basal and stimulated MAPK phosphorylation (Hartsough et al., 2002).

Four distinct activities have been reported for Nm23—NDP kinase (Biggs et al., 1990), histidine kinase (Freije et al., 1997), exonuclease (Ma et al., 2004), and maintenance of genomic stability (Kaetzel et al., 2009). Yet, it is still somewhat unclear which of these plays the critical role in suppressing cancer metastasis. NDP kinase- and exonuclease-disrupting mutants still suppress metastasis (to varying degrees), suggesting complex and overlapping roles in metastasis regulation (Kaetzel et al., 2006; MacDonald et al., 1993). Moreover, the activities associated with them vary by cell type, making extrapolation to other tumor histologic types risky.

Another approach to elucidate the mechanism of action for Nm23 has been to explore the proteome before and after Nm23 expression (Lee et al., 2009). Interestingly, pathway analysis revealed that posttranscriptional processing of RNA was the most commonly affected. Overexpression of Gemin5, which is involved in RNA splicing, affected the splicing patterns of several motility-, invasion-, and metastasis-associated gene-encoded RNA. Since it has been estimated that the human genome encodes approximately 30,000 genes with several hundred thousand potential splice variants (Black, 2000), this finding highlights the depressingly daunting task for sorting out the myriad changes that occur in metastatic cells.

Patricia Steeg and her laboratory continue to take the lead with regard to identifying ways in which metastasis suppressors, specifically Nm23, could help in the clinical management of cancer metastasis. Beyond the potential as a prognostic marker, they have shown that restored expression of a metastasis suppressor is a potentially viable therapeutic option (Marshall et al., 2010). The rationale for their strategy is based upon observations

that most, if not all, metastasis suppressors are infrequently mutated. Rather, their expression is downregulated in advanced cancers (Steeg, 2006; Steeg and Theodorescu, 2007). Therefore, administration of agents that selectively induce metastasis suppressor expression could be a therapeutic option. After promoter analysis, they proceeded to show that both dexamethasone and medroxyprogesterone acetate (MPA) induce Nm23 expression *in vitro*. Furthermore MPA-treated mice had significantly fewer metastases (Ouatas et al., 2002; Palmieri et al., 2005). These provocative and enticing experiments lead the way toward possible use of metastasis suppressor expression through external drug administration.

3.3.2.3. JNKK1/MKK7/p38 By combining MMCT and positional cloning, the Rinker-Schaeffer and Yamada laboratories identified JNKK1/MKK4 (SEK1/MEK4/MAP2K4) as a metastasis suppressor in prostate (Kim et al., 2001) and subsequently ovarian cancer (Yamada et al., 2002). Consistent with its role as a metastasis suppressor, expression has been found to be lower in poor prognosis patients with pancreatic, breast, or gastric cancers (Cunningham et al., 2006; Stark et al., 2004; Xin et al., 2004). However, clinical and experimental measurements of JNKK1 mRNA or protein expression have not always been consistent nor yielded similar conclusions. Expression is often higher in some high-grade tumor types or can promote tumorigenicity (Finegan and Tournier, 2010; Kim et al., 2001; Lotan et al., 2007). However, as emphasized by Taylor et al. (2008a,b), mere measurement of signaling protein levels is looking at the wrong parameter. Measurement of activation state (in this case phosphorylation status) is more relevant and informative.

In an experimental xenograft metastasis model of ovarian cancer, JNKK1/MKK4 activity correlated with growth arrest, but did not increase apoptosis (Lotan et al., 2008). In this model, JNKK1/MKK4 acted through the p38 arm of the SAPK pathway (Hickson et al., 2006). In a prostate cancer model, MKK7, a specific activator of JNK, caused reduced overt metastases more than 90%, compared with controls, just as was observed when the same AT6.1 cells were transfected with JNKK1/MKK4 alone (Vander Griend et al., 2005). However, ectopic expression of MKK6, a specific activator of p38, did not affect metastasis in the prostate model (Vander Griend et al., 2005).

Studies related to the role(s) of JNKK1/MKK4 in metastasis are challenging long-held notions that metastatic cells are selected for a universal ability to override growth inhibitory signals. Instead, the JNKK1/MKK4 data suggest that there may exert a reversible cell cycle arrest that occurs with signaling (in)activation state (Lotan et al., 2008). The very nature of the experimental models in which a small fraction of initially suppressed cells escape growth inhibition will be useful for defining the signals which enforce or oppose dormancy and, in a more clinically relevant way,

contribute to understanding how disseminated cells may break dormancy to form macroscopic metastases.

The careful, methodical studies of Rinker-Schaeffer and colleagues deserve reemphasis. Simplistic "-omic" measurements can, and often do, mislead. Moreover, the context of the cells and the stimuli impinging upon the tumor cell will alter the signaling cascades. Therefore, interpretation of all known variables and use of well-defined, specific reagents to measure the relevant activation states is essential.

3.3.2.4. RhoGDI2 RhoGDI2 is a member of a family of molecules that bind to Rho GTPases, sequester them in the cytosol keeping Rho proteins in the GDP-bound or inactive state. They do so by precluding Rho interaction with GTPase activating proteins (GAP) and guanine nucleotide exchange factors (GEF) (Ellenbroek and Collard, 2007; Karlsson et al., 2009). RhoGDI2 is a metastasis suppressor in bladder cancers (Theodorescu et al., 2004) and Hodgkin's lymphoma (Ma et al., 2007b), but has also been shown to promote metastasis in other cancers (Hu et al., 2007; Tapper et al., 2001; Wang et al., 2005; Zhang, 2006, 2005, 2009c).

In human bladder cancer, RhoGDI2 levels inversely correlate with development of metastatic disease. In fact, RhoGDI2 is an independent prognostic marker of recurrence following radical cystectomy (Theodorescu et al., 2004). However, approximately one-third of patients with high levels of RhoGDI2 protein develop metastases, suggesting that other mechanisms might regulate the metastasis suppressor functions of RhoGDI2, such as phosphorylation, protein complex partners, protein turnover, and subcellular localization.

RhoGDI2 has a relatively modest effect on RhoGTPase function; however, RhoGDI2 binds with Rac1 (Moissoglu et al., 2009), which can itself exert antimetastatic actions (Uhlenbrock et al., 2004), presumably by altering cytoskeletal structure and organization. However, when the associations of RhoGDI2 and the oncogene Src are taken into account, such as rare concurrent decreased levels, involvement of RhoGDI2 in Src signaling becomes an enticing possible mechanism of action (Wu et al., 2009). Src phosphorylation is known to modulate RhoGDI1– and RhoGDI2–RhoGTPase complex formation (Dermardirossian et al., 2006). Theodorescu and colleagues suggest that specific phosphorylation of RhoGDI2 by Src at Tyr^{153} may affect the metastasis suppressor function by specifically altering membrane-bound Rac1 (Wu et al., 2009).

3.3.2.5. DLC1 Deleted in liver cancer 1 (a.k.a. Rho-GAP 7 or START domain containing protein 12) was discovered in hepatocellular carcinoma (Yuan et al., 1998). Like RKIP and RhoGDI2, regulation of GTPase activity is presumably the mechanism by which DLC1 inhibits metastasis

(Goodison et al., 2005; Yuan et al., 2003), although this has yet to be proven. To term DLC1, a metastasis suppressor may be a misnomer because it can also act as a tumor suppressor, depending upon the cell line to which it is introduced (Yuan et al., 2003, 2004; Zhou et al., 2004b).

Even clinically, DLC1 gene expression is so commonly decreased in many human cancers (Durkin et al., 2007; Yuan et al., 2003) that its function is more likely as a tumor suppressor than as a metastasis suppressor. Yet, a recent report suggests that reduced expression in renal cell cancers correlates with increased invasion and poor prognosis (Zhang et al., 2009a). Thus, while DLC1 satisfies the definition of a metastasis suppressor in some cell lines and models, it does not in others.

3.3.2.6. DRG1 Drg1 (a.k.a. cap43/rit42/RTP/Ndrg1/TDD5) was discovered in colon carcinoma (Kurdistani et al., 1998) (van Belzen et al., 1997) and is found mostly in the cytoplasm. Following DNA damage, Drg1 expression increases and it translocates and accumulates in the nucleus (Kim et al., 2004; Kurdistani et al., 1998; Piquemal et al., 1999). Drg1 was first identified as a tumor suppressor in human bladder and pancreatic cancers (Kurdistani et al., 1998), but overexpression in breast, colon, and prostate cancer cell lines suppressed metastasis without suppressing tumorigenicity (Guan et al., 2000), a pattern which is supported in limited clinical studies in breast, prostate, and liver cancers (Bandyopadhyay et al., 2003; Chua et al., 2007).

DRG-1 appears to be downstream of many cancer- and metastasis-associated signaling pathways, such as p53 (Kim et al., 2004; Kurdistani et al., 1998), PI3K/PTEN (Bandyopadhyay et al., 2004), PKC (Fujii et al., 2008), hypoxia (Agarwala et al., 2000; Bandyopadhyay et al., 2003; Park et al., 2000), and in breast and prostate tumors, estrogens and androgens (Ulrix et al., 1999). Downstream mediators include VEGF and IL8 which are both involved in angiogenesis (Maruyama et al., 2006). Taken together, these findings suggest that DRG1 might control metastasis by controlling intravasation due to vessel integrity or by controlling colonization.

3.3.2.7. Gelsolin As discussed previously, cytoskeletal organization plays a major role in the ability of a cell to migrate. Gelsolin is a major actin-binding protein that has been shown to suppress metastasis in B16-BL6 mouse melanoma cells (Fujita et al., 2001). Depending on the cell line and model system, gelsolin may play either tumor suppressive or metastasis suppressive roles (Tanaka et al., 1995). More recently, gelsolin expression was correlated with metastatic potential in human colon adenocarcinoma cells (Litwin et al., 2009). Gelsolin is a member of a superfamily of calcium-dependent actin-binding proteins, and in response to extracellular stimuli, gelsolin modulates the reorganization of the actin cytoskeleton

(Dos Remedios et al., 2003). This regulation of cytoskeletal changes is most likely one of the key mechanisms for its ability to suppress metastasis.

3.3.2.8. SSeCKS/GRAVIN/AKAP12 Src is a well-known oncogene that alters cell signaling and cytoskeletal organization by direct phosphorylation of protein substrates and through transcriptional regulation of tumor-promoting or -suppressing genes (Dehbi and Bedard, 1992; Frame, 2004; Jove and Hanafusa, 1987). Src-suppressed protein Kinase C Substrate (SSeCKS) is the rodent ortholog of human GRAVIN and is an important regulator of cell signaling and cytoskeletal dynamics (Chapline et al., 1998; Gelman et al., 1998; Lin et al., 1996; Nelson et al., 1999). It was discovered using PCR-based subtractive hybridization in NIH3T3 mouse fibroblasts and was found to be suppressed by oncogenic src, ras, fos, and myc (Lin et al., 1996). Reexpression of SSeCKS in rat prostatic cancer MATLyLu cells resulted in significantly reduced lung metastasis in nude mouse models with only slightly decreased growth of the primary subcutaneous tumors (Su et al., 2006; Xia et al., 2001). Clinically, SseCKS/GRAVIN/AKAP12 is expressed in benign and well-differentiated prostate carcinomas, but not in highly aggressive and undifferentiated prostate lesions (Xia et al., 2001).

SSeCKS is phosphorylated by PKC and dynamically regulates cytoskeletal architecture by binding F-actin resulting in modulation of signaling pathways (Gelman et al., 1998; Lin et al., 1996). SSeCKS is also known as A-Kinase Anchor Protein 12 (AKAP12) since it has the ability to scaffold PKA through a C-terminal RII subunit binding motif (Nauert et al., 1997). In its dephosphorylated state, SSeCKS acts as a scaffolding protein where it binds signaling molecules such as PKC, PKA, calmodulin, and cyclins. Upon mitogenic signaling, SSeCKS is phosphorylated and translocates to the perinuclear membrane releasing signaling molecules to mediate changes within a cell (Lin et al., 2000). These changes induced from extracellular growth factors and adhesion of integrins to the ECM through SSeCKS has a significant impact on migration. Focal adhesions are also impacted by SSeCKS. Focal adhesions are formed at the plasma membrane when receptors bind ECM proteins and induce clustering of actin containing protein complexes leading to the recruitment and activation of the protein tyrosine kinase, FAK (Zhao and Guan, 2009). Phosphorylation of SSeCKS through stimulation by multiple growth factors has been shown to be FAK-dependent through mediation of an unidentified FAK-induced kinase (Xia and Gelman, 2002). This FAK-dependent phosphorylation of SSeCKS not only induces its release of signaling molecules, but reduces its F-actin-binding capability. Thus, SSeCKS acts to sequester growth factors and binds F-actin when in its dephosphorylated state during G_0 and early G_1 phases of the cell cycle. But upon mitogenic signaling, SSeCKS relinquishes actin binding and allows induction of signaling cascades leading to changes in the cytoskeleton.

In addition to cytoskeletal organization changes, SSeCKS also has been correlated with decreased levels of proangiogenic factors including HIF1, VEGF, FGF-7, angiopoietin, tenascin C, PDGF-R, and OPN and an increase in antiangiogenic factors vasostatin and col18 (Su et al., 2006; Xia et al., 2001). More recently, SSeCKS significantly decreased invasion through Matrigel that correlated with decreased MMP-2 levels and suppression of serum-induced activation of PKC-Raf/MEK/ERK pathway (Su et al., 2010). In that study, podosome formation was inhibited independent of the actin cytoskeleton, but inhibition of MEK/ERK activation required actin cytoskeletal remodeling. Taken together, SSeCKS suppression of metastasis appears to involve multiple mechanisms that impact cell motility, invasion, and angiogenesis.

3.3.2.9. HUNK The Hormonally Upregulated Neu-associated Kinase (HUNK) was recently identified as a suppressor of breast cancer metastasis by blocking actin polymerization leading to reduced cell motility (Quintela-Fandino et al., 2010), although it has previously been shown to promote metastasis (Wertheim et al., 2009). These discrepancies are likely the result of, as yet unresolved, context-dependent factors. HUNK was previously shown to be a kinase essential for mammary tumor metastasis through the restoration of invasion and migration (Wertheim et al., 2009). More recently, it has been shown to inactivate cofilin-1 (CFL-1) by preventing the binding of protein phosphatase 2-A (PP2A) to CFL-1 (Quintela-Fandino et al., 2010). In the latter report, no kinase activity was detected. Additional studies will be required to identify specific context-dependent functions of HUNK in metastasis.

3.3.2.10. Caspase 8 Death receptor-activated apoptotic pathways function by activation of caspase 8, which maps to human chromosome 2q33–q34. Addition of TNFα and FAS ligand activates caspase 8 by activating death domains which, in turn, cleave procaspase 8. Activated caspase 8 cleaves caspase 3, leading to apoptosis. Using a neuroblastoma model, Stupack et al. reexpressed caspase and found significant suppression of metastasis (Stupack et al., 2006). Subsequent analysis of relapsed glioblastoma multiforme tissues revealed hypermethylation and silencing of the caspase 8 gene (Martinez et al., 2007). Although not metastasis *per se*, the trend in glioblastoma is supportive of a metastasis-associated role.

Paradoxically, caspase 8 can promote migration in cells in which apoptotic machinery is compromised (Barbero et al., 2009). Caspase 8 was recruited to migration machinery following integrin ligation. While activity is not required for caspase 8-enhanced cell migration, association with FAK and calpain 2 was thought to be integrally involved. Thus, caspase 8 exemplifies the differential roles that a single molecule may play in cellular behavior, depending upon other molecules with which it interacts.

Additionally, caspase 8's role in anoikis is intriguing. Stupack has elegantly demonstrated that anoikis need not be exclusively the result of no adhesion. But rather, anoikis can be induced if cells adhere to a nonpreferred substrate (Cheresh and Stupack, 2002; Stupack and Cheresh, 2002). This observation has led to the hypothesis that analogous pathways might be involved in organotropism or failure to thrive following dissemination and arrest (Lahti et al., 2006).

3.3.2.11. Ribonucleotide reductase M1 The ribonucleotide reductase M1 (RRM1) gene maps to chromosome 11 and its reexpression in the murine lung carcinoma cell line, Line 1, met the functional definition for a metastasis suppressor (Gautam and Bepler, 2006). Genome comparisons showed that LOH was observed in approximately half of informative lung tumor specimens, but mutation was not observed (Pitterle et al., 1999), but correlation with human lung cancer metastasis was not determined. RRM1 converts ribonucleotides to deoxyribonucleotides for DNA synthesis, suggesting that it may regulate dNTP pools which, in turn, would determine proliferative capacity. However, this hypothesis has not been tested. Interestingly, RRM1 has been found to upregulate PTEN and reduce FAK phosphorylation. In addition, RRM2, a related ribonucleotide reductase, has been studied with regard to cellular responsiveness to DNA damage and repair. Whether similar functions might alter tumor aggressiveness has not yet, to our knowledge, been tested.

3.3.3. Extracellular signaling molecules and KISS1/kisspeptins

Metastasis is clearly determined, to a sizable extent, by tumor–host interactions, that is, the microenvironment participates in the induction and selective proliferation of malignant cells. Host physiology can foster or reject neoplastic cells. It stands to reason, then, that some metastasis suppressors might work because they render the ectopic microenvironment less hospitable. The most obvious example of cell–cell signaling relates to inflammatory cells that infiltrate the tissue adjoining a tumor or disseminated cell in response to secreted cytokines, chemokines, or hormones. The immune populations can produce pro- or antitumor molecules that alter the capacity of tumor cells to migrate, invade, or survive (Fridlender et al., 2009; Mantovani et al., 2006). However, it is imperative that other stromal populations not be ignored in the interplay.

The milieu surrounding a tumor cell undoubtedly explains metastatic organotropism. Yet, despite more than a century of attempts, clear-cut biochemical explanations still do not exist. Nonetheless, some clues may be forthcoming from understanding the mechanisms of action of extracellular metastasis suppressors.

High-frequency deletions or rearrangements involving chromosome 6q in late-stage melanoma prompted introduction of full-length chromosome

6 into the human metastatic melanoma cell line C8161 by MMCT (Welch et al., 1994b). *KISS1* was subsequently identified using subtractive hybridization between metastatic and nonmetastatic cell line variants (Lee and Welch, 1997a; Lee et al., 1996, 1997). Transfection of full-length KISS1 cDNA into melanoma (Lee and Welch, 1997a; Lee et al., 1996, 1997), breast carcinoma (Lee and Welch, 1997b), ovarian (Jiang et al., 2005), and pancreatic adenocarcinoma (McNally et al., 2010) cell lines suppressed metastasis in spontaneous and experimental metastasis assays.

Unexpectedly, the *KISS1* gene mapped to the long arm of chromosome 1. Using cDNA microarrays and chromosome 6 MMCT donors with defined deletions on the long arm of chromosome 6, Goldberg et al. found that *KISS1* was regulated by TXNIP and CRSP3 (see Section 3.1.2; Goldberg et al., 2003). The *KISS1* gene encodes a 154 amino acid protein but full-length KISS1 is rarely detectable since the nascent protein is proteolytically processed into numerous polypeptides, termed kisspeptins (Kotani et al., 2001; Ohtaki et al., 2001). An internal 54-amino acid polypeptide, termed kisspeptin (KP)-54 or metastin, binds to the KISS1 receptor (Kotani et al., 2001; Muir et al., 2001; Ohtaki et al., 2001). KISS1R expression is highest in placenta, pituitary gland, pancreas, brain, and spinal cord (Kotani et al., 2001; Muir et al., 2001), while *KISS1* expression is more restricted, located primarily in the placenta, pancreas, kidney, and the arcuate nucleus of the hypothalamus (Lee et al., 1996; Muir et al., 2001; Ohtaki et al., 2001).

KP processing is still not understood (Nash and Welch, 2006; Nash et al., 2007). However, KISS1 protein is thought to be cleaved by furins or prohormone convertases (Kotani et al., 2001; Ohtaki et al., 2001) based upon the amino acids at the ends of the KP. The preponderance of primary literature on KISS1 and KISS1R relates to the involvement of these molecules in pubertal development (Colledge, 2009; Gianetti and Seminara, 2008; Roa et al., 2008; Tena-Sempere, 2008). Since this is not the focus of this chapter, those aspects of KISS1 biology are not highlighted here.

Clinical reports from a variety of tumor types generally support a positive correlation between KISS1 expression and metastasis-free survival and other progression-associated phenotypes (Beck and Welch, 2010; Dhar et al., 2004; Guan-Zhen et al., 2007; Hata et al., 2007; Ikeguchi et al., 2004; Katagiri et al., 2009; Kostadima et al., 2007; Martin et al., 2005; Masui et al., 2004; Nagai et al., 2009; Prentice et al., 2007; Shirasaki et al., 2001; Stark et al., 2004). The key exception has been a positive correlation with hepatocellular carcinoma progression (Ikeguchi et al., 2003; Schmid et al., 2007), but a recent immunohistochemical study suggests that KISS1 might be significantly inversely associated with stage and intrahepatic metastasis from HCC (Shengbing et al., 2009). As described above, many of these studies measured only mRNA expression using *in situ* hybridization or PCR-based methods. The former are less ambiguous than studies in

which stromal cells contaminate the cell preparation, making it impossible to judge the origins of KISS1 or KISS1R. mRNA measurements were required because of difficulties in generating specific antibodies. Most commercially available antibodies used have not been validated or the data have not been published (Beck and Welch, 2010; Bordeaux et al., 2010). Since KISS1 is processed and (presumably, though not formally proven) not all KP are biologically active, nonprotein-based studies should be interpreted with caution. To the best of our knowledge, no one has yet evaluated KISS1 → KP processing in clinical samples.

Surprisingly, based upon the nature of the experimental studies showing KISS1 functionality as a metastasis suppressor, many tumor cells suppressed by KISS1 reexpression do not express KISS1R (Nash et al., 2007). This has led us to postulate a paracrine feedback loop in which KISS1/KP secreted by tumor cells acts upon one or more stromal populations which, in turn, respond with growth-inhibitory factors (Beck and Welch, 2010; Nash and Welch, 2006).

4. Conclusions and Perspectives

The process of metastasis is obviously very complex and involves intrinsic and extrinsic factors. In this chapter, we have focused on genetic changes, specifically metastasis suppressors, in tumor cells, but as the data were collated and as the field matured, awareness that the function of metastasis regulatory genes were not functioning autonomously became acute. Even with incomplete knowledge regarding the interrelationships of tumor cells with their microenvironments, some patterns are emerging. The basic observation that neoplastic cells expressing metastasis suppressors grow at orthotopic sites but fail to successfully complete the metastatic process illustrates that the mechanisms of action for the suppressors are context sensitive. Although many of the metastasis suppressors inhibit metastasis in different histiotypes, most have not been extensively evaluated (largely because there are so few metastasis models). Several examples of metastasis suppressors showing opposite effects (e.g., worse prognosis or more aggressive behaviors for Nm23 in neuroblastoma or KISS1 in hepatocellular carcinoma) further highlight that the cellular backgrounds are crucial variables for understanding mechanism.

Metastasis suppressor mutations are rare. Instead, expression is downregulated in advanced cancers. Notwithstanding the apparent position downstream of some oncogenic signaling pathways which may be complicit in driving tumor progression toward metastasis, the undeniable inference is that the expression of metastasis suppressors can change under varying circumstances. Among the potentially altered queues are the cofactors with which many metastasis suppressor interact. Such cofactors can interact

directly (e.g., BRMS1 with components of SIN3:HDAC complexes), indirectly as part of quaternary protein complexes (e.g., LSD1 with NuRD chromatin remodeling complex components or Nm23 as part of MAPK scaffolding) or indirectly as nodes within signaling cascades (e.g., SSeCKs or JNKK1 receiving and passing along signaling).

If we couple the observations involving metastasis suppressors with literature emphasizing that three-dimensional architecture (Debnath and Brugge, 2005; Nam et al., 2010) and cellular tension (Butcher et al., 2009) alter cellular behavior and the irrefutable fact that *in vitro* assays are inadequate to study metastasis, then a major priority needs to be developing experimental manipulable assays that more faithfully model different microenvironments in which tumor cells find themselves. Some recent advances in recapitulating elements of more complex *in vivo* milieus are chipping away at this problem (Mendoza et al., 2010), but more concerted efforts are needed.

This basic research effort has practical applications related to metastasis treatment. Recall that *all* treatments beyond surgical removal of the primary tumor are designed to prevent new or eliminate already established metastases. Since the disease is systemic, therapy must also be systemic. Unfortunately, most cancer treatments are extremely toxic; contribute to a poor quality of life during the therapy; and are only modestly effective. The natural products represented by metastasis suppressors might offer some opportunity for therapeutic development and improvements in one or more of these shortcomings. Of course, the usual caveats regarding gene replacement, drug targeting, and other pharmacokinetic parameters must be taken into account. However, use of intrinsic molecules would appear to offer some advantages.

Two potential treatments invoking metastasis suppressors include restoration of expression (Marshall et al., 2009; Ouatas et al., 2003; Palmieri et al., 2006) or to utilize the metastasis suppressor gene product(s) directly (Beck and Welch, 2010; Nash and Welch, 2006). The latter, if the kisspeptins remain as nontoxic as they currently appear in other clinical settings (Mead et al., 2007; Niida et al., 2006; Ramaswamy et al., 2007), look promising, especially with regard to the potential utility against seeded, but not yet overt metastases. Our current understanding of KISS1 is that it actually targets stromal populations, which means that the treatment would be directed to supposedly more genetically stable cell populations, which is good. However, similar thought processes were involved when designing antiangiogenic drugs (Folkman, 2006). The assumption of genetic stability may not have been entirely accurate (Hida and Klagsbrun, 2005; Hida et al., 2004). Moreover, relatively incomplete knowledge of full interplay between tumor cells and endothelial cells probably contributed to the unexpectedly enhanced metastatic progression observed in anti-VEGFR-treated mice (Ebos et al., 2009; Paez-Ribes et al., 2009).

In addition to highlighting deficiencies in current knowledge related to tumor–stromal interactions, those two papers illustrate the essential need to incorporate metastasis studies into preclinical testing of anticancer agents (Steeg et al., 2009). Regardless, the clinical need is enormous and unmet. The daunting complexity of the metastasis suppressor function upon which genetic background and extrinsic signals are superimposed should not paralyze nor discourage. We hope that, by assembling the facts, a level of clarity and perspective have emerged that will facilitate more rapid translation into clinical practice.

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CHAPTER FOUR

Dynamic Behavior of Double-Membrane-Bounded Organelles in Plant Cells

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Contents

Abstract

In plant cells, different kinds of single- and double-membrane-bounded cell organelles exhibit dynamic changes in their morphology, motility, and distribution patterns. The dynamic behavior of organelles plays crucial roles intimately associated with plant development and/or adaptive responses to environmental fluctuations. Recent progress in techniques for the visualization of cell organelles and cytoskeletal components has provided useful systems to dissect

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these complex processes, and revealed a number of striking features of plant organelle dynamics. This chapter summarizes recent findings on dynamic behavior of nuclei, mitochondria, and plastids in plant cells, focusing on imaging analyses and regulatory proteins.

Key Words: Cytoskeleton, Double-membrane-bounded organelle, Mitochondrion, Nucleus, Plant cell, Plastid.

1. Introduction

One unique characteristic of plant cells is the active movement of intracellular contents. Vigorous streaming of the cytoplasmic matrix is a basic feature of intracellular movement, and the mechanisms and significance of cytoplasmic streaming of multiple modes have long been attractive subjects for many researchers (Shimmen, 2007; Verchot-Lubicz and Goldstein, 2010). Together with, or independent of, cytoplasmic streaming, different kinds of single- and double-membrane-bounded cell organelles exhibit dynamic changes in their morphology, motility, and distribution patterns in plant cells. Recent progress in visualization techniques for intracellular components, including cell organelles and cytoskeleton, based on light microscopy of transformed cells expressing fluorescent fusion proteins, has enabled us to reevaluate and revisit the dynamic behavior of cell organelles. From the late 1990s, researchers have succeeded in visualizing not only single-membrane-bounded organelles such as the endoplasmic reticulum (ER) (Ridge et al., 1999), Golgi apparatus (Boevink et al., 1998), vacuoles (Di Sansebastiano et al., 1998), vacuolar membrane (tonoplast) (Saito et al., 2002; Uemura et al., 2002), and peroxisomes (Mano et al., 2002) but also double-membrane-bounded organelles, such as nuclei (Chytilova et al., 2000), mitochondria (Köhler et al., 1997a), and nongreen plastids (Köhler et al., 1997b), in living plant cells. These innovating methodologies combined with forward- and reverse-genetic approaches have reinforced our accumulated knowledge signifying that the dynamic behavior of organelles is intimately associated with plant development and/or short- and long-term adaptive processes to specific environmental conditions, as well as creating a method for investigating the molecular mechanisms underlying plant organelle dynamics.

Plants possess three prominent double-membrane-bounded organelles: nuclei, mitochondria, and plastids. Mitochondria (Lang et al., 1999) and plastids (Gould et al., 2008) are thought to evolutionally derived from α-proteobacteria and photosynthetic ancestral cyanobacteria, respectively, through endosymbiosis. The content of DNA in mitochondria and plastids is much less compared with that in their present prokaryotic relatives, which

has resulted from "intercompartmental DNA transfer"; the redistribution and rearrangement of genetic materials among nucleus, mitochondria, and plastids proceeded during and after the establishment of endosymbiosis (Kleine et al., 2009a; Timmis et al., 2004). However, the expression of many of the genes transferred into the nuclear genomes of host cells is in turn under the control of mitochondria- and plastids-mediated processes in multiple ways (Kleine et al., 2009b; Woodson and Chory, 2008; Yang et al., 2008a), called "retrograde regulation." For knowing more specifically how such genetic interactions enable plants to achieve an efficient life cycle and survival under acute environmental conditions, more sophisticated and precise analyses of the behavior of organelles at the cellular level may be indispensable. In this chapter, we summarize recent findings on dynamic behavior of double-membrane-bounded organelles in plant cells, especially focusing on nuclei and mitochondria. Since actin and microtubule cytoskeletons play central roles in the regulation of morphology and movements of organelles, we also survey recent information on plant cytoskeletal components involved in the organelle dynamics.

2. Nuclear Positioning in Plant Cells

In many textbooks, the nucleus is conveniently illustrated to be located in the center of the cell. However, nuclei in most eukaryotes have the ability to move to take a particular position within cells. From fungi to mammals, nuclear positioning underlies a variety of developmental processes, including fertilization, cell division, cell growth, cell migration, and the establishment of polarity (Starr, 2009). In filamentous fungi, it is well known that nuclei maintain a more or less regular distribution along the mycelium (Fischer, 1999; Xiang and Fischer, 2004; Xiang and Morris, 1999). Other good examples may be the migration of the nucleus into the bud during budding and during karyogamy in yeasts (Fischer, 1999; Gladfelter and Berman, 2009; Suelmann and Fischer, 2000), the movement of nuclei to the egg cortex during embryogenesis in flies (Wilhelmsen et al., 2006), the congression of male and female pronuclei during fertilization (Reinsch and Gönczy, 1998), and the elevator-like interkinetic nuclear migration observed in neuroepithelium (Norden et al., 2009). Also in plants, nuclear positioning is necessary for developmental processes, including the cell cycle progression and cell growth (Britz, 1979; Ketelaar et al., 2002). However, one of the most exciting findings in nuclear positioning in plants is that nuclei become relocated in response to environmental stimuli, such as mechanical and light stimulation (Iwabuchi and Takagi, 2008; Nagai, 1993). However, a large part of molecular mechanisms for nuclear positioning in plants remains to be elucidated. In this chapter, we first

introduce nuclear positioning and its regulation examined in plant cells, and then those in other organisms comparing with the knowledge obtained in plants.

2.1. Nuclear positioning associated with plant development

Nuclear positioning is observed with the cell cycle progression. Proper positioning of the nucleus is essential for cell division, especially for asymmetric divisions (Menke and Scheres, 2009; Smith, 2001), which are critical events in the normal pattern formation of plants. The asymmetric division, in which the future division plane is placed unevenly within a cell, generates two daughter cells with different sizes and fates to provide cell-type diversity for multicellular organisms. Stomatal formation is a good example performed through a series of asymmetric divisions and progressive fate transitions within a stem cell lineage in leaf epidermis. Prior to the asymmetric division of the meristemoid mother cells in *Arabidopsis thaliana* leaves, the nucleus and most of the cytoplasm become located unevenly on one side of the cell, near the future division site (Bergmann, 2006; Nadeau and Sack, 2003; Zhao and Sack, 1999). In dicotyledonous plants, many asymmetric divisions further occur in the neighboring cells adjacent to a stoma or stomatal precursor, the process in which nuclei are located away from the stoma or precursor, regulating the number and patterning of stomata (Geisler et al., 2003). Asymmetrical nuclear distribution is also seen in the subsidiary mother cells of monocotyledonous plants (Kennard and Cleary, 1997; Pickett-Heaps and Northcote, 1966; Smith, 2001). In subapical protonemata cells of the moss *Physcomitrella patens*, the position of the nucleus predicts the site of side branch formation (Doonan et al., 1986).

The importance of nuclear positioning is also obvious in tip-growing cells. During tip growth, the growing point is determined at one end of the cell, resulting in the formation of unidirectionally elongated cells (Baluska et al., 2003). Root hair cells, derived from specialized epidermal cells of the root termed trichoblasts, exhibit typical tip growth. In root hair cells of *A. thaliana*, nuclei are positioned at a fixed distance from the growing apex (Chytilova et al., 2000; Ketelaar et al., 2002). This positioning is disturbed by an artificial entrapment of the nucleus by a laser beam, consequently resulting in the cessation of cell growth (Ketelaar et al., 2002). In the *cow1-2* (can of worms) mutant defective in phosphatidylinositol transfer protein, which has several hairs in the individual trichoblasts (Böhme et al., 2004), the growth is normal in one hair with the nucleus but abnormal in other hairs without it (Ketelaar et al., 2002). This may suggest that nuclear positioning is essential for efficient tip growth; however, this idea is not applicable to ROP2 (Rho-related GTPase from plants) overexpressing plants (Jones and Smirnoff, 2006). Although its phenotype was similar to that of the *cow1-2* mutant, the growth rate of root hair cells in the ROP2

overexpressing plants was almost identical irrespective of the presence of the nucleus. Nuclear positioning with a constant space from the apex is retained until the cell fully grows, and after maturation of the cell, the nucleus moves randomly (Ketelaar et al., 2002; Van Bruaene et al., 2003). Other than root hair cells, nuclear positioning during cytomorphogenesis has been reported in pollen tubes (Åström et al., 1995; Heslop-Harrison and Heslop-Harrison, 1989a,b), protonemata in ferns (Kadota and Wada, 1995; Wada, 1995), and coenocytic algae (Takahashi et al., 2001).

2.2. Nuclear positioning in response to environmental stimuli in plants

2.2.1. Mechanical stimulation

Nuclear positioning in plant cells can be affected by changes in environmental conditions. Wounding, a serious mechanical stimulation that causes cell death at the wounded site, is known to induce nuclear migration in cells adjacent to the wounded site, called "traumatic nuclear migration." In general, the nucleus migrates in the direction of the wounded site and thereafter returns to the original position (Nagai, 1993). However, relatively moderate mechanical stimulation, such as touch, wind, and drought stress that causes changes in turgor pressure of cells, can also induce nuclear migration in stimulated cells. This movement is thought to be the first prominent intracellular response to mechanical stimulation. Upon a local application of pressure to the surface of *Tradescantia virginiana* leaf epidermal cells, the nucleus migrated toward the stimulation site within several tens of minutes and stayed there (Kennard and Cleary, 1997). In leaf hair cells of *Nicotiana tabacum*, touch-induced nuclear migration was repeatedly induced; the nucleus can respond to a second stimulation without loss of velocity, whether it was in a resting or moving state (Qu and Sun, 2007). During the course of studies on the nonhost resistance response to the fungus *Phytophthora sojae* in suspension cultured cells of *Petroselinum crispum*, Gus-Mayer et al. (1998) found that local mechanical stimulation by a needle without elicitor induced the translocation of nuclei as well as cytoplasm to the site of stimulation. These results indicate that plant nuclei have the ability to quickly respond to mechanical stimulation with a high sensitivity.

2.2.2. Light

The position of the nucleus in plant cells is also affected by light. Light-dependent nuclear positioning was found for the first time in the fern *Adiantum capillus-veneris*. Kagawa and Wada (1993) showed that nuclei in prothallial cells are located along the anticlinal walls in darkness, while under weak light they migrate toward the outer periclinal walls facing the light. In cells irradiated with strong light following weak light, nuclei move from the outer periclinal walls back to the anticlinal walls (Tsuboi et al., 2007).

Such nuclear movement was induced repeatedly and specifically by either blue or red light (Kagawa and Wada, 1993). Nuclear repositioning from the anticlinal to outer periclinal walls in weak light took less than 8 h, whereas that from the outer periclinal to anticlinal walls in darkness took about 36 h (Kagawa and Wada, 1993). Light-dependent nuclear positioning is also seen in seed plants. Iwabuchi et al. (2007) reported that, in epidermal and mesophyll cells of *A. thaliana* leaves, nuclei are distributed along inner periclinal walls in darkness, while under strong light, nuclei become relocated to anticlinal walls (Fig. 4.1A–D). Nuclear repositioning from the inner to anticlinal walls was induced repeatedly and specifically by blue light of high-fluence rates (more than 50 μmol m^{-2} s^{-1}). Interestingly, the weak light-induced relocation of nuclei toward the outer periclinal walls is obvious in *A. capillus-veneris* but not in *A. thaliana*.

Plants possess diverse and specific photoreceptor systems to sense light conditions. Experiments with polarized red or blue light have suggested that phytochrome and the blue light receptor function as photoreceptors mediating the light-dependent nuclear positioning in *A. capillus-veneris* (Kagawa and Wada, 1995). Phytochrome is equipped with a single linear tetrapyrrole chromophore and exhibits characteristic photoreversible conversion

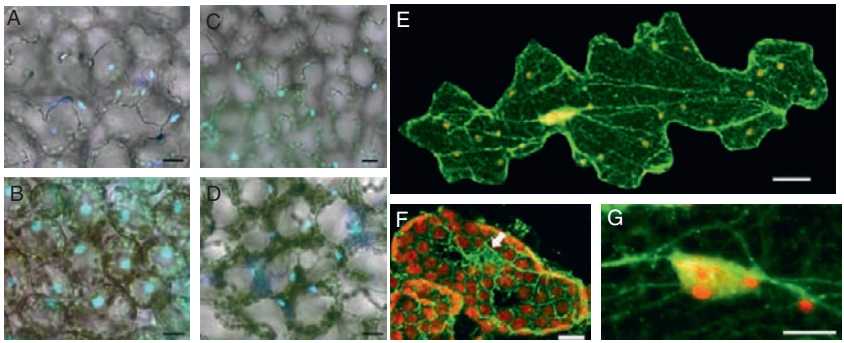


Figure 4.1 Nuclear distribution and actin organization in *Arabidopsis thaliana* leaf cells. (A–D) Nuclear distribution in darkness and light. (A and B) Dark-adapted epidermal and mesophyll cells for 16 h, respectively. (C and D) Epidermal and mesophyll cells continuously irradiated with blue light at 100 μmol m^{-2} s^{-1} for 5 h, respectively. Nuclei were stained with Hoechst 33342 and are shown in blue. All are merged images of bright-filed and fluorescence images. (E–G) Nuclei and actin organization. (E and F) Dark-adapted epidermal and mesophyll cells for 16 h, respectively. (G) Magnified nucleus and actin filaments in epidermal cell. Nuclei (red) were stained with propidium iodide and actin filaments (green) were visualized by immunofluorescence microscopy. In (E) and (G), nuclei appear yellow as a result of nuclear and actin staining. In (F), the nucleus was not stained but its position can be found (arrow). Red signals except for nuclei show autofluorescence of plastids (E and G) and chloroplasts (F). Bars = 20 μm.

between two spectrophotometrically and structurally distinct forms (Bae and Choi, 2008; Franklin and Quail, 2010). Phototropin is a blue-light receptor containing two light, oxygen, and voltage domains in the N-terminus, which bind a chromophore flavin mononucleotide and a serine/threonine kinase domain in the C-terminus, which is required for autophosphorylation of the protein (Briggs et al., 2001; Christie, 2007). Mutant analyses have demonstrated that phototropin and neochrome1, a chimeric protein consisting of the chromophore-binding domain of phytochrome and the full length of phototropin (Nozue et al., 1998; Suetsugu et al., 2005b), are the genuine photoreceptors for nuclear positioning in *A. capillus-veneris* (Tsuboi et al., 2007). Also in *A. thaliana*, it has been demonstrated that phototropin2 (Jarillo et al., 2001; Kagawa et al., 2001; Sakai et al., 2001), but not phototropin1 (Huala et al, 1997), mediates light-dependent nuclear positioning (Iwabuchi et al., 2007, 2010). Phototropin2 likely controls nuclear positioning in darkness both in *A. capillus-veneris* (Tsuboi et al., 2007) and in *A. thaliana* mesophyll cells (Iwabuchi et al., 2007), but not in *A. thaliana* leaf epidermal cells (Iwabuchi et al., 2010).

The physiological significance of light-dependent nuclear positioning remains to be elucidated. The response may have different role(s) from nuclear movement accompanying the cell division and cytomorphogenesis, as it has been observed in cells that exhibit neither cell division nor cell growth (Kagawa and Wada, 1993). In strong light, the mode of nuclear positioning in *A. thaliana* leaf cells (Fig. 4.1D) is very similar to that of chloroplast positioning (Trojan and Gabryś, 1996), in which the physiological role is believed to avoid photodamage induced by excess light (Kasahara et al., 2002). Consequently, we can assume that light-dependent nuclear positioning may serve to protect nuclear DNA from damage under excess light conditions, such as those experienced at midday during summer, which includes UV radiation—a potent mutagen (Britt, 1996).

The photoreceptor phototropin2 and the actin cytoskeleton (see below) are involved in positioning of both organelles, suggesting that plants may use similar mechanisms to position both nuclei and chloroplasts. However, organelle-specific positioning mechanisms should be present, considering the following data obtained in experiments using *A. capillus-veneris*. First, a brief irradiation can induce chloroplast movement (Kagawa and Wada, 1994), whereas a longer irradiation was required to induce nuclear movement (Kagawa and Wada, 1995). Second, chloroplasts took a shorter time than the nuclei to migrate from the anticlinal to outer periclinal walls (2 h vs. 8 h) under horizontally polarized red or blue light (Kagawa and Wada, 1995). Third, the chloroplasts lay just beneath the plasma membrane, whereas the nuclei were positioned beneath the chloroplast layer, namely, closer to the vacuole (Kagawa and Wada, 1993). Finally, Oikawa et al. (2003) isolated an *A. thaliana* mutant defective in chloroplast photorelocation movement, named *chup1* (chloroplast unusual positioning), possessing a

mutation in a plausible actin-binding protein. In the *chup1* mutant, chloroplasts formed an abnormal aggregate along the inner periclinal wall and no chloroplast movement was observed under any light conditions. Intriguingly, positioning of nuclei appeared normal. This further supports the idea that the positioning of the nucleus and chloroplast is independently controlled by largely similar, but partly distinct, mechanisms (Iwabuchi and Takagi, 2010).

2.3. Regulation of nuclear positioning by cytoskeleton

2.3.1. Developmental processes

In general, the cytoskeleton is the main factor controlling organelle dynamics. Three fundamental protein components, microtubules, actin filaments, and intermediate filaments, build up the intricate cytoskeletal network throughout the cell. Cell organelles appear to be linked to the cytoskeleton and are transported along the filamentous structures of the network with aid of the interaction with motor proteins (Gross et al., 2007; Vale, 2003). Kinesins are plus-end-directed and dyneins are minus-end-directed microtubule-based motors, while myosins are barbed-end-directed actin-based motors. Kinesins and kinesin-related proteins constitute a superfamily, containing over 60 genes in *A. thaliana*, and play vital roles in a wide spectrum of cellular activities (Lee and Liu, 2004; Reddy and Day, 2001a). Dynein genes appear to be absent from the genome of *A. thaliana* (Lawrence et al., 2001). One of the possible reasons for this conspicuous absence may be the presence of numerous kinesin species of minus-end directed motor types in *A. thaliana* (Lee and Liu, 2004). Myosins are ubiquitous motor proteins that have diversified in different evolutionary lineages with specific functions among eukaryotic organisms (Richards and Cavelier-Smith, 2005). Flowering plants possess two major classes of myosin: myosin VIII and XI (Berg et al., 2001; Reddy and Day, 2001b).

Taking advantage of specific cytoskeleton-modifying chemicals such as amiprophos-methyl and oryzalin, microtubule-disrupting reagents, and cytochalasins and latrunculins, actin-depolymerizing reagents, many researchers have demonstrated the involvement of microtubules and actin filaments in nuclear positioning during plant development, for example, cell division (Britz, 1979) and tip growth (Chytilova et al., 2000; Ketelaar et al., 2002). The mode and extent of the contribution of the microtubule and actin cytoskeletons to those processes differ from species to species, as well as from response to response. Premitotic nuclear migration during asymmetric cell division is actin dependent in *Allium cepa* (Mineyuki and Palevitz, 1990), *Secale cereale* (Cho and Wick, 1991), and *T. virginiana* (Kennard and Cleary, 1997), while microtubules and actin filaments are both required in *Cucumus sativus* (Kazama et al., 1995). Premitotic nuclear migration in tobacco BY-2 (*N. tabacum* "Bright Yellow 2") suspension cultured cells is

microtubule dependent (Katsuta et al., 1990), whereas postmitotic nuclear migration is actin dependent (Katsuta and Shibaoka, 1988). In the case of nuclear migration during tip growth, basipetal nuclear migration in most, but not all, of the species is likely an actin-dependent process. Acropetal nuclear migration is microtubule dependent in *Vicia hirsuta* root hair cells (Lloyd et al., 1987) and *Funaria hygrometrica* protonema (Schmiedel and Schnepf, 1980), while the dual involvement of mictotubules and actin filaments was reported in *A. capillus-veneris* protonemal cells (Kadota and Wada, 1995).

Nuclear movement in *A. thaliana* root hair cells appears to be actin dependent (Chytilova et al., 2000; Ketelaar et al., 2002). Furthermore, Ketelaar et al. (2002) showed that the injection of an antibody against plant villin, a Ca^{2+}-sensitive bundling protein of actin filaments (Yokota et al. 2005), into the root hairs of *A. thaliana* led to the unbundling of the actin filaments and displacement of the nucleus closer to the apex. Bundling of actin filaments may be required for the maintenance of the nucleus at the close proximity to the growing tip of the cell. Other research showed that nuclear movement in *A. thaliana* root hair cells is an energy- and magnesium-dependent process (Sliwinska et al., 2002). Several reports have suggested the implication of myosin activities in nuclear movement during tip growth of angiosperm pollen tubes (Heslop-Harrison and Heslop-Harrison, 1989b), *A. thaliana* root hair cells (Chytilova et al., 2000), and in tobacco BY-2 cells (Hoffmann and Nebenführ, 2004). These results raise the possibility that the actomyosin system serves to drive nuclear movement not only during tip growth but also during the cell cycle progression.

2.3.2. Responses to environmental stimuli

There have been also a number of studies investigating the role of the cytoskeleton in nuclear positioning induced by touch or injury (Kennard and Cleary, 1997; Nagai, 1993). Recently, it has been reported that light-dependent nuclear positioning in *A. thaliana* leaf cells is dependent exclusively on the actin cytoskeleton (Iwabuchi et al., 2010). Disruption of actin filaments inhibited both nuclear positioning from the inner to anticlinal walls in strong blue light and repositioning from the anticlinal to inner periclinal walls following return to darkness. Immunofluorescence microscopy of actin organization revealed that the nucleus in the dark-adapted epidermal cell was associated with thick actin bundles running along the inner periclinal wall roughly parallel to the longest axis of the cell (Iwabuchi and Takagi, 2010; Fig. 4.1E and G). The actin bundles, especially in the vicinity of the nuclei, were rearranged close to the anticlinal walls in a blue-light-dependent manner (Iwabuchi et al., 2010). In the case of mesophyll cells, numerous actin filaments can be seen running in a meshwork fashion and seem to be associated with the nucleus and encircling each chloroplast; however, it is difficult to distinguish actin filaments that are specifically associated with the

nucleus from others, due to the presence of plenty of chloroplasts within the cells (Fig. 4.1F). In epidermal cells of the phototropin2-deficient mutant, no apparent changes in actin reorganization were visible (Iwabuchi et al., 2010). Thus, blue-light-dependent nuclear positioning in *A. thaliana* leaf epidermal cells is thought to be regulated by phototropin2-mediated reorganization of the actin cytoskeleton. Phototropin-dependent signaling pathway involved in regulation of nuclear positioning requires further investigation.

How does the nucleus take a particular position within a cell? Katsuta and Shibaoka (1988) argued that actin filaments connect the nucleus and the plasma membrane, and generate tension between them. Tension in the perinuclear scaffold of actin and microtubule cytoskeletons was also invoked to explain nuclear positioning in the green alga *Spirogyra crassa* (Grolig, 1998). In tip-growing hyphae of the fungus *Basidiobolus magnus*, McKerracher and Heath (1986) reported that UV microbeam irradiation to the anterior and posterior regions to the nucleus caused depolymerization of microtubules at the irradiated site, and consequently induced backward and forward movements of the nucleus, respectively. From this result, the authors proposed that tension on microtubules plays a key role in positioning the nucleus. In an *in vitro* study, when centrosomes isolated from cultured Chinese hamster ovary cells and tubulin purified from bovine brain were placed in a microfabricated chamber, the centrosomes migrated toward the geometrical center of the chamber, which was attributed to the microtubule assembly (Holy et al., 1997). Therefore, the position of the nucleus may represent an equilibrium position where tension forces in cytoskeletal elements balance. Concerning these hypotheses, it is noteworthy that the dark position of nuclei in *A. thaliana* leaf epidermal cells appears to be strictly determined around the center of inner periclinal region of the cells (Iwabuchi et al., 2010).

To date, we have almost no information about specific proteins or genes involved in microtubule- and actin-mediated plant nuclear positioning. In fungi and animals, there are two basic mechanisms for microtubule-dependent nuclear positioning: one is positioning of the nucleus following a microtubule-organizing center (MTOC), or a centrosome or spindle pole body, when the MTOC is attached to the nucleus, and the other is nuclear migration along microtubules, where the MTOC is absent (Reinsch and Gönczy, 1998). Although higher plant cells lack discrete MTOC-like structures, there are several reports demonstrating that isolated plant nuclei can organize microtubules *in vitro* (Bartolini and Gundersen, 2006; Schmit, 2002). GCP2 and GCP3 (γ-tubulin complex proteins) of *A. thaliana*, possible candidates for members of the γ-tubulin small complex which constitutes a ubiquitous core complex of MTOCs together with γ-tubulin (Raynaud-Messina and Merdes, 2007; Wiese and Zheng, 2006), were localized at the nuclear envelope (Seltzer et al., 2007). Independently, as another possible candidate involved in microtubule organization at the nuclear envelope,

Nakayama et al. (2008) identified histone H1 in tobacco BY-2 cells. Although its role in nuclear movement has not yet been examined, histone H1 appeared to be localized at the nuclear surface, where the continuous incorporation of tubulin occurred, and mediates the nucleation of microtubules in a radial array (Hotta et al., 2007). On the other hand, *A. thaliana* has 17 myosin sequences in its genome, with four types of myosin VIII and 13 types of myosin XI (Berg et al., 2001; Reddy and Day, 2001b). Among them, myosin XI-I has been localized at the nuclear envelope (Avisar et al., 2009), raising the possibility that this myosin species might function in the motive force generation for nuclear movement in *A. thaliana*.

2.4. Nuclear positioning in other eukaryotes and its regulatory systems

2.4.1. Genetic analyses in fungi

Fungi, such as filamentous fungi and yeasts, have been used as model organisms for the molecular study of nuclear positioning. Especially, filamentous fungi such as *Aspergillus nidulans* and *Newrospora crassa* have made tremendous contributions to this field. Nuclear positioning can be seen during vegetative hyphal growth and asexual spore production (Fischer, 1999; Gladfelter and Berman, 2009; Xiang and Fischer, 2004; Xiang and Morris, 1999). In growing hyphae, multiple nuclei enter the hyphae and migrate to achieve relatively equal spacing of the individual nuclei. The nucleus closest to the hyphal tip moves at a speed similar to that of hyphal tip extension, meaning that the distance between the nucleus and the hyphal tip is kept more or less constant. The remaining nuclei in the hyphae are evenly distributed along the hyphae. Nuclear positioning has been shown to depend on microtubules (Oakley and Morris, 1980, 1981; Willins et al., 1995).

About 30 years ago, Morris (1975) started genetic research on nuclear positioning using *A. nidulans* and succeeded in isolating a series of heat-sensitive mutants—designated *nud* (nuclear distribution) mutants—that exhibited abnormal distribution patterns of nuclei at restrictive temperature. To date, several *nud* genes have been identified and characterized (Fischer 1999; Morris, 2000; Xiang and Morris, 1999). The *nudA* gene encodes the cytoplasmic dynein heavy chain that comprises a subunit of the microtubule-based motor protein dynein (Xiang et al., 1994, 1995b). Other mutants with mutations in genes for the intermediate chain (*nudI*) and light chain (*nudG*) of cytoplasmic dynein exhibited a similar phenotype (Beckwith et al., 1998; Zhang et al., 2002), confirming that dynein is involved in nuclear migration. The *nudC* gene encodes a protein of 22 kDa (Osmani et al., 1990) and the *nudF* gene encodes a WD (tryptophan-aspartic acid)-repeat protein that is homologous to LIS1 (Miller-Dieker lissencephaly) protein (Xiang et al., 1995a), the product of the causal gene for Miller-Dieker lissencephaly, in which neuronal migration is compromised (Morris et al., 1998a). LIS1

protein was shown to interact with NUDC protein (Morris et al., 1998b). NUDF protein was localized at the plus end of microtubules forming comet-like structures, with the protein level controlled by NUDC protein levels (Xiang et al., 1995a). Both proteins are believed to function in the cytoplasmic dynein regulatory pathway, although their exact roles require further investigation (Xiang and Fischer, 2004). The *nudK* gene encodes Arp1 (actin-related protein) of the dynactin (dynein activator) complex (Xiang et al., 1999), which is required for dynein functions (Holleran et al., 1998). KINA (kinesin) protein, the conventional kinesin motor protein of *A. nidulans*, was shown to play a role in nuclear migration, conceivably by affecting microtubule stability (Requena et al., 2001). Zhang et al. (2003) demonstrated that KINA is necessary for plus-end accumulation of cytoplasmic dynein and dynactin along microtubules, while accumulation of these proteins at the plus end of microtubules is an interdependent process. The authors also suggested that NUDF protein facilitates minus-end-directed dynein movement.

In *A. thaliana*, *BOBBER1*, a homolog of the *nudC* gene of *A. nidulans*, has been recently shown to play an essential role in normal development of the apical domain of the embryo (Jurkuta et al., 2009). BOBBER1 may be a small heat shock protein functioning as a molecular chaperone (Perez et al., 2009), while its potential involvement in nuclear positioning has not been examined.

Mutants with similar phenotypes to the *nud* mutants of *A. nidulans* have been screened in another filamentous fungus *N. crassa* and designated as *ro* (ropy) mutants (Plamann et al., 1994). The *ro-1* gene encodes the heavy chain of cytoplasmic dynein (Plamann et al., 1994). The *ro-3* encodes a protein homologous to $p150^{Glued}$ of *Drosophila melanogaster*, the largest subunit of dynactin, and the *ro-4* gene encodes Arp1 (Plamann et al., 1994; Tinsley et al. 1996). Genetic analyses using the *ro* mutants suggested that dynein interacts with dynactin *in vivo* (Tinsley et al., 1996). The role of dynein in nuclear migration was also shown in another fungus *Ashbya gossypii* (Alberti-Segui et al., 2000).

In the budding yeast *Saccharomyces cerevisiae*, in which the nucleus migrates from a random position in the mother cell to the budding neck prior to mitosis to provide each cell with a nucleus, microtubules and dynein are required for nuclear migration (Huffaker et al., 1988; Jacobs et al., 1988; Yeh et al. 1995). Mutant studies on *ACT3* and *ACT5* genes, *S. cerevisiae* homologs of the Arp1 gene, indicated that dynactin is involved in nuclear positioning (Clark and Meyer, 1994; Muhua et al., 1994), while kinesin also seems to play an essential role (Suelmann and Fischer, 2000). Although the specific roles were not clarified, NUM1 (nuclear migration), a 313-kDa protein equipped with a potential Ca^{2+}-binding site and a central domain containing 12 almost identical tandem repeats of a 64-amino acid polypeptide (Kormanec et al., 1991), and JNM1 (just nuclear migration), a

44-kDa protein containing three coiled-coil domains (McMillan and Tatchell, 1994), were also demonstrated to participate in nuclear migration. In summary, nuclear positioning in fungi is dependent on microtubule-dynein and -kinesin systems that are regulated by dynactin complex.

2.4.2. Linkage proteins connecting the nucleus and cytoskeletons

Nuclear migration has been extensively studied in cells within the embryo and larvae of the nematode *Caenorhabditis elegans*. For example, the nuclei of the dorsal hypodermis migrate past the dorsal midline toward the opposite side of the embryo (Sulston et al., 1983). In the young larva, the precursor nuclei that lie ventrolaterally begin to migrate into the ventral cord and the preanal ganglion about 8 h after hatching (Sulston, 1976; Sulston and Horvitz, 1977). It has been shown that these movements are dependent on microtubules, dynein, and dynactin (Fan and Ready, 1997; Reinsch and Gönczy, 1998).

Several important findings have been reported in *C. elegans* with regard to proteins indispensable for nuclear migration. Mutant analyses of *unc-83* and *unc-84* (uncoordinated) strikingly revealed that the *unc-83* and *unc-84* genes encode the outer and inner nuclear membrane protein, respectively. The UNC-83 has a conserved C-terminal Klarsicht/ANC-1 (nuclear anchorage defective)/Syne-1 (synaptic nuclear envelop) homology domain (KASH domain) and a transmembrane region (McGee et al., 2006), and the UNC-84 contains Sad1p/ UNC-84 domain (SUN domain) at the C-terminus, homologous to the yeast protein Sad1p (spindle architecture disrupted) (Malone et al., 1999; McGee et al., 2006). In the embryo of *unc-84* mutant worms, the nuclei of the dorsal hypodermis still possessed the ability to move but failed to migrate past the dorsal midline to the opposite side of the embryo, while in the precursor cells of young *unc-84* mutant worms, the nuclei did not move (Malone et al., 1999). The *unc-83* mutant worm exhibited a similar phenotype to the *unc-84* mutant worm with respect to these two types of nuclear movement (McGee et al., 2006). UNC-84 was localized to the inner nuclear membrane without requiring UNC-83, whereas UNC-83 was localized to the outer nuclear membrane with the aid of UNC-84 (Malone et al., 1999; McGee et al., 2006). The membrane-bound-yeast two-hybrid system demonstrated that these proteins interact with each other (McGee et al., 2006). Thus, these results have provided the model that KASH and SUN proteins bridge the nuclear envelope, thereby connecting the nuclear lamina to cytoplasmic cytoskeletons. Members of KASH and SUN proteins identified in fungi, worms, and mammals are summarized elsewhere together with their roles in nuclear positioning in those organisms (Burke and Roux, 2009; Starr, 2007, 2009; Starr and Han, 2003; Wilhelmsen et al., 2006). At present, KASH proteins are thought to function in multiple ways to position nuclei; they mediate

nuclear anchorage in *C. elegans* and mammals, nuclear migration via centrosome attachment in *D. melanogaster* and *C. elegans*, nuclear migration without centrosome in *C. elegans*, and association of nuclei with intermediate filaments in mammals (Starr, 2009).

SUN homologs of *A. thaliana*, AtSUN1 and AtSUN2, have been partially characterized (Graumann et al., 2010; Meier and Brkljacic, 2009). Both AtSUN1 and AtSUN2 seemed to be localized at the nuclear envelope, especially the inner nuclear membrane (Graumann et al., 2010), which is consistent with the localization of SUN proteins in the yeast and animals (Starr, 2009). These two proteins were ubiquitously expressed in various tissues at low levels, suggesting housekeeping roles, but currently unknown functions. It may be worth testing whether the SUN proteins are required for nuclear positioning in plant cells.

3. Dynamic Behavior of Plant Mitochondria

Mitochondria form a dynamic intracellular network responsible for energy production, amino acid metabolism, fatty acid oxidation, calcium homeostasis, and cell signaling. The intracellular movement and distribution of mitochondria play indispensable roles in the maintenance and adaptation of a large number of cellular activities. In 1898, Benda first introduced the term mitochondrion, coming from the Greek words *mitos* (thread) and *chondron* (grain). At the beginning of the twentieth century, advances in bright-field light microscopy allowed the first reliable observations of dynamic behavior of mitochondria in living cells (Lewis and Lewis, 1914). Since then an enormous number of studies have been carried out to characterize mitochondrial behavior in a variety of cell types including mammals, yeast, and plants. In this chapter, we briefly reviewed recent findings on the dynamic behavior of plant mitochondria comparing with the knowledge obtained in animals and fungi. We focused on dynamic images of plant mitochondria visualized by ameliorating techniques of fluorescence microscopy. There are comprehensive reviews describing similar as well as different aspects of plant mitochondria (Linke and Bőrner, 2005; Logan, 2003, 2006a,b, 2010; Rhoads and Subbaiah, 2007; Sakai et al., 2004).

3.1. Regulation of mitochondrial morphology

3.1.1. Morphology of plant mitochondria

Mitochondria are generally oblong organelles, which range in size between 1 and 10 μm in length, and are distributed throughout the cytoplasm. Regardless of whether movement occurs, they are typically discrete, long,

and snake-like tubules and occasionally form branched reticula (Bereiter-Hahn and Vöth, 1994; Yaffe, 1999). There is some evidence that the number, size, and shape of mitochondria are maintained through the opposing processes of their fusion and fission (Cerveny et al., 2007; Logan, 2003; Okamoto and Shaw, 2005). The number of mitochondria in a cell varies widely between organisms and cell types. A single, highly branched mitochondrion was described in the unicellular alga *Polytomella agilis* (Burton and Moore, 1974), whereas thousands of mitochondria are present in the giant amoeba *Chaos chaos* (Bereiter-Hahn and Vöth, 1994). Concerning plant cells, a typical mesophyll cell of the model plant *A. thaliana* contains approximately 200–300 discrete mitochondria (Sheahan et al., 2004). In pollen of *A. thaliana*, by vegetative-cell- and sperm-cell-specific expression of mitochondrion-targeted fluorescent proteins, Matsushima et al. (2008) revealed that each sperm cell contained 8.3 ± 1.6 mitochondria. These paternal mitochondria entered the egg and central cells of the embryo sac upon double fertilization, and then disappeared after several hours, indicating the maternal inheritance of mitochondria.

As exemplified in the study of Matsushima et al. (2008), from 1990s to 2000s, fluorescence microscopy using transgenic plants that express mitochondrion-targeted fluorescent protein allowed detailed visualization of the size, shape, and distribution of mitochondria as well as their structural rearrangements in living plant cells (Köhler et al., 1997a; Logan and Leaver, 2000; Niwa et al., 1999). Those analyses revealed that there is an extreme heterogeneity in the morphology and behavior of mitochondria in different plant species, even in different cell types of the same plant species, and in different developmental stages (Zottini et al., 2006). Typically in epidermal (Arimura and Tsutsumi, 2002; Logan and Leaver, 2000) and leaf mesophyll cells (Islam et al., 2009; Köhler et al., 1997a) as well as in suspension cultured cells (Arimura et al., 2004b; Köhler et al., 1997a; Zottini et al., 2006), plant mitochondria exhibit spherical structure with uniform diameter, while in some cases, they form sausage-shaped to longer worm-like structures under rapid movement.

In animal cells, it has been postulated that the intracellular morphology of mitochondria is intimately related to their energy status. Mitochondrial morphology can be categorized into one of two typical conformations: orthodox or condensed (Hackenbrock, 1966). Orthodox mitochondria take bacilliform shapes and exhibit higher motility, whereas condensed mitochondria are spherical and less mobile. These changes could be observed both *in vivo* and *in vitro* through modulation of metabolic status of mitochondria (Bereiter-Hahn and Vöth, 1994; Hackenbrock, 1966; Van der Klei et al., 1994). Similar changes in mitochondrial behavior were reported in leaf cells or protoplasts of *A. thaliana* subjected to treatment with chemicals inducing the production of reactive oxygen species or heat

shock (Scott and Logan, 2008; Yoshinaga et al., 2005). Although mechanisms underlying these responses have not been examined in plant cells, the occurrence of orthodox and condensed mitochondria has been noticed in a variety of cell types (Logan and Leaver, 2000).

3.1.2. Genetic analyses of morphology of plant mitochondria

Plant mitochondria dynamically undergo the processes of fusion and fission (Arimura et al., 2004b; Sheahan et al., 2005). If any of the steps in these processes are impaired, the morphology of mitochondria should become abnormal. Using forward genetic approaches, several critical factors involved in the regulation of mitochondrial morphology have been identified in plants. For example, Feng et al. (2004) isolated 17 lines of mutants of *A. thaliana* harboring mitochondria with aberrant morphology. Since all these mutants exhibited elongated and larger mitochondria than normal ones, the relevant genes might be involved in the fission of mitochondria. To date, there is no report of any plant mutant that has been unequivocally demonstrated to be deficient in the fusion processes of mitochondria.

Logan et al. (2003) isolated five distinct mutants of *A. thaliana*: *mmt1* (motley mitochondrial), *mmt2*, *bmt* (big mitochondrial), *nmt* (network mitochondrial), and *fmt* (friendly mitochondrial), all exhibiting drastically altered morphology or distribution of mitochondria. Among them, the *fmt* mutant was characterized by the presence of aggregated clumps of mitochondria in every cell type. The *FMT* gene was identified as a homolog of the *cluA* gene of *Dictyostelium discoideum* (Zhu et al., 1997). The CluA protein has no homology to other proteins of known functions except for a short tetratricopeptide repeat (TPR), which is assumed to function in protein–protein interactions. Disruption of the *cluA* gene in *D. discoideum* brought about clustered mitochondria near the center of cells (Zhu et al., 1997), in addition, disruption of the *CLU1* gene in the yeast *S. cerevisiae*, another homolog of the *cluA* gene, also induced aberrant mitochondrial phenotypes (Fields et al., 1998). Since clustered mitochondria in the *cluA* disruptants of *D. discoideum* were interconnected, Zhu et al. (1997) concluded that the mutants were deficient in the stage of outer membrane scission during the fission of mitochondria. However, since such interconnected structures could not be observed in the aggregated mitochondria in the *fmt* mutant of *A. thaliana*, Logan et al. (2003) hypothesized that the FMT protein might be involved in regulation for the association of mitochondria with motor proteins interacting with microtubules through its TPR domain.

Other than the aberrant phenotypes in mitochondria, intriguingly, the size of chloroplasts in *mmt1* was much larger than that in wild-type plants, and the intrastructure of chloroplasts in *mmt2* was abnormal (Logan et al., 2003). Although the aberrant shapes of chloroplasts were similar, *mmt1* was not allelic to *arc5*, *6*, *11*, or *12* (accumulation and replication of chloroplasts),

the series of mutants in which the morphology of chloroplasts becomes abnormal because of defects in chloroplast division process (Marrison et al., 1999; Pyke and Leech, 1994).

3.1.3. Fission of plant mitochondria regulated by dynamin-related GTPase proteins

Since mitochondria cannot be created *de novo*, they must be formed through fission of preexisting mitochondria. Mitochondrial fission is conducted, at least partially, by the division apparatus named "fission machinery" consisting of three primary components: evolutionarily conserved dynamin-related GTPase proteins, their adaptor proteins, and transmembrane proteins localized at the outer membrane of mitochondria (Cerveny et al., 2007; Chan, 2006). In the yeast *S. cerevisiae*, the dynamin-related protein Dnm1p is recruited to the mitochondria fission sites through the interaction with adaptor protein Mdv1p, which can bind to the outer membrane protein Fis1p (Hoppins et al., 2007; Okamoto and Shaw, 2005). Functional assembly of division apparatus for mitochondria and its evolutional diversity were reviewed elsewhere (Kuroiwa et al., 2006).

DRP3A (dynamin-related protein) (Arimura et al., 2004a; Logan et al., 2004) and DRP3B (Arimura and Tsutsumi, 2002) were identified in *A. thaliana* as the GTPase proteins involved in mitochondrial fission and also of peroxisomes (Fujimoto et al., 2009; Mano et al., 2004; Zhang and Hu, 2009). In the *drp3a/drp3b* double mutant, mitochondria formed a highly complicated tubular network, which was most probably due to impairment of outer membrane fission (Fujimoto et al., 2009). Fujimoto et al. (2009) further demonstrated that DRP3A and DRP3B are redundantly involved in mitochondrial fission, while DRP3A plays a major role and DRP3B plays a minor but distinct role in the fission of peroxisomes in leaf epidermal cells of *A. thaliana*. From an evolutionary point of view, after the gene duplication of *DRP3A* and *DRP3B* (Fujimoto et al., 2004), mutations in *DRP3B* might have made it function in the fission of peroxisomes. Other than the members of family 3 of DRPs in *A. thaliana*, two members of family 1, DRP1C (ADL1C) and DRP1E (ADL1E), have also been demonstrated to participate in the fission of mitochondria (Jin et al., 2003).

On the other hand, Arimura et al. (2008) revealed that a plant-specific protein ELM1 (elongated mitochondria) may function as the adaptor protein, which recruits DRP3A and DRP3B through direct interaction with the fission sites of mitochondria, but not of peroxisomes. ELM1 might be targeted to the mitochondria surface through the interaction with FISSION1A (BIGYIN; Scott et al., 2006) and FISSION1B, possible orthologs of yeast Fis1p, although these orthologs were localized to peroxisomes as well as mitochondria likely to participate in the fission of these organelles (Zhang and Hu, 2009).

In the *drp3a* mutant, mitochondria became longer while peroxisomes became larger with long tails (Fujimoto et al., 2009; Mano et al., 2004). In addition, the numbers of both organelles reduced. Importantly, plant growth of the *drp3a* mutant was repressed (Mano et al., 2004; Zhang and Hu, 2009) and this could be attributable, at least partially, to decreased photorespiratory rates (Mano et al., 2004). Efficient exchanges of metabolic intermediates among organelles are required for the operation of photorespiration (Foyer et al., 2009; Raghavendra and Padmasree, 2003; Tolbert, 1997). Since peroxisome proteins including those involved in photorespiration appeared to be properly transported to peroxisomes in the *drp3a* mutant, the aberrant morphology of mitochondria and peroxisomes might affect their mutual interactions and/or metabolites exchange normally conducted in wild-type plants (Mano et al., 2004).

3.2. Regulation of mitochondrial movement

3.2.1. Movement of plant mitochondria and its regulation by cytoskeleton

Mitochondria in plant cells exhibit small oscillations of less than 1 μm to a large-scale displacement over 10 μm s^{-1}. There are several studies mentioning the velocity of mitochondrial movement ranging from 0.2 to 3.0 μm s^{-1}, such as in tip-growing cells (Heslop-Harrison and Heslop-Harrison, 1987; Romagnoli et al., 2007; Zheng et al., 2009) and leaf epidermal and mesophyll cells (Doniwa et al., 2007; Islam et al., 2009). Along the longitudinal axis of elongating root hair cells of *A. thaliana*, a gradient in the velocity of mitochondrial movement was observed (Zheng et al., 2009). In the subapical region, mitochondria moved more slowly and in an irregular manner. The velocity of mitochondrial movement increased as the distance from the apex increased and mitochondria moved more linearly in the shank region. However, mitochondria visualized in sperm cells moved more slowly than the velocity of cytoplasmic streaming in pollen tubes of *A. thaliana* (Matsushima et al., 2008).

Changes in the shape, motility, and distribution pattern of mitochondria are regulated by the cytoskeleton (Anesti and Scorrano, 2006; Boldogh and Pon, 2007; Yaffe, 1999). Consequently, mitochondria may be organized into lengthy traveling chains, packed tightly into relatively stable groups, or take on various other morphologies. In neurons, filamentous fungi, and fission yeast, mitochondria movement is driven by the microtubule-dependent system (Frederick and Shaw, 2007), while in neurons (Hollenbeck and Saxton, 2005) and budding yeast (Altmann et al., 2008; Boldogh et al., 2005), the crucial involvement of actomyosin system has also been extensively investigated. In plant cells, the involvement of microtubule- and actin-dependent systems in regulation of mitochondrial movement and distribution has been reported, though the actin cytoskeleton

seems to play more prominent roles as described below. There has been no report on the involvement of intermediate filaments in the behavior of plant mitochondria, as demonstrated in animal cells (Anesti and Scorrano, 2006; Tang et al., 2008; Toivola et al., 2005).

In elongating cells derived from leaf protoplasts of *N. tabacum*, mitochondrial movement in the transvacular cytoplasmic strands was actin dependent, while their positioning on the cortical cytoplasm appeared to be microtubule dependent (Van Gestel et al., 2002). On the other hand, using this material, microtubule-dependent fusion of mitochondria in the initial phase of protoplast culture (Sheahan et al., 2005) and actin-dependent redistribution and symmetrical partitioning of mitochondria during cell division (Sheahan et al., 2004) were described. In Characean cells, the involvement of actin and microtubule cytoskeletons in regulation of the shape, motility, and distribution pattern of cortical mitochondria was suggested (Foissner, 2004). Mitochondrial movement in *A. thaliana* leaf epidermal and tobacco BY-2 cells was sensitive to actin-depolymerizing reagent but not to the microtubule-disrupting reagent (Doniwa et al., 2007). In living leaf epidermal cells of transgenic *A. thaliana*, in which both mitochondria and actin filaments were visualized with different kinds of fluorescent proteins, Doniwa et al. (2007) unambiguously demonstrated that mitochondria move along the bundles of actin filaments, but independent of microtubules. In a similar way, using evanescent-wave microscopy equipped with high spatiotemporal resolution, intimate correlations between mitochondrial movement and dynamic behavior of actin filaments were described in root hair cells of *A. thaliana* (Zheng et al., 2009). The effects of cytoskeleton-modifying reagents suggested the involvement of myosin and microtubules in the actin-dependent movement of mitochondria.

3.2.2. Motor proteins associated with plant mitochondria

Romagnoli et al. (2007) biochemically isolated mitochondria from the pollen tubes of *N. tabacum* and investigated their motile activity *in vitro*. Mitochondria moved more slowly along microtubules, while more rapidly and in an irregular manner along actin filaments. In the presence of both cytoskeletal components, mitochondria tended to move slowly, similar to the situation observed in intact pollen tubes. By immunoelectron microscopy, Romagnoli et al. (2007) demonstrated that the same mitochondrion was associated simultaneously with 170-kDa myosin, one of the myosin XI members first identified in *Lilium longiflorum* pollen tubes (Shimmen and Yokota, 2004; Yokota and Shimmen, 1994), and with 90-kDa kinesin-like protein, one of the polypeptides interacting with microtubules in an ATP-dependent manner found in *N. tabacum* pollen tubes (Cai et al., 2000). The association of the 170-kDa myosin did not seem to be mitochondrion-specific because it was also localized to the Golgi vesicles. This study raised possibilities that plant mitochondria are equipped with motor proteins to

actively move interacting with cytoskeletal components, and that plant mitochondria can use dual motor activities, myosin and kinesin.

A possible association of myosin XI with mitochondria and plastids was also reported in *Zea mays* (Wang and Pesacreta, 2004). Moreover, using dominant-negative, knocked-down, and knock-out transgenic plants, prominent and partially overlapping roles of several members of myosin XI in mitochondrial movement were investigated in *Nicotiana benthamiana*, *N. tabacum*, and *A. thaliana* (Avisar et al., 2008, 2009; Peremyslov et al., 2008; Prokhnevsky et al., 2008). A possible association of kinesin-like proteins with mitochondria-enriched fractions was examined biochemically in *N. tabacum* pollen tubes (Romagnoli et al., 2003) and *A. thaliana* leaves (Ni et al., 2005). Itoh et al. (2001) reported the presence of kinesin-related proteins with an N-terminal mitochondrion-targeting signal, which carries proteins into mitochondria, although the localization and functions of endogenous proteins have not been elucidated.

In animal cells and budding yeast, motor proteins are thought to be targeted to the mitochondria through their interaction with adaptor proteins, which are associated with receptor proteins located at the outer membrane (Frederick and Shaw, 2007). Miro GTPases, equipped with two GTPase domains that flank Ca^{2+}-binding motifs and with a C-terminal transmembrane domain, are known to function as such receptor proteins to recruit the motor proteins kinesins. Microtubule-based motor proteins are not equipped with Ca^{2+}-binding sites (Vale, 2003) and it has been recently revealed that Miro GTPases play a pivotal role through their Ca^{2+}-binding activities in Ca^{2+}-dependent regulation of microtubule-driven mitochondrial movement in neurons (MacAskill et al., 2009; Wang and Schwarz, 2009). In these cases, Milton family proteins function as the adaptor proteins mediating the interaction between Miro GTPases receptor and kinesin heavy chains (Liu and Hajnóczky, 2009; Reis et al., 2009), though no Milton homologs have been identified in plants.

Miro GTPases are widely conserved and constitute a unique member of the major branches in the Ras GTPase superfamily (Reis et al., 2009). MIRO1 (EMB2473; Tzafrir et al., 2004) was identified in *A. thaliana* as an essential GTPase, which associates with mitochondria via its C-terminal transmembrane domain (Yamaoka and Leaver, 2008). The homozygous *miro1* mutant was lethal during embryogenesis. In the heterozygous *miro1* mutant, pollen development was normal, whereas pollen germination and pollen tube elongation were severely impaired. In such pollen tubes, mitochondria exhibited abnormally enlarged or tube-like morphology. Although its function as the receptor protein for recruitment of the microtubule-based motor proteins and regulation of their activities has not been fully elucidated, MIRO1 in *A. thaliana* likely plays some essential roles in developmental processes.

3.3. Ca^{2+} regulation mediated by plant mitochondria

One of the important roles of mitochondria in intracellular signaling is a participation in Ca^{2+} regulation functioning as a Ca^{2+}-transporting compartment (Rimessi et al., 2008; Saris and Carafoli, 2005). In neurons, mitochondria-mediated Ca^{2+} regulation appeared to depend on the microtubule cytoskeleton (Mironov et al., 2005). Both microtubule-stabilizing and -disrupting reagents induced depolarization of the mitochondrial membrane and a Ca^{2+} release from mitochondria. Since mitochondria are located in the vicinity of the ER in a microtubule-dependent manner in those cells, a Ca^{2+} efflux from mitochondria may result in the Ca^{2+}-induced Ca^{2+} release from the ER. All of these processes were inhibited by pretreatment with blockers of mitochondrial permeability transition pore, a multiprotein complex localized at the contact sites between outer and inner membrane, which contains voltage-dependent nonselective anion channel with high conductance on the outer membrane, adenine nucleotide translocator on the inner membrane, and cyclophilin D in the matrix (Zoratti et al., 2005). This complex is assumed to play important roles in programmed cell death in animals (Tsujimoto et al., 2006). The occurrence of mitochondrial permeability transition pore was reported in plants (Arpagaus et al., 2002), and similar roles have also been suggested, at least partially, in stress-induced plant cell death (Lin et al., 2005; Saviani et al., 2002; Scott and Logan, 2008; Yao et al., 2004), the subject that was comprehensively reviewed elsewhere (Gadjev et al., 2008; Logan, 2008; Reape et al., 2008; Williams and Dickman, 2008).

Although only a couple of studies have focused on the role of plant mitochondria in the intracellular Ca^{2+} regulation (Logan and Knight, 2003; Subbaiah et al., 1998), Wang et al. (2010) has recently examined the role of the actin cytoskeleton in mitochondria-mediated Ca^{2+} regulation in tip-growing cells in a similar way as described above in neurons (Mironov et al., 2005). In tip-growing cells, such as root hair cells and pollen tubes, there is a characteristic polarized zonation of cytoplasm and of the ionic environment along the longitudinal axis (Companoni and Blatt, 2007; Hepler et al., 2001). For example, the distribution patterns of organelles and a tip-directed gradient in cytoplasmic Ca^{2+} concentration are strictly maintained both spatially and temporally.

During elongation of lily pollen tubes (Lovy-Wheeler et al., 2007) and *A. thaliana* root hair cells (Wang et al., 2010; Zheng et al., 2009), mitochondria accumulated in the subapical region; there was a smaller number of mitochondria in the shank region and almost no mitochondria in the most apical region. Such distribution patterns may be produced through different modes of mitochondrial movement along the longitudinal axis of cells, as demonstrated in root hair cells (Zheng et al., 2009). Actin-depolymerizing reagent impaired the distribution pattern of mitochondria,

but the microtubule-disrupting reagent did not (Lovy-Wheeler et al., 2007). Furthermore, in *A. thaliana* root hair cells, mitochondria in the subapical region were located in the vicinity of ER and contained higher concentrations of Ca^{2+}, while those in the shank region contained lower concentrations of Ca^{2+} (Wang et al., 2010). Treatments with the depolymerizing or stabilizing reagents of actin filaments induced a disruption of the Ca^{2+}concentration gradient in mitochondria, concomitantly with a transient increase in the cytoplasmic concentration of Ca^{2+}. These effects were suppressed by pretreatment of the cells with a blocker of mitochondrial permeability transition pore. Taken together, as demonstrated for the roles of mitochondria in Ca^{2+} signaling in myoblasts (Yi et al., 2004), cytoskeleton-dependent Ca^{2+} regulation mediated by mitochondria may be one of the processes tightly integrated in a highly complex network among cytoskeleton, membrane cycling, and ionic regulation, which is indispensable for the maintenance of tip-growth machinery.

3.4. Colocalization of plant mitochondria with other organelles

The maintenance of proper distribution patterns of mitochondria is essential for animal development (Chan, 2006), and mitochondria are positioned at the intracellular sites of high energy consumption. Plant mitochondria occasionally exhibit characteristic distribution patterns. In tip-growing cells as described above (Wang et al., 2010; Zheng et al., 2009) and elongating zygotes of *A. thaliana* (Mansfield and Briarty, 1990), mitochondria accumulate in the subapical or apical region, respectively. In the cortical cytoplasm of internodal cells of Characean algae, where chloroplasts are firmly anchored, mitochondria exhibit an asymmetric distribution pattern, which may have some relation to the level of the operation of photosynthesis (Foissner, 2004). However, the physiological and/or developmental significance and underlying mechanisms of those phenomena have not been assessed so intensively in plant cells compared with animal cells. Instead, colocalization of plant mitochondria with other cell organelles and their role in a wide spectrum of cellular activities have been noticed for a long time.

Microscopic studies suggested that physical contacts might be present between mitochondria and different cell organelles, for example, mitochondria–nucleus (Ornstein, 1956; Smart et al., 1994; Southworth et al., 1997), mitochondria–ER (Stachelin, 1997; Wang et al., 2010), mitochondria–peroxisomes (Frederick and Newcomb, 1969), and mitochondria–chloroplasts (Frederick and Newcomb, 1969; Islam et al., 2009; Logan and Leaver, 2000). In sunflower anther cells, electron microscopic observations revealed the association of mitochondria with the nucleus at the later stages of meiotic divisions. A possible explanation for

this association is that mitochondria might be that this facilitates their equal distribution in the four microspores following meiotic divisions (Smart et al., 1994). Other than the mutual involvement in Ca^{2+} regulation described above (Wang et al., 2010), colocalization of mitochondria with the ER may mediate the transfer of lipid molecules (Stachelin, 1997). Transfer of such molecules was documented in unicellular organisms, mammals, and plants (Browse and Somerville, 1991; Wirtz, 1991). During cell division of the primitive unicellular red alga *Cyanidioschyzon merolae*, vacuoles appeared to be bound to a dividing mitochondrion, resulting in their symmetrical partitioning into two daughter cells (Yagisawa et al., 2007). A close distribution of mitochondria and lysosomes, which are equivalent intracellular compartments to vacuoles, was also reported in animal cells (Beard and Novikoff, 1969). A novel coiled-coil protein VIG1 (vacuole inheritance gene) has been recently identified in *C. merolae*, functioning to tether vacuoles to the outer membrane of mitochondrion specifically at the G_2 and M phases of cell cycle (Fujiwara et al., 2010). Finally, the association of mitochondria with peroxisomes and chloroplasts in light-grown plants has been postulated to facilitate exchange of metabolic intermediates during glycolate metabolism (Tolbert, 1997; Trelease et al., 1971). These interactions of mitochondria with various organelles take place at the particular stages of cell development and/or in response to fluctuations of specific environmental factors, indicating the presence of precise regulatory mechanisms.

Recently, light-induced colocalization of mitochondria with chloroplasts has been described in *A. thaliana* leaf mesophyll cells (Islam et al., 2009). The pattern of light-dependent redistribution of mitochondria was essentially identical to that of chloroplasts (Trojan and Gabryś, 1996); mitochondria occupied the periclinal regions of the cells under weak blue light (Fig. 4.2A and B) while the anticlinal regions under strong blue light (Fig. 4.2C and D). Redistributed mitochondria seemed to be rendered immobile through the adhesion with chloroplasts, and these processes were induced by blue light in a reversible manner (Islam and Takagi, 2010). It has become increasingly evident that plant mitochondria function as fundamental elements for metabolic interactions with photosynthesizing chloroplasts. Mitochondrial metabolism plays essential roles not only for photosynthetic carbon assimilation but also for nitrogen assimilation, photorespiration, and dissipation of excess reducing equivalents generated from the photochemical reactions in chloroplasts (Noctor et al., 2007; Noguchi and Yoshida, 2008; Nunes-Nesi et al., 2007; Raghavendra and Padmasree, 2003). A dissection of light-dependent colocalization of mitochondria with chloroplasts should provide important information concerning the mode of regulation and signaling pathway of organelle interactions in plant cells.

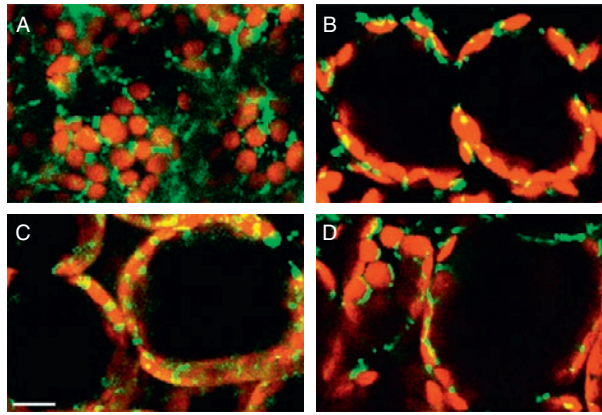


Figure 4.2 Redistribution of mitochondria and chloroplasts in mesophyll cells of *Arabidopsis thaliana* expressing mitochondrion-targeted GFP under different light conditions. Fixed cells were visualized by confocal microscopy after illumination with weak blue light (470 nm, 4 μmol m^{-2} s^{-1}) (A and B) or strong blue light (470 nm, 100 μmol m^{-2} s^{-1}) for 4 h (C and D), respectively. Left panels (A and C) indicated the outer periclinal regions of the cells (top view), while right panels (B and D) indicated the transverse sections of the leaf (side view). Green: GFP signals. Red: chlorophyll autofluorescence. Bar = 50 μm.

4. Overview of Recent Investigation on Plastids–Cytoskeleton Interactions

Plant cells bear a plant-specific double-membrane-bounded compartment: plastids. Since many aspects of dynamic behavior of plastids have been comprehensively reviewed, for example, plastid motility (Gunning, 2005; Morita, 2010; Suetsugu and Wada, 2007; Takagi, 2003), chloroplast positioning (Takagi et al., 2009; Wada and Suetsugu, 2004), plastid division (Miyagishima, 2005; Yang et al., 2008b), and plastid differentiation/interconversion (Kato and Sakamoto, 2010; Lopez-Juez and Pyke, 2005), we have merely added here several recent findings on plastids–cytoskeleton interactions.

Probably the best-analyzed plant organelle movement is the photorelocation movement of chloroplasts (Suetsugu and Wada, 2007; Wada et al., 2003), which has been investigated by multiple ways including extensive screening of various lines of mutants in *A. thaliana* (DeBlasio et al., 2005; Luesse et al., 2006; Oikawa et al., 2003; Suetsugu et al., 2005a, 2010). Among them, CHUP1 was identified as a unique actin-binding protein localized at the outer envelope to mediate the interaction between chloroplasts and the actin cytoskeleton (Oikawa et al., 2003, 2008; Schmidt von Braun and Schleiff, 2008). A characteristic actin-based motile apparatus of chloroplast movement, in

which CHUP1 plays the crucial role, has been proposed (Kadota et al., 2009); however, the molecular mechanism for motive force generation still has not been clarified. An involvement of myosin activities in the photorelocative movement of chloroplasts is still controversial (Avisar et al., 2008; Paves and Truve, 2007), while it has been recently suggested that certain species of myosin XI are involved in the formation and characteristic movement of stromules (stroma-filled tubules) in *A. thaliana* and tobacco plants (Natesan et al., 2009; Sattarzadeh et al., 2009). Stromules are enigmatic structures found on the surface of a variety of plastids, dynamically extending and shrinking tubules interconnecting neighboring plastids (Gunning, 2005; Hanson and Sattarzadeh, 2008). Although genuine functions of those structures *in planta* are elusive, mitochondrial metabolism might affect the formation of filamentous leucoplasts, probably equivalent structures to stromules, in root cortical cells of *A. thaliana* (Itoh et al., 2010).

There have been relatively few studies on the association between plastids and the microtubule cytoskeleton, while such associations have been frequently observed in the meiotic stage of sporogenesis in several species of liverworts (Brown et al., 2010). γ-Tubulin was localized at the plastid surface during this stage in the liverwort *Dumortiera hirsuta*, and the isolated plastids can organize microtubules *in vitro* (Shimamura et al., 2004). The underlying mechanisms of this ability should be investigated in further detail.

Another typical example of the dynamic behavior of plastids is that of starch-containing amyloplasts as statoliths in gravitropic responses (Morita 2010). The direction of gravity is sensed in specially differentiated cells named statocytes: endodermal cells in shoots (Fukaki et al., 1998) and columella cells in roots (Blancaflor et al., 1998), respectively. Endodermal cells are in a radial tissue of shoots, in which amyloplasts are enclosed by vacuolar membrane in the cytoplasmic strands. Columella cells are in a conical tissue in the root caps, in which amyloplasts are located in the vicinity of sheet-like or tuberous ER residing at the bottom. Precise observations of living statocytes during gravistimulation revealed that gravity-induced characteristic behavior of amyloplasts, which is assumed to be an indication of gravisensing process, was detected very rapidly, within 1 min or 1 s of gravistimulation in endodermal (Saito et al., 2005) or columella cells (Leitz et al., 2009), respectively. In both cases, intimate interactions between amyloplast movement and membrane components, vacuolar membrane in the endodermal cells and ER in the columella cells, may be crucial for gravisensing, while the mode of involvement of the actin cytoskeleton in the signal transduction pathway is controversial (Morita and Tasaka, 2004).

On the other hand, Sedbrook et al. (1999) identified an *arg1* (altered response to gravity) mutant of *A. thaliana* with reduced gravitropism of hypocotyls and roots. The *ARG1* gene encodes a type-II DnaJ (DNA)-like protein equipped with the conserved "J" domain, a hydrophobic region, and a coiled-coil region. ARG1 may be associated with the actin cytoskeleton

and membrane compartments involved in vesicular trafficking and appears to play an important role in the early processes in gravitropic signal transduction (Boonsirichai et al., 2003). Stanga et al. (2009a) further isolated *mar* (modifier of *arg1*) mutants from mutagenized lines of the *arg1* mutants. Strikingly, the *mar1* and *mar2* mutants, exhibiting extreme phenotypes of shoot and root grown in random directions only in the background of *arg1* mutation, possessed mutations in different components of the TOC (translocon at the outer envelope membrane of chloroplasts) complex (Andrès et al., 2010; Jarvis, 2008), *TOC75-III* (*mar1*) and *TOC132* (*mar2*) genes, respectively. Independently, using a detergent-solubilized fraction of chloroplast envelope membranes prepared from *Pisum sativum* seedlings, Jouhet and Gray (2009a) demonstrated that TOC159, another component of the TOC complex, can be immunoprecipitated by anti-actin antibodies together with actin. Recombinant TOC159 interacted with actin filaments *in vitro*. The *in vitro* interaction between chloroplasts and actin was also reported in spinach (Kumatani et al., 2006) and *A. thaliana* (Schmidt von Braun and Schleiff, 2008). Taken together, these studies may raise two attractive hypotheses on previously unexpected features of the TOC complex. One is its association with the actin cytoskeleton implicated in the regulation of import of photosynthesis proteins (Jouhet and Gray, 2009b). The other is its role in the gravitropic signal transduction pathway (Stanga et al., 2009b), which is mediated by interactions between the statoliths, amyloplasts, and other membrane components, plasma membrane, vacuolar membrane, and ER. In the latter case, the actin cytoskeleton appears to be involved in the modulation of interactions among the different membrane components, although its mode of action is not known.

5. Perspectives

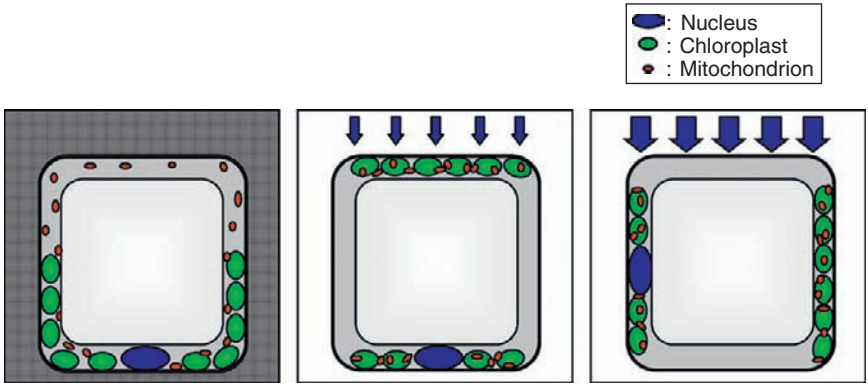
Now, no one doubts that plant organelles dynamically change their shapes, motility, and distribution patterns during development and/or in response to environmental fluctuations, as in fungi and animal cells. The cytoskeleton and its associated proteins play central roles in these dynamic behaviors of plant organelles. Although our knowledge of cytoskeleton-associated proteins and their modes of action is increasing, we do not yet fully understand the complex and diverse regulation pathways involved in plant organelle dynamics. Especially, in plant cells, regulatory factors mediating the interactions between organelles and cytoskeleton are largely unsolved.

Plants share some important regulatory factors with other eukaryotes, such as kinesin (Lee and Liu, 2004; Reddy and Day, 2001a) and myosin (Berg et al., 2001; Reddy and Day, 2001b) motor proteins, γ-tubulin complex (Seltzer et al., 2007), and Miro GTPases (Yamaoka and Leaver, 2008), while

apparently lacking some other factors, such as dynein motor proteins, Milton families, KASH domain and lamin homologs. Accordingly, for example, the mode of Ca^{2+} regulation of movement of double-membrane-bounded organelles has not been assessed satisfactorily in plant cells. However, several plant-specific factors, such as CHUP1 associated with chloroplasts (Oikawa et al., 2003, 2008) and ELM1 associated with mitochondria (Arimura et al., 2008), have been identified and their unique natures have been partially characterized. To deepen understanding of the dynamic behavior of plant organelles and the evolutionary processes of its establishment, forward- and reverse-genetic approaches may have to be conducted more extensively to reveal unidentified common or plant-specific regulatory factors.

In general, plant cells have a thick cell wall, transparent nuclei and mitochondria, and many plastids. These features have prevented researchers from observing each organelle in detail in living cells. This is one of the main reasons why studies on organelle dynamics in plant cells have been hampered so much. Fluorescent-protein techniques have opened the possibility to visualize individual organelles and cytoskeletal components in living plant cells as well as in animal cells (Higaki et al., 2007; Staiger et al., 2009), and have been disclosing the remarkable behavior of nuclei (Chytilova et al., 2000), mitochondria in specifically differentiated cells (Matsushima et al., 2008), colorless plastids (Itoh et al., 2010), and so on. In the near future, this technique in combination with genetic approaches is expected to reveal surprising, new information regarding the nature of plant organelles as well as providing significant insights into molecular mechanisms for dynamic behavior of plant organelles.

Finally, to understand the significance of organelle behavior, whole-plant level analyses of development, growth, and metabolism are indispensable. A good example may be a study on the *drp3a* mutant (Mano et al. 2004), which demonstrated that decreased photorespiration caused by aberrant morphologies of mitochondria and peroxisomes may result in plant growth repression. It has become evident that, in *A. thaliana* mesophyll cells, the nuclei, mitochondria, and chloroplasts change their intracellular positions in response to illumination (Islam et al. 2009; Iwabuchi et al., 2007; Trojan and Gabryś, 1996), and moreover, their distribution patterns under illumination are quite similar (Fig. 4.3). The positioning of each organelle is obviously under the precise and reversible control of light and dark conditions. The movement itself of each organelle appears to be driven by the actin-based cytoskeleton (Doniwa et al., 2007; Iwabuchi et al., 2010; Kadota et al., 2009). However, why these organelles exhibit such an intriguing behavior together is totally unknown. To answer this question, not only relevant photoreceptor systems and cytoskeletal components but also possible metabolic and spatial interactions among these organelles and their significance in plant development should be dissected using multidisciplinary approaches.



Organelles	Darkness	Weak blue light	Strong blue light
Nucleus	Inner periclinal wall	Inner periclinal wall	Anticlinal walls
Chloroplast	Inner periclinal and lower half of anticlinal walls	Outer and inner periclinal walls	Anticlinal walls
Mitochondrion	Random	Outer and inner periclinal walls	Anticlinal walls

Figure 4.3 Schematic summary of light-induced redistribution of the nucleus, mitochondria, and chloroplasts in *Arabidopsis thaliana* mesophyll cells. In darkness, the nucleus and chloroplasts are located mainly along the inner periclinal wall, while mitochondria keep high motility and exhibit a random distribution in the cytoplasm. Under dim light, the nucleus remains along the inner periclinal wall, while both chloroplasts and mitochondria are along the inner and outer periclinal walls. Under strong light, all three organelles come to accumulate along the anticlinal walls. Blue light has prominent effects inducing the redistribution of the organelles.

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CHAPTER FIVE

The Biology of the Desmosome-Like Junction: A Versatile Anchoring Junction and Signal Transducer in the Seminiferous Epithelium

Pearl P.Y. Lie, C. Yan Cheng, *and* Dolores D. Mruk

Contents

Abstract

Mammalian spermatogenesis, a complex process that involves the movement of developing germ cells across the seminiferous epithelium, entails extensive restructuring of Sertoli–Sertoli and Sertoli–germ cell junctions. Presently, it is

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not entirely clear how zygotene spermatocytes gain entry into the adluminal compartment of the seminiferous epithelium, which is sealed off from the systemic circulation by the Sertoli cell component of the blood–testis barrier, without compromising barrier integrity. To begin to address this question, it is critical that we first have a good understanding of the biology and the regulation of different types of Sertoli–Sertoli and Sertoli–germ cell junctions in the testis. Supported by recent studies in the field, we discuss how crosstalk between different types of junctions contributes to their restructuring during germ cell movement across the blood–testis barrier. We place special emphasis on the emerging role of desmosome-like junctions as signal transducers during germ cell movement across the seminiferous epithelium.

Key Words: Blood–testis barrier, Tight junction, Ectoplasmic specialization, Desmosome-like junction, Gap junction, Testis.

1. Introduction

Mammalian spermatogenesis is a continuous process that involves division of type B spermatogonia and spermatocytes (i.e., mitosis and meiosis I/II, respectively), followed by morphogenesis of spermatids (i.e., spermiogenesis) into spermatozoa which are released into the lumen of the seminiferous tubule (i.e., spermiation). Spermatogenesis takes place in the seminiferous epithelium under the strict control of endocrine, paracrine, and autocrine factors (Clermont, 1972; Parvinen, 1982). The stepwise development of spermatozoa from spermatogonia, which takes ~54 days to complete in the rat, occurs in association with Sertoli cells—"nurse-like" cells that are known to extend from the basal lamina to the luminal edge and to provide nutritional and structural support to differentiating germ cells. Moreover, spermatogenesis and germ cell development within the seminiferous epithelium is not at all random. Instead, it is organized into unique cellular associations defined as stages of the seminiferous epithelial cycle (Fig. 5.1). Fourteen stages can be identified in the rat, 12 stages in the mouse, eight stages in the dog, and six stages in the human, and each distinct stage is denoted by a roman numeral. Throughout spermatogenesis, germ cells also traverse the entire height of the seminiferous epithelium. As such, the development of germ cells, the movement of these cells across the seminiferous epithelium, and the remodeling of Sertoli–Sertoli and Sertoli–germ cell junctions throughout spermatogenesis are synchronized (Lie et al., 2009). Thus, it is not surprising that a compromise in any one of these critical cellular events may result in transient or even permanent infertility.

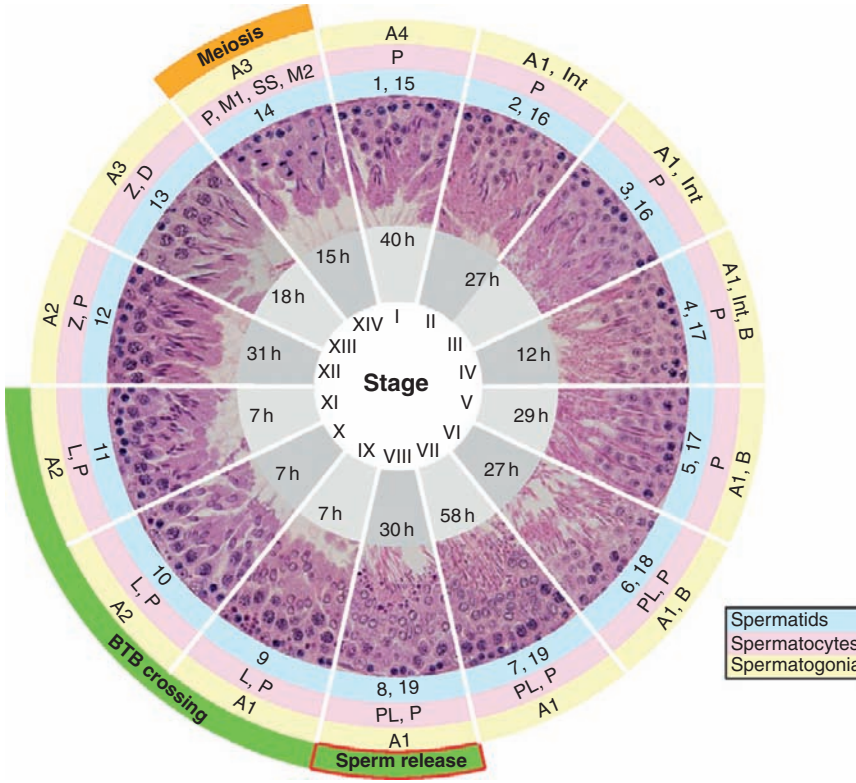

Figure 5.1 Seminiferous epithelial cycle in the rat testis. These 14 images represent stages of the seminiferous epithelial cycle obtained from paraffin-embedded cross-sections of the adult rat testis stained with hematoxylin and eosin. Stages are noted as roman numerals. Annotations in gray shaded areas indicate the approximate duration of each stage in hours (h). Germ cells are divided into spermatogonia (outer yellow circle), spermatocytes (middle pink circle), or spermatids (inner blue circle). Spermatogonia include types A1–A4, intermediate (Int) and B (yellow circle). Spermatocytes (i.e., preleptotene (PL), leptotene (L), zygotene (Z), pachytene (P), and diplotene (D)), primary spermatocytes in meiosis 1 (M1), secondary spermatocytes (SS), and secondary spermatocytes in meiosis 2 (M2) are also shown (pink circle). Finally, spermatid differentiation spans steps 1–19 (blue circle). Important cellular events are noted in the outermost layer as orange and green shaded areas. Spermiation takes place at stage VIII, concurrent with the transit of preleptotene spermatocytes across the BTB during stages VIII–XI. M1 and M2 take place at stage XIV.

Cell–cell interactions are essential for spermatogenesis, and several different junction types have been described to exist between Sertoli cells, as well as between Sertoli and germ cells (Fig. 5.2). Sertoli cell junctions

form an important aspect of the blood–testis barrier (BTB) which physically divides the seminiferous epithelium into a basal and an adluminal compartment. The BTB is located basally within the seminiferous epithelium (i.e., above preleptotene spermatocytes), and it is largely composed of coexisting tight junctions, basal ectoplasmic specializations, desmosome-like junctions, and gap junctions (Fig. 5.2). The BTB is believed to restructure transiently beginning at late stage VIII of the seminiferous epithelial cycle to allow the passage of preleptotene/leptotene spermatocytes across the BTB and the entry of zygotene spermatocytes into the adluminal compartment for continued development, and recent studies have begun to pinpoint several

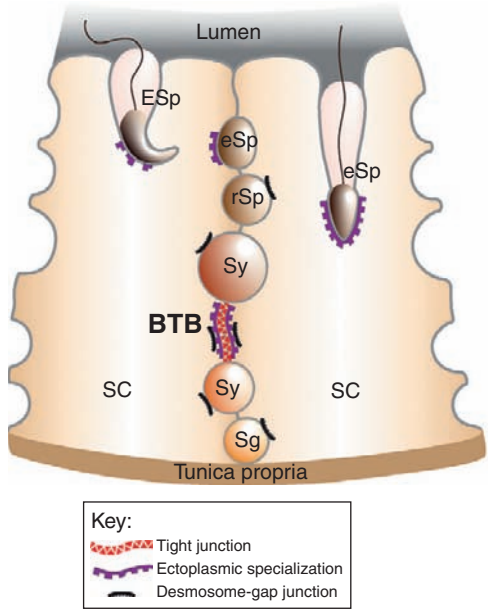

Figure 5.2 Sertoli–Sertoli and Sertoli–germ cell junctions. Two columnar Sertoli cells are shown sitting atop the tunica propria in the seminiferous epithelium. The BTB is constituted by adjacent Sertoli cells and composed of coexisting tight junctions, basal ectoplasmic specialization, and desmosome–gap junctions. Desmosome–gap junctions are found between Sertoli cells and all germ cells up to, but not including, step 8 spermatids, whereas the apical ectoplasmic specialization is found between Sertoli cells and all step 8–19 spermatids. Gap junctions and hemidesmosomes (a type of cell–matrix junction) are not illustrated since these junction types were not discussed in great detail. Also, it is also worth noting that two different stages of the seminiferous epithelial cycle are shown within a single panel (i.e., left, stage VII; right, stage VI) for the sake of simplicity, but this does not accurately represent the *in vivo* situation. Abbreviations: BTB, blood–testis barrier; SC, Sertoli cell; Sg, spermatogonium; Sy, spermatocyte; rSp, round spermatid; eSp, elongating spermatid; ESp, elongated spermatid.

key molecules that are involved in BTB restructuring and germ cell movement. However, the type of adhesive junction present between Sertoli and germ cells depends on the developmental stage of the germ cell: desmosome-like junctions exist between Sertoli cells and pre-step 8 germ cells, whereas apical ectoplasmic specializations exist between Sertoli cells and step 8/post-step 8 spermatids (Fig. 5.2).

In this chapter, we will focus on the biology of desmosome-like junctions in germ cell movement and spermatogenesis. As mentioned above, desmosome-like junctions are present at the BTB and at the Sertoli cell–pre-step 8 germ cell (i.e., spermatogonia, spermatocytes, and round spermatids) interface. At both sites, their morphology is not characteristic of mature desmosomes such as those found in stress-bearing tissues (hence the name "desmosome-like"; alternatively, they can be defined as "desmosome-gap"), but characteristic of wound-edge desmosomes which exhibit considerably less adhesive strength, suggesting that desmosome-like junctions may have an unconventional function in the testis (Russell, 1977a). For instance, two recent reports have described crosstalk between proteins at the desmosome and proteins at the tight junction or gap junction (Li et al., 2009; Lie et al., 2010), and these reports essentially showed that desmosome-like junctions—in addition to conferring adhesion—can also function as a platform for signal transduction. As the basis of our review, we will first summarize what is known about the structure, function, and regulation of conventional desmosomes. Next, we will examine point-by-point how desmosome-like junctions found in the seminiferous epithelium are different from conventional desmosomes found in other epithelia, followed by a general update of cell junction dynamics in the testis. Finally, we will discuss how crosstalk among desmosome-like junctions, tight junctions, ectoplasmic specializations, and gap junctions facilitates the movement of preleptotene/leptotene spermatocytes across the BTB from stages VIII to XI of the seminiferous epithelial cycle.

2. Structure, Function, and Regulation of Conventional Desmosomes

Desmosomes are intermediate filament-based junctions known to confer robust cell–cell adhesion. They are particularly prominent in tissues subjected to enormous mechanical stress such as the heart and skin, but their presence in other organs has also been reported (Delva et al., 2009; Garrod and Kimura, 2008; Green and Gaudry, 2000; Green and Simpson, 2007; Stokes, 2007). By electron microscopy, desmosomes are easily identifiable as a pair of electron dense plaques with each plaque lying adjacent to the plasma membrane and an intermediate dense line marking the extracellular

space. Desmosomal adhesion between adjacent epithelial cells is mediated by *trans*-interactions of single-pass transmembrane proteins known as the desmosomal cadherins (i.e., desmogleins and desmocollins). The cytoplasmic tails of desmosomal cadherins are then tethered to intermediate filaments via proteins from the armadillo (i.e., plakoglobin and plakophilin) and plakin (i.e., desmoplakin, periplakin, and envoplakin) families. Together, proteins from these three families assemble a functional desmosome (Table 5.1; Fig. 5.3). It is also important to note that the basic architecture of the desmosome closely resembles that of the adherens junction, an actin-based cell–cell junction. In the following sections, we will discuss the molecular components (Section 2.1), unique structural features (Section 2.2), regulation (Section 2.3), and signaling role (Section 2.4) of desmosomes.

2.1. Molecular components of desmosomes

Members of the three desmosomal protein families (i.e., desmosomal cadherins, armadillo proteins, and plakins) exhibit variable and unique tissue expression patterns. For instance, desmoglein-1 and desmocollin-1 are expressed abundantly in the superficial differentiated layers of the epidermis, whereas desmoglein-3 and desmocollin-3 are expressed mainly in the basal layer (Green and Simpson, 2007). As for the seminiferous epithelium in the testis, constitutive desmosomal proteins such as desmoglein-2, plakoglobin, and plakophilin-2 are expressed by both Sertoli and germ cells (Li et al., 2009; Lie et al., 2010; Section 3). In addition to the three desmosomal protein families, we will also briefly discuss intermediate filaments.

2.1.1. Desmosomal cadherins

The cadherin superfamily is composed of type I and II cadherins, desmosomal cadherins, protocadherins, atypical cadherins, and large cadherins (Halbleib and Nelson, 2006; Shapiro and Weis, 2009). Type I cadherins (i.e., E-, N-, P-, and R-cadherins)—the classic cadherins—are well-studied transmembrane proteins that promote Ca^{2+}-dependent cell–cell adhesion via *cis*- (i.e., homodimerization of cadherins on the same cell) and *trans*- (i.e., homodimerization of cadherins on opposing cells) interactions. Relatively less is known about type II cadherins (i.e., cadherins 5–12), except that they appear to be structurally related to type I cadherins. The desmosomal cadherin family includes desmogleins-1 to -4 and desmocollins-1 to -3, and both desmogleins and desmocollins are critical for desmosome function. Each desmocollin gene can also give rise to variants (i.e., a and b forms) which are the result of differential splicing. Moreover, desmosomal cadherins are closely related in both structure and function to classic cadherins. For example, desmosomal cadherins possess five N-terminally located extracellular cadherin repeats (i.e., EC1 to 5; EC1 is membrane distal, and EC5 is

Table 5.1 Tissue expression and functions of desmosomal proteins

	Expression in seminiferous epithelium (Lie et al., 2010)	Expression in other tissues[a]	Phenotypes in knockout animals	Related hair/skin diseases	Related heart disease
Desmosomal cadherins					
Desmoglein-1	GC	E, I (γ isoform) (Brennan et al., 2004)		Pemphigus foliaceus (Emery et al., 1995), pemphigus vulgaris (Emery et al., 1995), SPPK (Rickman et al., 1999)	
Desmoglein-2	SC, GC	E, H, I, L (Schafer et al., 1994)	Apparently normal blastocysts with altered desmoplakin distribution which die shortly after implantation (Eshkind et al., 2002)		ARVC (Pilichou et al., 2006)
Desmoglein-3	nil	E (Tsunoda et al., 2003)	Hair loss, lesions in the oral cavity and traumatized skin (Koch et al., 1997)	Pemphigus vulgaris (Amagai et al., 1996)	
Desmoglein-4	GC	E (Whittock and Bower, 2003)		Localized autosomal recessive hypotrichosis (Kljuic et al., 2003)	
Desmocollin-1	GC	E (Legan et al., 1994)	Fragile epidermis, abnormal epidermal proliferation and differentiation, hair loss (Chidgey et al., 2001)		

(*continued*)

Table 5.1 (*continued*)

	Expression in seminiferous epithelium (Lie et al., 2010)	Expression in other tissues[a]	Phenotypes in knockout animals	Related hair/skin diseases	Related heart disease
Desmocollin-2	SC, GC	E, H, I, L (Lorimer et al., 1994; Nuber et al., 1995; Wang et al., 2010)			ARVC (Heuser et al., 2006)
Desmocollin-3	SC	E (Legan et al., 1994)	Conditional KO in epidermis—intraepidermal blistering, hair loss (Chen et al., 2008)		
Armadillo proteins					
Plakoglobin	SC, GC	E, H, I, L (Cowin et al., 1986)	Embryonic lethal due to heart defect; skin blistering in late survivors (Bierkamp et al., 1996; Ruiz et al., 1996)	Naxo's disease—SPPK (McKoy et al., 2000)	Naxo's disease—ARVC (McKoy et al., 2000)
Plakophilin-1	SC	E (Kapprell et al., 1988)		Skin fragility syndrome (Ersoy-Evans et al., 2006)	
Plakophilin-2	SC, GC	E, H, I, L (Mertens et al., 1996)			ARVC (Gerull et al., 2004)

Plakophilin-3	nil	E, H (fetal), I (Bonne et al., 1999; Schmidt et al., 1999)	Defective hair formation, abnormal proliferation, apoptosis, and differentiation in epidermis (Sklyarova et al., 2008)		
Plakins					
Desmoplakin	SC, GC	E, H, I, L (Angst et al., 1990; Wang et al., 2010)	KO—embryonic lethal due to defective desmosomal assembly (Gallicano et al., 1998); Conditional KO in epidermis—epithelium peeling after mild mechanical stress, desmosomes lack intermediate filament attachment (Vasioukhin et al., 2001)	SPPK (Norgett et al., 2000)	ARVC (Norgett et al., 2000)
Envoplakin	n.d.	E (Kim et al., 1997; Ruhrberg et al., 1996)	Slight delay in barrier formation in the epidermis during embryonic development (Maatta et al., 2001)	Paraneoplastic pemphigus (Sonnenerg and Liem, 2007)	
Periplakin	n.d.	E (Ruhrberg et al., 1997)	No discernible abnormalities (Aho et al., 2004)	Paraneoplastic pemphigus (Sonnenerg and Liem, 2007)	

Abbreviations: ARVC, arrhythmogenic right ventricular cardiomyopathy; E, epidermis; GC, germ cell; H, heart; I, intestine; KO, knockout; L, liver; n.d., not determined; SC, Sertoli cell; SPPK, striate palmoplantar keratoderma.

[a] These are only selected examples of tissues expressing desmosomal genes/proteins, and this table is not meant to be exhaustive.

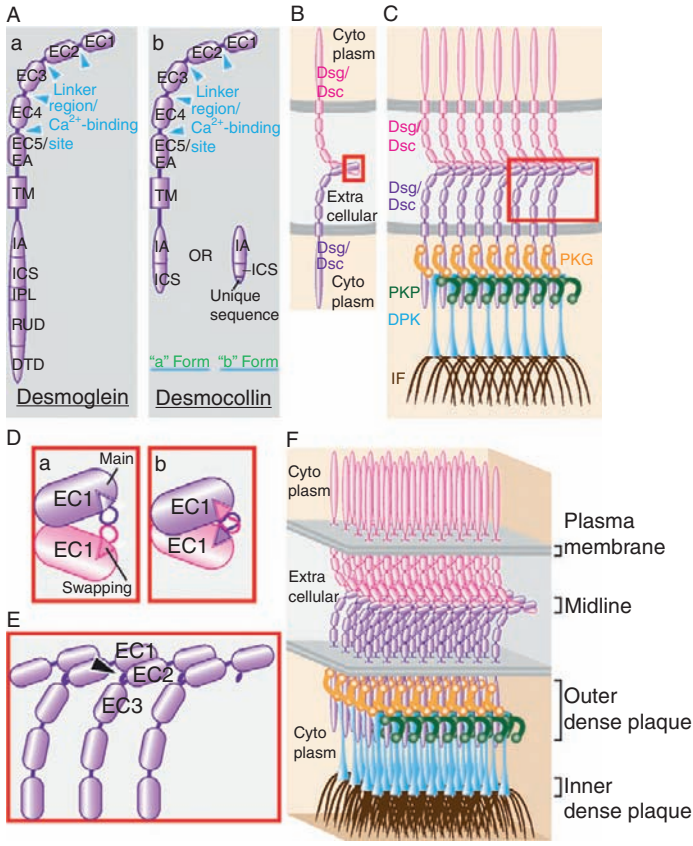

Figure 5.3 Domain structure of desmosomal cadherins and the structure of desmosomes. (A) Different domains in desmosomal cadherins. From the N-terminus, desmogleins (a) and desmocollins (b) are composed of the following domains: extracellular (extracellular cadherin repeat, EC 1–5; connected by linker regions which contain Ca^{2+}-binding sites), transmembrane (TM), and cytoplasmic (intracellular anchor (IA), intracellular cadherin-like sequence (ICS), intracellular proline-rich linker (IPL), repeat unit domain (RUD), desmoglein terminal domain (DTD)). Desmocollins do not contain IPL, RUD, and DTD, and the shorter "b form" has a truncated ICS followed by a unique sequence. It is worth noting that EC5 is sometimes known as the extracellular anchor (EA). (B) *Trans*-interaction of desmogleins (Dsg) or desmocollins (Dsc) between two opposing membranes. The boxed area represents the site of adhesion and is magnified in (D). (C) Organization of desmosomal cadherins (Dsg/Dsc), cytoplasmic plaque proteins and intermediate filaments (IF) at a desmosome. Plakoglobin (PKG) links desmosomal cadherins to desmoplakin (DPK), while plakophilins (PKP) link individual DPK molecules together laterally, which in turn tethers the desmosomal plaque to intermediate filaments. Boxed area is magnified in (E). (D) Strand-swap mechanism of cadherin adhesion. This diagram shows the EC1 domains of two *trans*-interacting cadherins (see B), before (a) and after (b) they undergo symmetrical strand swapping. Each molecule inserts its "swap domain," also known as A strand, into the "main domain" of its partner. (E) Mechanism of Ca^{2+}

membrane proximal) that are linked together by Ca^{2+}-binding sites similar to classic cadherins. Ca^{2+} binding (the concentration of Ca^{2+} in the extracellular milieu is in the millimolar range) is critical for cell adhesion, as it rigidifies the connection between cadherin repeats, resulting in a curved and rod-like ectodomain (Holthofer et al., 2007; Shapiro and Weis, 2009; Fig. 5.3). The accepted idea is that cell–cell adhesion is mediated by two *trans*-interacting EC1 domains, one from each cadherin molecule (Boggon et al., 2002). While Ca^{2+} binding is critical for cadherin function *per se*, mature desmosomes are in fact Ca^{2+} independent (i.e., they are resistant to disruption by divalent cation chelation; Garrod et al., 2005). Therefore, conventional desmosomes are defined as hyperadhesive, which essentially means that these structures are capable of facilitating strong adhesion (Garrod and Kimura, 2008; Section 2.2). Because of structural and functional homologies between classic and desmosomal cadherins, the following sections will also include discussions on classic cadherins when applicable.

Presently, it is not entirely clear whether stable desmosomal adhesion is mediated by homophilic or heterophilic interactions, or whether they involve a combination of both types of interactions. To better understand interactions between different desmosomal cadherin ectodomains, Waschke and colleagues performed a series of *in vitro* experiments at the molecular biology level. These studies utilized atomic force microscopy to examine the adhesive force between two interacting desmosomal cadherin molecules. It was shown that desmogleins-1 (Waschke et al., 2005) and -3 (Heupel et al., 2008) were each capable of mediating homophilic *trans*-interactions. In addition, a heterophilic interaction between desmoglein-1 and desmocollin-3 was noted (Spindler et al., 2009). These results were expanded at the cellular level by an *in vitro* adhesive force assay (i.e., laser tweezer trapping) in which microbeads coated with a desmosomal cadherin were seeded atop keratinocytes, followed by application of a laser beam to displace microbeads. In this experiment, specific interactions between desmoglein-1−desmoglein-1, desmoglein-3−desmoglein-3, and desmoglein-1−desmocollin-3 were demonstrated when deadhesion was induced by

independence. Within the compact arrangement in desmosomes (see C), the Ca^{2+}-binding site between EC2 and 3 on each molecule is protected by a small β-strand in EC1 of the neighboring molecule (arrowhead). This results in the entrapment of bound Ca^{2+}, even in low Ca^{2+} medium. (F) Compact arrangement within a desmosome. This dense arrangement of proteins leads to a few desmosomal-specific features discernable under the electron microscope. The midline in the extracellular space consists of the ends of desmosomal cadherin where the EC1 domains engage with each other. The outer dense plaque includes desmosomal cadherin cytoplasmic tails, plakoglobin, plakophilins, and the plakin domain of desmoplakin. The inner dense plaque is made up of the plakin repeat domains (PRD) of desmoplakin. The area in between outer and inner dense plaques consists of the rod domain of desmoplakin.

pemphigus (an autoimmune disease that causes skin and mucosa to blister) autoantibodies (Heupel et al., 2008; Spindler et al., 2009; Waschke et al., 2005). In other studies, aggregation assays were used to better understand *trans*-interactions in cells expressing desmosomal cadherins. For instance, cells expressing desmoglein-1 and desmocollin-2a failed to aggregate (Kowalczyk et al., 1996), whereas those expressing desmoglein-3 only aggregated weakly (Amagai et al., 1994). Furthermore, overexpression of desmocollin-1a in desmoglein-2-expressing cells caused the latter protein to transit from the cytoplasm to the cell surface, demonstrating that desmoglein-2 can be recruited to the plasma membrane by desmocollin-1a. However, the nature of their interaction (i.e., *cis* or *trans*) was not defined (Chitaev and Troyanovsky, 1997).

By far, the best approach used to investigate how cadherin molecules interact is to visualize their three-dimensional structures. To date, the ectodomains (either partial or full) of several classic cadherins have been studied by X-ray crystallography, but some of these results remain mootable (Al-Amoudi and Frangakis, 2008). At present, the structure of the C-cadherin (a maternally encoded cadherin found in *Xenopus laevis* cleavage-stage embryos and oocytes; Choi et al., 1990; Ginsberg et al., 1991; Levine et al., 1994) ectodomain obtained by X-ray crystallography at 0.31-nm resolution reveals that it assumes a curved conformation and that *trans*-interacting cadherin molecules engage in symmetrical strand swap (Boggon et al., 2002). This means that in the presence of Ca^{2+}, two cadherin monomers present on opposing plasma membranes swap their EC1 N-terminal β-strands, thereby resulting in *trans*-dimer formation. Adhesion is stabilized by a conserved tryptophan side chain (Trp^2) that is inserted into the hydrophobic pocket in the other molecule (Boggon et al., 2002; Fig. 5.3). While strand swapping appears to be characteristic of classic cadherins, it is probably also used by desmosomal cadherins (Posy et al., 2008; Shapiro and Weis, 2009). However, in the absence of Ca^{2+}, cadherin molecules interact with each other in *cis*, instead of in *trans*. Regardless, *cis*-dimers utilize the same strand swapping mechanism as *trans*-dimers to mediate adhesion through their N-terminal ends (Troyanovsky et al., 2003). Moreover, the flexibility of the linker regions when Ca^{2+} is depleted is likely to be an important factor as well (Pokutta et al., 1994). While *cis* dimer formation induced by Ca^{2+}-depletion has little significance *in vivo* because Ca^{2+} can only be depleted under experimental *in vitro* conditions, desmosomal cadherins are hypothesized to participate in lateral *cis*-interactions *in vivo* under a back-to-back arrangement, and this may be important for the tight arrangement of cadherins which is needed for robust cell adhesion (Garrod and Chidgey, 2008; Section 2.2).

The next questions we ask are: how are cadherin molecules arranged laterally within the plasma membrane of one cell, and how does this arrangement contribute to desmosomal adhesion? Based on the three--dimensional visualization of the C-cadherin ectodomain, cadherin

molecules within the plasma membrane of one cell are arranged back-to-back at regular intervals, and they interact with each other in *cis* (Boggon et al., 2002). The combination of *cis*-interactions within one cell with *trans*-interactions between two opposing cells results in a lattice which is somewhat similar to a stack of zippers arranged in parallel at the cell–cell interface (Boggon et al., 2002). Interestingly, when homology models for desmoglein-2 and desmocollin-2 were generated using the C-cadherin crystal structure as a template, it was shown that the three-dimensional arrangement of desmosomal cadherins also generated a similar lattice (Garrod et al., 2005). As such, this arrangement of desmosomal cadherins is believed to confer hyperadhesion in desmosomes (Section 2.2). It is hoped that studies in the future make available the crystal structures of desmosomal cadherins so that these hypothetical models can be validated.

On a final note relating to desmosomal cadherin distribution, useful information has also been obtained from cryoelectron tomography experiments which helped to define the arrangement of desmosomal cadherin molecules within desmosomes *in situ*. Desmosomes visualized in close-to-native conditions using vitreous sections of human epidermis displayed a regular but periodic arrangement of cadherin molecules along the midline (Al-Amoudi et al., 2004, 2007). In contrast, when freeze-substituted resin-embedded sections of newborn mouse epidermis were used, an irregular arrangement of desmosomal cadherin molecules was observed with clustering of their N-terminal ends (He et al., 2003). Owen et al. (2008) sought to settle this discrepancy in data by using the same tissue but processing it in two different ways. Tomographic slices were obtained from both frozen and freeze-substituted sections of cow snout epidermis. In both cases, desmosomes exhibited irregular midlines in the extracellular space, illustrating that discrete groups of molecules are present rather than a uniform arrangement (Owen et al., 2008). At this point, additional studies using a higher resolution are needed to further reconcile these conflicting observations.

2.1.2. Armadillo proteins

Proteins from the armadillo family that constitute the desmosome include plakoglobin and members of the p120 catenin subfamily, namely plakophilins-1 to -3 (Delva et al., 2009; Green and Gaudry, 2000; Green and Simpson, 2007). Another member of the p120 catenin subfamily, p0071 (also known as plakophilin-4), has also been hypothesized to be a component of both desmosomes and adherens junctions (Hatzfeld and Nachtsheim, 1996; Hatzfeld et al., 2003). However, its presence at the desmosome is somewhat arguable at this point (Hatzfeld, 2007; Hofmann et al., 2008, 2009). Thus, this protein will not be discussed any further. Armadillo proteins are unique because they are characterized by a series of armadillo repeats which are flanked by N-terminal head and C-terminal tail

domains. These proteins display variable tissue expression patterns, and they localize to desmosomes, as well as to nuclei. Therefore, they are defined as multifunctional scaffolding proteins with dual roles in cell adhesion and signal transduction (Bass-Zubek et al., 2009; Hatzfeld, 2007; Schmidt and Jager, 2005).

2.1.2.1. Plakoglobin Plakoglobin (also known as γ-catenin) is an adaptor protein found in both desmosomes and adherens junctions, and it also localizes to nuclei in various cells (Delva et al., 2009; Green and Gaudry, 2000). Proteins that interact with plakoglobin include classic and desmosomal cadherins, α-catenin, plakophilins, and desmoplakin (Choi et al., 2009; Hatzfeld, 2007). Plakoglobin is critical for the early stages of desmosomal assembly. For example, in keratinocytes isolated from plakoglobin knockout mice, the incorporation of desmosomal proteins such as desmogleins-1 and -2 into the plasma membrane was impaired (Yin et al., 2005a). This phenotype, which could not be rescued by β-catenin, was reversed by the reexpression of plakoglobin. In another related study published by a separate group of investigators, keratinocytes from plakoglobin knockout mice were still capable of clustering desmosomal cadherins on the cell surface and forming desmosome-like junctions, except that in this case β-catenin appeared to compensate for the loss in plakoglobin. However, β-catenin still failed to recruit sufficient amounts of plakophilin-1 and desmoplakin to desmosomes, thereby resulting in thin and sparse cytoplasmic plaques that were weakly connected to intermediate filaments (Acehan et al., 2008). Moreover, during Ca^{2+}-induced junction assembly, plakoglobin was found to first associate with desmocollin-3, which preceded binding of desmocollin-3 to desmoglein-3 (Aoyama et al., 2009). These studies exemplify the important role of plakoglobin in recruiting desmosomal components to the desmosome which are needed for junction assembly.

Desmosomal cadherins are known to bind to plakoglobin's armadillo domain, and they generally do not bind to β-catenin (Choi et al., 2009). Thus, plakoglobin is thought to be largely responsible for the vertical linkage of desmosomal cadherins to desmoplakin, in contrast to plakophilin which facilitates lateral linkage between desmoplakins (Hatzfeld, 2007; Kowlczyk et al., 1999; Section 2.1.2.2). Plakoglobin is also an important regulator of cell adhesion and motility. For instance, the adhesive strength of desmosomes was shown to be downregulated by phosphorylation of plakoglobin following activation of the epidermal growth factor receptor (EGFR). This resulted in the dissociation of desmoplakin, which is an important prerequisite for cell movement during wound healing (Yin et al., 2005a). Finally, plakoglobin also plays an important role in several signaling cascades during the processes of cell motility, cell proliferation, and apoptosis, as well as in the pathogenesis of pemphigus vulgaris (de Bruin et al., 2007; Yin et al., 2005b; Section 2.3). All these processes involve

the interaction of cytoplasmic plakoglobin with various kinases and the inhibition of Wnt−β-catenin signaling by nuclear plakoglobin (Garcia-Gras et al., 2006).

2.1.2.2. Plakophilins Plakophilins-1 to -3 have been shown to localize to the nuclei of various cells irrespective of the presence of desmosomes, but their incorporation into these structures was found to be tissue specific (Bass-Zubek et al., 2009; Hatzfeld, 2007; Schmidt and Jager, 2005). For instance, plakophilin-1 is expressed predominantly in the differentiated layers of the epidermis (Bass-Zubek et al., 2009), whereas plakophilin-2 is found in desmosomes in almost all desmosome-bearing cells, including those in simple epithelia (e.g., colon) and the proliferating layer of complex epithelia, as well as in nonepithelial cells such as cardiomyocytes (Bass-Zubek et al., 2009). Finally, plakophilin-3 is found in desmosomes of both simple and complex epithelia (Bonne et al., 1999). The functional significance of plakophilin in the structural integrity of desmosomes is demonstrated by several human diseases. For instance, mutations in plakophilin-1 result in ectodermal dysplasia/skin fragility syndrome (McGrath et al., 1997), whereas mutations in plakophilin-2 associate with arrhythmogenic right ventricular cardiomyopathy (Gerull et al., 2004).

Coimmunoprecipitation, *in vitro* binding assays, and yeast two-hybrid systems have been used to identify plakophilin-interacting proteins. Plakophilins are unique in that they bind to almost all other proteins within the desmosome, including desmosomal cadherins, plakoglobin, desmoplakin, and intermediate filament proteins (Bonne et al., 2003; Chen et al., 2002; Hatzfeld et al., 2000; Hofmann et al., 2000; Kowlczyk et al., 1999). However, intermediate filaments do not appear to be decorated by plakophilins *in vivo* (Hofmann et al., 2000). Plakophilin-2 also binds to the adherens junction adaptors β-catenin (Chen et al., 2002) and αT-catenin (Goossens et al., 2007). In essence, this broad myriad of interacting proteins enables plakophilins to function as important hubs within desmosomes by stabilizing other desmosomal components at the cell–cell interface (Hatzfeld et al., 2000; Kowlczyk et al., 1999).

The interaction between plakophilins and desmoplakin is of particular importance because the plakophilin head domain contains at least two desmoplakin-binding sites (Bonne et al., 2003). This is thought to confer lateral linkage between individual desmoplakin molecules, which are multidomain proteins that tether the whole desmosome to the intermediate filament network. Indeed, plakophilin-1-deficient keratinocytes formed fewer Ca^{2+}-independent desmosomes (South et al., 2003), a cellular phenomenon that requires a tight array of desmosomal proteins (Garrod and Kimura, 2008). A downregulation of plakophilin also resulted in the dissociation of desmoplakin and in the disassembly of the junctional complex (Grossmann et al., 2004; Pieperhoff et al., 2008). Furthermore,

plakophilin-1 is known to compete with plakoglobin for desmoplakin binding. Interestingly, both armadillo proteins are required for the clustering of desmosomal components into punctate structures, which otherwise would display a continuous distribution along the cell surface and possibly abrogate robust desmosomal adhesion (Bornslaeger et al., 2000).

As versatile adaptors with several binding partners, it is not surprising that plakophilins also interact with the actin cytoskeleton, as well as with the actin-based adherens junction. Plakophilin-1 binds to actin filaments via its armadillo domain, which is different from the domain required for desmoplakin interaction (i.e., the N-terminal head domain; Hatzfeld et al., 2000). Moreover, decoration of actin filaments by plakophilin-1 has been observed in both normal and overexpressed cells, and the upregulation of plakophilin-1 induced the formation of actin-containing structures such as filopodia and cell protrusions (Hatzfeld et al., 2000). As mentioned above, plakophilin-2 also binds to αT-catenin in the area composita, a specialized junction found at intercalated disks in the heart that contains both adherens junction and desmosome components (Goossens et al., 2007). This interaction may strengthen cell–cell adhesion by providing the actin-based cadherin–catenin complex with an additional opportunity to link to the intermediate filament network. It is also worth noting that plakophilin-2 interacts with noncadherin-bound β-catenin and promotes β-catenin−T cell factor (TCF) signaling (Chen et al., 2002).

Plakophilins also localize to cell nuclei, but their exact roles within this structure have yet to be defined. One study showed that plakophilin-2 was present in nuclear particles containing the largest subunit of RNA polymerase III and that it was also found in the holoenzyme (Mertens et al., 2001). RNA polymerase III is responsible for the transcription of rRNA and tRNA, which may explain in part the constitutive nuclear localization of plakophilin-2. This is in contrast to the transient nuclear localization of another armadillo protein, β-catenin, which is imported to the nucleus only upon the induction of Wnt signaling that activates the transcription of Wnt target genes (Clevers, 2006). Also, recent studies have suggested a role for plakophilins in translation. Plakophilin-3 was detected in cytoplasmic particles containing poly (A)-binding, fragile-X-related, and ras-GAP-SH3-binding proteins, all of which are RNA-binding proteins. When cells were exposed to stress, these proteins together with plakophilin-1 or -3 were found within stress granules that are known to accumulate stalled translational initiation complexes (Hofmann et al., 2006). In addition, plakophilin-1 was shown to associate with eukaryotic translation initiation factor 4A1 (eIF4A1), and plakophilin-1 overexpression enhanced its translational activity by promoting adenosine triphosphatase activity (Wolf et al., 2010). Taken collectively, these findings show the involvement of plakophilins in translation.

2.1.3. Plakins

Plakins are large multidomain proteins that link junction proteins to the cytoskeleton. They comprise an N-terminal plakin domain for binding adaptor proteins, a central coiled-coil domain for dimerization and several C-terminal plakin repeat domains for intermediate filament binding. The plakin repeat domain comprises a series of plakin repeat motifs that are characteristic of this family (Jefferson et al., 2004; Leung et al., 2002; Sonnenerg and Liem, 2007). In addition to this basic structure, nondesmosomal plakins can also contain an actin-binding domain, spectrin repeats, and a Gas-2-related domain for microtubule binding, which enable plakins to mediate crosstalk between cytoskeletal networks. Similar to members of the armadillo protein family, plakins are involved in both cell adhesion and signaling. For instance, as a constitutive component of the desmosome, desmoplakin is needed for the late stages of desmosomal assembly. Moreover, desmoplakin is downregulated during wound healing and morphogenesis, which destabilizes desmosomes. Interestingly, this is mediated by the proteolytic action of caspases (Aho, 2004). Plakins are also known to participate in important signaling pathways by acting as platforms for kinase activity (van den Heuvel et al., 2002; Sections 2.3 and 2.4).

To date, desmoplakin, periplakin, and envoplakin have been reported to localize to desmosomes, plectin, and bullous pemphigoid antigen 1 (BPAG-1) to associate with hemidesmosomes and microtubule actin cross-linking factor (MACF) to associate with focal adhesions. However, the localization of epiplakin is presently unclear (Jefferson et al., 2004; Leung et al., 2002; Sonnenerg and Liem, 2007). Desmoplakin is a putative constituent of the desmosomal plaque and is found in all epithelia and in cardiomyocytes, while periplakin and envoplakin are found in complex epithelia but not in simple epithelia and nonepithelial cells such as cardiomyocytes.

The function of desmoplakin is to tether cytoplasmic plaque proteins (e.g., plakoglobin) and the cytoplasmic tails of desmosomal cadherins to intermediate filaments. As mentioned above, individual desmoplakin molecules are linked laterally by plakophilins (Leung et al., 2002). To investigate the role of desmoplakin in desmosomal assembly, Godsel et al. (2005) studied the dynamics of desmoplakin incorporation by visualizing desmoplakin tagged to green fluorescent protein using time lapse imaging. Their findings demonstrated that desmoplakin binding to intermediate filaments was not required for the early stages of desmosomal assembly. Specifically, it was reported that desmoplakin was incorporated into discrete puncta in a *de novo* manner, followed by their aggregation. Thereafter, cytoplasmic particles containing desmoplakin, as well as plakophilin-2, were visible, and these were shuttled toward the cell surface via the actin network. In cells expressing desmoplakin with a deleted plakin repeat domain, which affected binding to intermediate filaments, the translocation of cytoplasmic particles

to the plasma membrane was interrupted at first but was then restored to normal. Conversely, phosphorylation-deficient desmoplakins, which showed an increase in intermediate filament binding, were somewhat delayed in their recruitment to the cell surface (Godsel et al., 2005), illustrating that premature binding to intermediate filaments can affect the assembly of desmosomes.

Another study reported similar findings when desmosome-like junctions were formed in keratinocytes from epidermis-specific desmoplakin knockout mice. In this case, desmosomes were improperly connected to intermediate filaments, lacked the inner dense plaque, and associated with few desmosomal cadherins and plaque proteins (Vasioukhin et al., 2001). This scenario was equivalent to an early stage of normal desmosome assembly during which time cadherin engagement and recruitment of adaptors occurs but not the attachment of plaque proteins to intermediate filaments. Progression through this stage would require desmoplakin-mediated binding to intermediate filaments, as reflected by the formation of partial desmosomes in desmoplakin-null keratinocytes. Furthermore, these cells were incapable of forming an epithelium because adherens junction assembly was halted after the engagement of E-cadherin at cell–cell contacts. The formation of adhesion zippers and actin organization was also adversely affected (Vasioukhin et al., 2001). In essence, these studies emphasize the requirement of desmoplakin for intermediate filament linkage which completes desmosome assembly, as well as the critical role of desmosomes in stabilizing adherens junctions by acting as spot welds to hold apposing membranes together. This facilitates remodeling of the actin cytoskeleton and the sealing of membranes via the formation of mature adherens junctions (i.e., the adhesion belt).

2.1.4. Intermediate filaments

To conclude this section on desmosomal proteins, we include a brief discussion on intermediate filaments. In the cytoplasm, intermediate filaments extend from the perinuclear region to the cell periphery where they link to cell junctions, namely desmosomes and hemidesmosomes. Intermediate filaments are filamentous hetero- or homopolymers, and they do not exhibit polarity, a characteristic of actin microfilaments and microtubules. This is because intermediate filament protein dimers are arranged in antiparallel (Herrmann et al., 2007, 2009; Kim and Coulombe, 2007). Intermediate filaments belong to a large family of proteins comprising $\sim$70 genes in the human which exhibit tissue-specific expression, and they are categorized into five subtypes (Herrmann et al., 2007). For instance, keratins belong to subtypes 1 and 2, but vimentin belongs to subtype 3. Keratins are generally expressed by epithelial cells, whereas vimentin is generally expressed by mesenchymal cells (Eriksson et al., 2009; Kim and Coulombe, 2007). In this regard, it is worth noting that

Sertoli cells in the seminiferous epithelium are an exception to this rule: Sertoli cell intermediate filaments comprise vimentin, while keratins are only expressed during their development (Vogl et al., 2008).

2.2. Unique features of desmosomes

2.2.1. Ca^{2+} independence

Ca^{2+} independence is a unique characteristic of mature desmosomes, and this feature is not shared by other types of junctions such as adherens and tight junctions (Garrod and Kimura, 2008). Ca^{2+} dependence is an inherent characteristic of cadherin molecules because their rod-like conformation can only be maintained when Ca^{2+} ions are bound to their Ca^{2+} binding sites at linker regions (Leckband and Prakasam, 2006). This conformation favors *trans*-dimer formation between opposing cell surfaces to confer cell–cell adhesion. Otherwise, Ca^{2+}-free cadherin molecules would become flexible. This relaxed conformation favors the formation of *cis*- instead of *trans*-dimers (Troyanovsky et al., 2003). The mechanism of Ca^{2+} independence was revealed by structural information relating to the cadherin ectodomain. As discussed previously, a homology model of the desmocollin-2 ectodomain structure was obtained (Garrod et al., 2005) by using the C-cadherin crystal structure as a template (Boggon et al., 2002). From this model, the three-dimensional packing of the desmocollin-2 ectodomain resulted in rows of back-to-back *cis*-interacting desmosomal cadherin molecules distributed at regular intervals on each cell surface. Within this arrangement, the Ca^{2+}-binding site present between EC2 and EC3 on one cadherin molecule was protected by a small β-helix found in EC1 from the neighboring cadherin molecule, thereby trapping the bound Ca^{2+} ions even in a low Ca^{2+} environment (Garrod et al., 2005). Nevertheless, Ca^{2+} independence *per se* is only the end product of this tight cadherin arrangement, and this property has no relevance *in vivo* because a Ca^{2+}-depleted environment can only be created *in vitro*. Hyperadhesion, however, is the endpoint of the compact arrangement of cadherins (Section 2.2.2), and it can only be attained by mature desmosomes which are also Ca^{2+} independent. Thus, Ca^{2+} independence can be a useful tool to experimentally study the adhesive status of desmosomes (Garrod and Kimura, 2008).

2.2.2. Hyperadhesion

It is hypothesized that hyperadhesion (and thus Ca^{2+} independence) is a characteristic of mature desmosomes *in vivo* such as those found in the epidermis and heart, thus enabling desmosomes in these tissues to endure great physical stress (Garrod et al., 2005). Mature desmosomes are typified by a dense midline in the extracellular space, indicative of the regular arrangement of tightly packed cadherin molecules. During morphogenesis

and wound healing, desmosomal adhesion is downregulated by intracellular signals to allow cell motility, giving rise to desmosomal plaques with no discernable midlines due to the irregular arrangement of cadherin molecules. However, both halves of the desmosome were still attached. The connection between hyperadhesion and Ca^{2+} independence was demonstrated by Garrod et al. (2005). Mature desmosomes in the epidermis *in vivo* were resistant to disruption after prolonged exposure to low-Ca^{2+} medium, while wound-edge desmosomes split into two halves. Interestingly, wound-edge desmosomes associated with protein kinase C (PKC)-α, suggesting that PKC-α-mediated inside–out signals downregulate desmosomal adhesion (Garrod et al., 2005). Epithelial cells cultured at high density *in vitro* could also attain Ca^{2+} independence over time after reaching confluency. The adhesive strength of desmosomes before and after the acquisition of Ca^{2+} independence was experimentally determined by an adhesion assay using intact cell sheets (Kimura et al., 2007). Indeed, when HaCaT cell sheets cultured for 2 days were subjected to rotational shear force, sheets were fragmented into several more pieces, as compared to cell sheets cultured for 6 days which had acquired Ca^{2+} independence (Kimura et al., 2007). This validated the association between adhesive strength and Ca^{2+} dependence. Furthermore, in agreement with *in vivo* findings, the use of a PKC-α/β inhibitor or PKC-α antisense oligonucleotides could rapidly reverse desmosomes to the Ca^{2+}-independent state without affecting the levels of desmosomal proteins, which is an extremely useful method for manipulating desmosomal adhesion (Kimura et al., 2007; Wallis et al., 2000).

Hyperadhesion not only strengthens cell–cell adhesion in stress-bearing tissues but has also recently been shown to attenuate the disruptive effects of pemphigus vulgaris serum (Cirillo et al., 2010). When keratinocytes were treated with the PKC-α/β inhibitor Go6976, a hyperadhesive state was rapidly induced. Interestingly, subsequent exposure of treated cells to pemphigus vulgaris serum resulted in significantly less cell–cell detachment then in non-Go6976-treated cells. It was proposed that the compact cadherin arrangement in hyperadhesive desmosomes helped to protect the epitopes from being targeted by autoantibodies, thereby attenuating their disruptive effects. To investigate whether hyperadhesion is solely responsible for this protective effect, the authors prevented hyperadhesion by pretreating cells with blocking peptides of cadherin adhesion recognition sites. In this case, the PKC inhibitor could still partially attenuate the effects of the autoantibodies, illustrating that other aspects were also responsible for the pathogenesis of pemphigus vulgaris (Cirillo et al., 2010). These results illustrate that the maintenance of hyperadhesion can provide new insights into the treatment of pemphigus vulgaris.

2.3. Regulation of desmosomal adhesion

Desmosome function is under strict regulation by multiple factors. For instance, desmosomal adhesion can be downregulated at the wound edge during healing to prepare cells for migration. Desmosomal adhesion is also regulated by numerous signaling cascades that involve kinases and proteases, and many of these molecules are known to directly interact with desmosomal plaque proteins. Cirillo and colleagues compiled interactions between desmosomal and cellular proteins in keratinocytes by a systems biology approach, and classified these interactions into six functional subnets (Cirillo and Prime, 2009). Apart from membrane proteins, adaptors, and cytoskeletal proteins, the three main types of proteins that desmosomal proteins are known to interact with are kinases, phosphatases, and proteases, illustrating that desmosome function is regulated by these protein families. For example, phosphorylation of cytoplasmic plaque proteins by kinases was shown to introduce negative charges on the former. These proteins then repelled each other, thereby perturbing adhesive strength (Garrod and Chidgey, 2008). Indeed, there are other important examples in which kinases were able to downregulate desmosomal adhesion, and these are discussed in Section 2.3.1.

2.3.1. Regulation by kinases

2.3.1.1. PKC PKC-mediated downregulation of desmosomal adhesion and its reversion to Ca^{2+} dependence were discussed previously, and readers are asked to refer to Section 2.2.2. After treating urinary bladder carcinoma cells with the PKC-α/β inhibitor Go6976, cell adhesion was shown to be strengthened. Interestingly, this was accompanied by an increase in the number of desmoplakin-positive spot welds at the cell–cell interface (Koivunen et al., 2004), findings which may suggest that more desmoplakin molecules were recruited to desmosomes or that the actual number of desmosomes increased. Since these effects—which appeared to be specific to desmosomes—were not affected by the actin disruptor cytochalasin-D, it was concluded that PKC regulates desmosomes (Koivunen et al., 2004). On the contrary, desmoplakin failed to be recruited to desmosomes when it was not phosphorylated by PKC-α (Bass-Zubek et al., 2008). Moreover, in SCC-9 and A431 epithelial cell lines, desmoplakin recruitment required the formation of a multiprotein complex comprising plakophilin-2, desmoplakin, and PKC-α. The disruption of this multiprotein complex by plakophilin-2 silencing rendered desmoplakin less phosphorylated which then associated with the intermediate filament network (Bass-Zubek et al., 2008).

2.3.1.2. Src Like many adherens and tight junction proteins, desmosomal proteins can also be regulated by Src. Remarkably, phosphorylation of plakoglobin by Src appears to elicit opposite effects in adherens junctions and in desmosomes. When plakoglobin was phosphorylated on Tyr-643 (equivalent to Tyr-654 on β-catenin), its association with E-cadherin and α-catenin decreased, whereas its association with desmoplakin increased (Miravet et al., 2003). The role of Src in the regulation of desmosomes was also illustrated by the pathogenesis of the autoimmune disease pemphigus vulgaris (Miravet et al., 2003). Exposure of keratinocytes to desmosomal protein autoantibodies led to the activation of Src which peaked at ~30 min, followed by the activation of EGFR and p38 mitogen-activating protein kinase (MAPK). Contrary to the hypothesis that acantholysis (the loss of adhesion between keratinocytes) is induced by antidesmoglein-1 or -3 antibodies, these investigators demonstrated that these proteins were not involved in the initial events of deadhesion. Instead, they showed that cell–cell detachment was triggered by nondesmoglein antibodies, eliciting a signaling cascade involving Src, which downregulated desmosomal adhesion via inside–out signaling (Miravet et al., 2003).

2.3.1.3. EGFR In squamous cell carcinoma cells cultured in low Ca^{2+} medium which only permitted the formation of E-cadherin-based adhesion, the inhibition of EGFR resulted in a shift from a fibroblastic to a more epithelial cell phenotype resulting from an induction of desmosomal assembly (Lorch et al., 2004). This was demonstrated by the expression and recruitment of desmoglein-2 and desmocollin-2 to the cell surface, a decrease in the phosphorylation of desmoglein-2 and plakoglobin, and an increase in desmoglein-2 and desmoplakin in the triton-insoluble fraction. These effects were specific to desmosomes because EGFR activation affected neither the phosphorylation states nor the localization of E-cadherin and β-catenin (Lorch et al., 2004).

2.3.2. Regulation by proteases

2.3.2.1. Caspases Caspases are a family of cysteine proteases that serve as primary effectors during apoptosis to proteolytically dismantle most cellular structures, including the cytoskeleton, cell junctions, mitochondria, endoplasmic reticulum, Golgi, and the nucleus (Taylor et al., 2008). They exist as inactive zymogens in normal cells, but they are rapidly processed into active proteases in response to apoptotic signals. The initial activation of major caspases such as caspases 3 and 7 leads to the activation of another set of caspases, which in turn activate more caspases in this cascade. Together, they mediate proteolysis of cellular proteins (Taylor et al., 2008). Many desmosomal proteins are targets of caspases during apoptosis, including desmogleins-1 to -3 (Cirillo et al., 2008; Nava et al., 2007; Weiske et al., 2001), plakoglobin (Weiske et al., 2001), plakophilin-1 (Weiske et al., 2001), and desmoplakin

(Aho, 2004; Nava et al., 2007; Weiske et al., 2001). Cleavage of these proteins produces two or more intracellular and extracellular fragments, which are then further processed into smaller fragments and internalized from the cell surface, resulting in the disintegration of desmosomal structure. These cleaved proteins can have biological activity, and they appear to be required for the propagation of apoptotic signals, illustrating that desmosomal proteolysis plays a role in the regulation of pathogenesis and normal development (Section 2.4.4).

2.3.2.2. Matrix metalloproteases and ADAMs The matrix metalloprotease (MMP) family of proteases—with collagenase being the founding member—plays an important role in disease and in development. Some MMPs such as MMP 14 (also known as membrane type 1-MMP) are membrane bound (Page-McCaw et al., 2007), whereas A Disintegrin And Metalloproteases (ADAMs) comprise a related family of proteases which specialize in the shedding of membrane-bound proteins (Huovila et al., 2005). Desmosomal proteins that are cleaved by MMPs or ADAMs include desmogleins-1 to -3 and desmocollin-3 (Dusek et al., 2006; Klessner et al., 2009; Santiago-Josefat et al., 2007; Weiske et al., 2001). Moreover, desmoglein-2 was found to be targeted by a broad spectrum of proteases, including MMPs and ADAMs 9, 10, 15, and 17, as well as caspase 3. While MMP and ADAM 17 generated a 100-kDa fragment encompassing the cytoplasmic tail, transmembrane, and extracellular juxtamembrane domains, further processing of this fragment required ADAM 10, whose knockdown led to the accumulation of this 100 kDa fragment (Klessner et al., 2009). In normal cells, ADAM 17 is located at the dorsal cell surface or within intracellular vesicles. Thus, it is sequestered away from desmoglein-2 which is present at the cell surface. Interestingly, EGFR can stabilize internalized desmoglein-2 during apoptosis, which would otherwise become available for ADAM-mediated proteolytic cleavage (Klessner et al., 2009; Santiago-Josefat et al., 2007).

2.3.2.3. Serine proteases In the epidermis, terminal differentiation is a process that produces the outermost cornified layer of apoptotic cells. These cells possess modified desmosomes called corneodesmosomes, which contain desmoglein-1 and desmocollin-1, as well as another extracellular glycoprotein known as corneodesmosin (Candi et al., 2005). The desquamation of these apoptotic cells is mediated by the cleavage of corneodesmosomal proteins by serine proteases, including stratum corneum chymotryptic enzyme (SCCE) and stratum corneum tryptic enzyme (SCTE), until cells no longer retain their adhesive properties and detach (Ovaere et al., 2009). Desmoglein-1, desmocollin-1, and corneodesmosin are all targeted by these two serine proteases (Caubet et al., 2004; Simon et al., 2001). Interestingly, SCTE was unable to cleave recombinant corneodesmosin *in vitro*, suggesting that this

enzyme may act through the activation of another protease. Indeed, SCTE is capable of proteolytically processing pro-SCCE into its mature active form, which in turn cleaves corneodesmosin (Caubet et al., 2004). The activity of these serine proteases is under the regulation of inhibitors such as lympho-epithelial kazal-type inhibitor (LEKTI), whose deficiency led to increased SCCE and SCTE activity and the Netherton syndrome, a skin disease (Descargues et al., 2005).

2.4. Desmosome-mediated signaling

Emerging evidence suggests that desmosomes also function as signaling centers (Delva et al., 2009; Green and Gaudry, 2000; Green and Simpson, 2007), especially since not all tissues possess mature desmosomes. However, many investigators prefer not to use the terminology "desmosome signaling" because it may be confused with the type of signaling triggered by integrins. Nevertheless, desmosomal proteins are capable of transducing signals via both genomic and nongenomic pathways, as well as indirectly affecting the localization of signaling molecules.

2.4.1. Desmosomal proteins as scaffolds for kinases

Many desmosomal proteins, especially adaptors such as plakoglobin and plakophilin-2, are known to physically interact with kinases (Cirillo and Prime, 2009). While some of these proteins are substrates for kinases, they can also tether kinases to desmosomes or intermediate filaments. The interaction between PKC-α and desmosomal plaque proteins was discussed previously (Garrod et al., 2005; Wallis et al., 2000), and readers are asked to refer to Section 2.2.2. Among the several plaque proteins that bind to PKC-α, plakophilin-2 was reported to form a complex with PKC-α and desmoplakin in SCC-9 and A431 epithelial cell lines (Bass-Zubek et al., 2008). Disruption of this complex by plakophilin-2 knockdown not only blocked desmoplakin recruitment but also resulted in the release of PKC-α and in the increase in phosphorylation of PKC-α substrates, indicating a role for plakophilin-2 in the sequestration of PKC-α (Bass-Zubek et al., 2008). In addition, the C-terminal domain of the desmosomal plakin, periplakin, was shown to interact with protein kinase B (PKB, also known as Akt; van den Heuvel et al., 2002). Overexpression of this domain resulted in a decrease in nuclear PKB, thereby reducing PKB-dependent Forkshead transcription factor activity.

Downstream of these kinases, desmosomes can also regulate other important signaling molecules such as Src and MAPK through which numerous pathways converge and diverge. For instance, an increase in cell motility was noted in keratinocytes isolated from the skin of plakoglobin knockout mice. This effect was suppressed by using PP2, a Src family

inhibitor or U0126, a MAPK kinase 1/2 inhibitor (Yin et al., 2005b). Furthermore, desmoglein-2 was found to associate with Src in a recent study from our lab when Sertoli cell and seminiferous tubule lysates were used for coimmunoprecipitation. Interestingly, simultaneous knockdown of desmoglein-2 and desmocollin-2 by RNA interference (RNAi) led to the relocation of Src from the Sertoli cell surface to the cytoplasm, suggesting that desmoglein-2 is a docking site for Src (Lie et al., 2010; Fig. 5.4). Concurrent with the mislocalization of Src, a decrease in the integrity of the tight junction barrier was noted, possibly resulting from the disruptive effects of Src on occludin–zonula occludens-1 (ZO-1; Kale et al., 2003) and connexin-43–ZO-1 (Gilleron et al., 2008) interactions, which are critical for barrier dynamics (Li et al., 2009). Another example of cadherin–kinase interaction is that between desmoglein-1 and EGFR. Desmoglein-1 was required for the suppression of the EGFR-Erk1/2 (a MAPK) signaling pathway during the terminal differentiation of keratinocytes, which exemplifies the importance of desmosomal signaling in tissue development (Getsios et al., 2009).

2.4.2. Desmosomal proteins as signal propagators for kinases

While many desmosomal proteins are regulated by kinases (Section 2.3.1), they can still propagate signals via downstream pathways and feedback loops. Plakoglobin is a good example, acting both as a substrate and as an effector of kinases (Berkowitz et al., 2005; Chernyavsky et al., 2007). During the pathogenesis of pemphigus vulgaris, nondesmoglein antibodies were shown to trigger acantholysis, followed by the activation of Src and EGFR (Berkowitz et al., 2005; Chernyavsky et al., 2007). This in turn downregulated desmosomal adhesion by phosphorylating desmosomal proteins (Garrod and Chidgey, 2008; Yin et al., 2005b; Section 2.3.1). One of the targets of these kinases is plakoglobin because plakoglobin-deficient keratinocytes were unaffected by pemphigus antibodies (Dusek et al., 2007). In addition, interesting changes relating to the phosphorylation state and intracellular localization of plakoglobin were also noted. For instance, depletion of nuclear plakoglobin was shown to mediate the transcription of c-myc (Williamson et al., 2006) and Wnt target genes (Garcia-Gras et al., 2006). Thus, desmosomes are not only targets of kinases (Section 2.3.1); they can also act as their effectors by eliciting downstream signaling.

2.4.3. Desmosomal proteins as regulators or components of transcriptional machinery

Many desmosomal proteins such as plakoglobin and plakophilin are also found in the nucleus, even in nondesmosome-bearing cells where they appear to participate in the regulation of gene expression (Delva et al.,

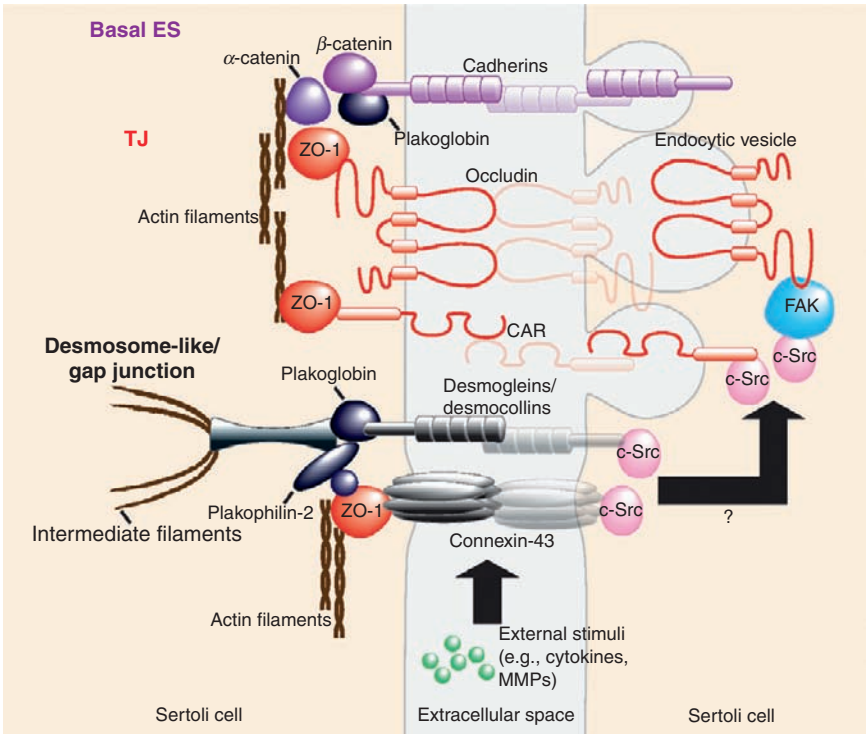

Figure 5.4 Crosstalk between different junction types at the BTB as a mechanism to regulate and to maintain the integrity of the immunological barrier. The BTB is constituted by adjacent Sertoli cells near the basement membrane and is composed of coexisting tight junctions (i.e., occludin), basal ectoplasmic specializations (i.e., N-cadherin), desmosome-like junctions (i.e., desmogleins, desmocollins, and

2009; Green and Gaudry, 2000; Green and Simpson, 2007). As discussed previously, nuclear plakophilin-2 is a component of RNA polymerase III holoenzyme which is responsible for the synthesis of rRNA and tRNA, thereby explaining its constitutive nuclear localization (Mertens et al., 2001). However, a balance exists among the distribution of armadillo proteins in junctions, the cytoplasm, and the nucleus in desmosome-bearing cells which essentially prevents gene expression from being disrupted (Bass-Zubek et al., 2009; Hatzfeld, 2007; Schmidt and Jager, 2005). For instance, plakoglobin is a target of multiple kinases (Cirillo and Prime, 2009). During pemphigus vulgaris, the increased rate of plakoglobin turnover at the desmosome, possibly mediated by Src and EGFR, led to the depletion of plakoglobin from the nucleus (Williamson et al., 2006). However, phosphorylation of Tyr-549 by Fer kinase increased plakoglobin's association with adherens junctions (Miravet et al., 2003).

The transit of plakoglobin to the nucleus is known to inhibit two genomic pathways. First, plakoglobin is required for the suppression of mitogenic c-myc transcription. Depletion of nuclear plakoglobin—and thus c-myc transcription—appeared to cause proliferation, as well as a weakening of cell–cell adhesion (Williamson et al., 2006). This illustrates that desmosomal proteins are capable of eliciting downstream signals and generating feedback loops to regulate cell–cell adhesion. Similar effects were also seen in plakoglobin knockout keratinocytes, which failed to accumulate plakoglobin in their nuclei and did not undergo acantholysis in response to pemphigus vulgaris antibodies (de Bruin et al., 2007; Dusek et al., 2007). Second, plakoglobin antagonizes with β-catenin in the canonical β-catenin−Wnt signaling pathway by inhibiting the transcriptional activity of β-catenin−Tcf/Lef1. As discussed previously, the armadillo proteins plakoglobin and β-catenin are homologous, both in their sequences and in their function as adaptors in desmosomes and/or adherens junctions. However, nuclear plakoglobin and β-catenin are antagonistic in

plakophilins), and gap junctions (i.e., connexin-43). The physiological significance for the coexistence of these junctions at the BTB has remained unknown for decades. Recent studies, however, have demonstrated that these junctions provide an efficient and effective means to induce restructuring of the BTB to facilitate the transit of preleptotene spermatocytes at stage VIII of the seminiferous epithelial cycle of spermatogenesis. It is likely that external stimuli (e.g., cytokines and MMPs) initiate disruption of two multiprotein complexes: (i) desmoglein-2/desmocollin-2 at the desmosome-like junction and (ii) connexin-43/plakophilin-2 at the gap junction. Since c-Src is an integrated component of both junctions (black arrow), this activates the c-Src signaling cascade, thereby eliciting changes in protein distribution (e.g., occludin, CAR, and N-cadherin) at the Sertoli–Sertoli cell interface as demonstrated in recent studies using RNA.

that they compete for the transcription factors Tcf/Lef1 (Garcia-Gras et al., 2006). By binding to different domains of Tcf-4, they can elicit opposite effects on its transcriptional activity (Solanas et al., 2004). This ligand specificity exhibited by plakoglobin and β-catenin is determined by their N- and C-terminal tails, whereas the binding site is found in the armadillo domain (Solanas et al., 2004). While β-catenin supports transcription of Wnt target genes via Tcf/Lef1 binding, plakoglobin renders these transcription factors less efficient in DNA binding, thereby reducing their transcriptional activity (Garcia-Gras et al., 2006). Taken collectively, these results illustrate that desmosomes also act as signaling centers to transduce signals outside–in and inside–out and that these involve genomic or nongenomic pathways and feedback loops.

2.4.4. Biologically active fragments from the proteolysis of desmosomal proteins

Desmosomal proteins are proteolytically cleaved during apoptosis and tissue remodeling by caspases, MMPs, ADAMs, and serine proteases (Section 2.3.2), and some of these cleavage products have biological activity. For instance, the cleavage of desmoglein-2 during apoptosis by caspase 3 yields 100 and 60 kDa products. However, during this time, full-length desmoglein-2 was shown to increase (Nava et al., 2007), and this appeared to be mediated by the 60 kDa product because its overexpression in normal cells was found to upregulate desmoglein-2 and caspase activity, thereby enhancing apoptotic signals (Nava et al., 2007). The necessity of these proteolytic products for the propagation of apoptotic signals was also reflected by the knockdown of desmoglein-1 (Dusek et al., 2006) and desmoglein-2 (Nava et al., 2007), two caspase 3 targets which protected epithelial cells from apoptosis. Furthermore, desmoplakin was also shown to be cleaved by caspases 2 and 4 during terminal differentiation of the epidermis, and one of these cleavage products was found to remain in the cornified envelope, suggesting that it may possess a yet unknown function (Aho, 2004). Thus, desmosomal proteins participate in the regulation of apoptosis during disease and development.

3. Desmosome-Like Junctions in the Seminiferous Epithelium

Desmosomes were first described in the testis in 1977, but ultrastructurally they do not resemble *bona fide* desmosomes such as the ones found in the skin and heart (Russell, 1977a). As such, they were classified as "desmosome-like" junctions, in part because they lacked a clearly defined dense midline which is characteristic of conventional desmosomes (Russell, 1977a). At the

time, this observation seemingly implied that desmosomes in the seminiferous epithelium are of the Ca^{2+}-dependent type and not likely to mediate robust cell adhesion—features that may be needed to facilitate germ cell movement across the BTB, as well as partially across the seminiferous epithelium. Desmosome-like junctions exist between Sertoli cells at the BTB, and between Sertoli and all germ cell types up to, but not including, step 8 spermatids (Fig. 5.2). Sertoli–germ cell desmosome-like junctions first appear within the seminiferous epithelium at the start of spermatogenesis on postnatal day ~5 in rodents. Once spermatids begin to elongate (i.e., step 8) on postnatal day ~30, the desmosome-like junction is completely replaced by the apical ectoplasmic specialization, revealing that there is no functional overlap between these two junction types at the Sertoli–germ cell interface (Russell, 1977a). At the BTB, however, desmosome-like junctions have been shown to coexist and to cofunction with tight junctions, basal ectoplasmic specializations, and gap junctions (Fig. 5.2).

A recent study from our laboratory aimed to better understand the biology of desmosome-like junctions at the BTB. A survey of desmosomal gene expression indicated that Sertoli and germ cells are equipped with all the necessary components to form functional desmosomes. Constitutive desmosomal proteins such as desmoglein-2, plakophilin-2, plakoglobin, and desmoplakin were shown to be abundantly expressed, while differentiation-specific proteins such as desmoglein-1 were absent in Sertoli cells (Lie et al., 2010). The immunofluorescent staining of desmoglein-2 in the testis showed a punctate pattern of localization at the BTB with some colocalization with the putative BTB protein N-cadherin, illustrating that the desmosome-like junction is an integral component of the BTB. Furthermore, desmoglein-2, desmocollin-2, and plakoglobin were indeed capable of forming a multi-protein complex, which also contained Src as shown by coimmunoprecipitation experiments (Lie et al., 2010).

Interestingly, it has been suggested that gap junctions are incorporated into the desmosomal plaque based on the observation that the width of the extracellular space within desmosomes occasionally converged from 14–18 to 3–5 nm (McGinley et al., 1979; Russell, 1977a). This observation led to the use of the terminology "desmosome-gap junction" to describe desmosomes in the testis (Russell et al., 1983). The notion that desmosomes and gap junctions are physically intermixed at the BTB was addressed by one of our recent studies. The desmosomal plaque protein plakophilin-2 was found to associate with the gap junction protein connexin-43 at the BTB where it mediated crosstalk with tight junctions and basal ectoplasmic specializations during junction reassembly (Li et al., 2009). Moreover, functional RNAi studies of desmosomal proteins at the BTB have been conducted, and they illustrated that desmosome-like junctions can regulate cell adhesion and junction reassembly by interacting with tight junctions, basal ectoplasmic specializations, and gap junctions (Section 5).

4. Junction Complexes in the Seminiferous Epithelium

4.1. Tight junctions

The ultrastructural presence of tight junctions between Sertoli cells at the BTB was reported in the 1970s, but it took until 1998 for occludin to be first identified as a putative BTB protein in the mouse testis (Moroi et al., 1998). Since then, however, other transmembrane proteins, namely claudin, junctional adhesion molecule (JAM), and coxsackie and adenovirus receptor (CAR, a tight junction and ectoplasmic specialization protein), as well as their cytoplasmic adaptor protein ZO-1, have also been identified as constituent proteins of the BTB (Mruk and Cheng, 2004b). Occludin is by far the best studied tight junction protein in different epithelia and endothelia, but its exact function within this junction is not yet known because of contradictory results from *in vitro* and *in vivo* experiments (Shin et al., 2006). While occludin has been linked to several signal transduction cascades such as those involving Raf-1 (Li and Mrsny, 2000; Wang et al., 2007) and RhoA (Matter et al., 2005), it does not appear to be indispensable for tight junction function because occludin-deficient mice possessed morphologically normal tight junctions (Saitou et al., 2000). Moreover, the significance of occludin in BTB function and mammalian spermatogenesis is not entirely known owing to the fact that occludin-deficient mice were found to be sterile, but also because occludin is not expressed by the human testis (Moroi et al., 1998). Recently, an occludin–ZO-1–focal adhesion kinase (FAK) protein complex was found to regulate BTB integrity (Siu et al., 2009; Fig. 5.4). Interestingly, knockdown of FAK, a nonreceptor tyrosine kinase, by RNAi was shown to desensitize Sertoli cells from the adverse effects of cadmium on the permeability barrier (Siu et al., 2009), demonstrating that FAK is an important regulator of BTB restructuring *in vivo*. It is worth noting, however, that FAK does not phosphorylate proteins *per se*. Instead, activated FAK is autophosphorylated, thereby binding to Src, which can then phosphorylate additional sites on FAK (Ilic et al., 1997) or possibly its binding proteins such as occludin (Siu et al., 2009). Indeed, FAK silencing would render occludin non- or less-phosphorylated (Siu et al., 2009), possibly sequestering it to the basolateral membrane. Additional studies are needed to investigate the role of Src within the occludin–ZO-1–FAK multiprotein complex.

Claudins are structural proteins responsible for creating charge-selective pores within tight junctions, and the overall paracellular ion permeability characteristics of an epithelium are defined by the pattern of claudin expression. Claudin 2, for example, is typically found in leaky epithelia, most notably in proximal renal tubules (Kiuchi-Saishin et al., 2002) but not in

seminiferous tubules of the testis (Mruk and Cheng, 2004b) which is well known for its tight blood–tissue barrier. In the seminiferous epithelium, claudin expression (i.e., claudins 1, 3, 4, 5, 8, and 11) has been reported by several investigators, and up until recently, claudin expression in the testis was restricted to Sertoli and endothelial cells (Morita et al., 1999; Morrow et al., 2009). Morrow et al. (2009) now report that spermatogonia and preleptotene spermatocytes also express claudin 5. Claudin 5 immunoreactivity was highest during stage VIII of the seminiferous epithelial cycle, coinciding with an early stage of preleptotene spermatocyte transit across the BTB. This is in agreement with another related study which also reported an elevated level of claudin 3 at stage VIII (Meng et al., 2005). Morrow et al. (2009) also demonstrate that claudin 5 expression in the seminiferous epithelium depended on the presence of germ cells. These findings are intriguing for several reasons. First, germ cells lack tight junctions. Thus, it is not immediately known why germ cells would express a tight junction protein, but it is possible that claudin is participating in some aspect of germ cell movement across the BTB and/or contributing to the maintenance of the immunological barrier. As hypothesized by Morrow et al., germ cell claudin 5 may be working in concert with membrane-type matrix metalloproteases (MT-MMPs) to activate soluble-type MMPs which may be needed to cleave proteins at the BTB during preleptotene spermatocyte transit (Fritz et al., 1993; Longin et al., 2001; Oku et al., 2006; Siu et al., 2003; Fig. 5.4). A stimulation of MT-MMP-mediated pro-MMP-2 activation has also been noted with claudins 1, 2, and 3 in 293 T cells (Miyamori et al., 2001). Second, the observation that Sertoli cell expression of claudin 5 is dependent on the presence of germ cells within the epithelium (Morrow et al., 2009) may suggest that germ cells (i.e., preleptotene spermatocytes) facilitate their transit across the BTB by regulating claudin. Indeed, germ cells are known to regulate several Sertoli cell proteins (Boitani et al., 1981; Morrow et al., 2009; Nicholls et al., 2009). The role of germ cells in Sertoli cell junction restructuring is an exciting new area that needs further investigation.

4.2. Ectoplasmic specializations

The ectoplasmic specialization is a testis specific, actin-based junction that is found at two sites within the seminiferous epithelium: (i) between opposing Sertoli cells at the BTB (defined as the basal ectoplasmic specialization) and (ii) between Sertoli cells and elongating/elongated spermatids in the adluminal compartment (defined as the apical ectoplasmic specialization; Mruk and Cheng, 2004a; Vogl et al., 2008; Fig. 5.2). In the rat, the apical ectoplasmic specialization appears when spermatids reach step 8 of spermiogenesis (i.e., stage VIII), which marks the elongation of round spermatids, and it disappears when spermatids reach step 19 prior to spermiation

(Russell, 1977b). The apical ectoplasmic specialization is known to structurally and functionally replace the desmosome-like junction as the only adhesive structure found between Sertoli and germ cells at these developmental stages. Ultrastructurally, the ectoplasmic specialization consists of a layer of hexagonally packed actin microfilaments found between the Sertoli cell plasma membrane and a network of endoplasmic reticulum, a hallmark feature observed in electron micrographs of the apical and basal ectoplasmic specialization. However, the ectoplasmic specialization has not been observed to exist in elongating/elongated spermatids. In recent years, the ectoplasmic specialization has been the focus of several studies because of its unique hybrid-like character. Although generally defined as an adhesive structure constituted by anchoring junction proteins (i.e., cadherin and nectin), the apical ectoplasmic specialization has also been shown to be composed of proteins normally found within tight junctions and focal contacts (a type of cell–matrix junction; Mruk and Cheng, 2004a; Mruk et al., 2008). In the remainder of this section on the ectoplasmic specialization, we will discuss recent findings relating to classic cadherins.

There are numerous studies reporting cadherin function to be regulated by cytokines, androgens, estrogens, protein kinases, and phosphatases, and small GTPases, as well as by phosphorylation, changes in protein–protein interactions and endocytosis (Braga et al., 1997; Delva and Kowalczyk, 2009; Le et al., 1999; Lee et al., 2003; MacCalman et al., 1997; Mosesson et al., 2008; Mruk and Cheng, 2004b; Nagafuchi et al., 1993). Recent studies demonstrate a novel mechanism for cadherin regulation via the proteolytic cleavage of type I and type II cadherins by metalloproteases (i.e., ADAMs) and γ-secretase, resulting in the generation of biologically active peptides having diverse functions including roles in cell adhesion (Marambaud et al., 2002, 2003; McCusker and Alfandari, 2009; Reiss and Saftig, 2009; Section 2.3.2). So far, more than 50 cell surface proteins have been shown to undergo ectodomain shedding by proteases. For instance, the cleavage of E- or N-cadherin by ADAM10 was shown to produce an extracellular N-terminal fragment (defined as NTF) and a membrane-bound C-terminal fragment (defined as CTF1). Interestingly, CTF1 was processed rapidly by the γ-secretase complex to produce a cytoplasmic fragment (defined as CTF2), which caused the relocation of β-catenin from the cell surface to the cytoplasm (Marambaud et al., 2002, 2003). Furthermore, this cytoplasmic fragment was found to maintain its association with β-catenin and to activate cyclin D1 (a protein known to promote progression through the G1–S phase of the cell cycle) transcription in the nucleus via the Wnt signaling pathway (Maretzky et al., 2005). Cyclins D1 and D2 upregulation was also demonstrated in another study when a soluble E-cadherin fragment generated by ADAM15 was shown to interact with

ErbB (a receptor tyrosine kinase) and to activate downstream signaling (Najy et al., 2008), suggesting a connection between cell adhesion and cell differentiation/division. In terms of biological activity, the NTF of N-cadherin has been shown to associate with the extracellular matrix, suggesting a role in cell movement (Cifuentes-Diaz et al., 1994; Paradies and Grunwald, 1993; Utton et al., 2001). Indeed, the NTF of cadherin 11 (generated via ADAMs 9 and 13) was demonstrated to promote cell migration in the *X. laevis* neural crest (McCusker et al., 2008). Equally important, ADAMs were found to colocalize and coimmunoprecipitate with cadherins in various cell types (Ham et al., 2002). At this point, studies are warranted to determine if ADAM-mediated cadherin cleavage has any role in BTB restructuring and germ cell movement.

4.3. Gap junctions

The study of gap junctions at the BTB and at the Sertoli–germ cell interface was investigated recently, and this study showed that connexin-43 is a primary component of gap junctions at the BTB (Li et al., 2009). In line with earlier observations which showed gap junctions to be intermixed with desmosomes in the testis (McGinley et al., 1979; Russell, 1977a), connexin-43 was shown to interact with plakophilin-2. Together they regulate junction reassembly at the BTB by interacting with tight junctions and basal ectoplasmic specializations (Li et al., 2009; Section 5.4).

5. Junctional Interplay at the BTB: Tight Junctions, Ectoplasmic Specializations, Desmosome-Like and Gap Junctions

A unique feature of the seminiferous epithelium is the coexistence of different junctions at the BTB, namely tight junctions, basal ectoplasmic specializations, desmosome-like junctions and gap junctions, and these are all located basally in the seminiferous epithelium. This is in contrast to other epithelia where the apical junctional complex contains a belt of tight junctions, followed by a discrete belt of adherens junctions. The coexistence of different junction types at the BTB represents an important mechanism for protecting tight junction barrier integrity while permitting the transit of spermatocytes across the BTB. Evidence to support crosstalk among these junction types at the BTB is becoming increasingly available.

5.1. Crosstalk between adherens and tight junctions

Based on elegant studies in other *in vitro* and *in vivo* systems, the general consensus is that tight junction organization requires prior assembly of adherens junctions and that, once assembled, adherens junctions can influence tight junction function (Rajasekaran et al., 1996). A good example of this is the upregulation of claudin 5 (Taddei et al., 2008) and the control of tight junction permeability (Corada et al., 1999) by vascular endothelial (VE)-cadherin in endothelial cells. While it is not yet known if cadherin can upregulate claudin or other tight junction proteins at the BTB, these findings would provide needed insight on the mechanism behind germ cell transit across the BTB. Interestingly, tight junction and basal ectoplasmic specialization junctional complexes at the BTB were shown to interact via their adaptors. It comes as little surprise that ZO-1 is involved in this crosstalk because it is known to transit between both junction types (Ikenouchi et al., 2007; Itoh et al., 1999). Moreover, tight junctions and basal ectoplasmic specializations are engaged via the association between ZO-1 and α-catenin, and this protein–protein interaction is believed to contribute to tight junction barrier impermeability in basal conditions (Yan and Cheng, 2005). However, when the seminiferous epithelium was being restructured following the administration of adjudin (a chemical entity that specifically affects Sertoli–germ cell adhesion in the testis, leading to germ cell loss and transient infertility), tight junction, and basal ectoplasmic specialization adaptors dissociated from each other, which protected the integrity of tight junction barrier while permitting restructuring of ectoplasmic specializations (Yan and Cheng, 2005).

5.2. Crosstalk between adherens and desmosome-like junctions

Evidence is also emerging to support crosstalk between adherens junctions and desmosomes. For decades, the regulation of actin-based cell junctions (i.e., tight junctions and adherens junctions/ectoplasmic specializations) was presumed to be separate from the regulation of intermediate filament-based junctions (i.e., desmosomes), but it is now well accepted that stable cell adhesion requires cues from both actin and intermediate filaments (Getsios et al., 2004; Huen et al., 2002). Similar to tight junctions in epithelial cells, the assembly of desmosomes requires preexisting adherens junctions. Indeed, in an epithelial cell line null for classic cadherins, overexpression of both E-cadherin and plakoglobin was required for desmosome formation (Lewis et al., 1997), illustrating the significance of these proteins in desmosome organization. Interestingly, plakoglobin was shown to localize to both junction sites in epithelial cells and reported to mediate crosstalk between adherens junctions and desmosomes (Cowin et al., 1986). In the testis,

restructuring of basal ectoplasmic specializations and desmosome-like junctions during the movement of preleptotene spermatocytes may be mediated by plakoglobin since this protein was found to localize to the BTB where it was incorporated into desmosome-like junctions together with desmoglein-2 and desmocollin-2 (Lie et al., 2010; Mruk et al., 2008), but additional functional studies would be needed to support this notion.

5.3. Crosstalk between tight and desmosome-like junctions

While considerably less is known regarding crosstalk between tight junctions and desmosomes in other epithelia, a recent study in the intestinal epithelium has demonstrated JAM-C (a tight junction protein) localization at the desmosome and proposed JAM-C involvement in neutrophil transepithelial migration at this site (Zen et al., 2004). This is in agreement with our recent findings which demonstrated a compromise in tight junction permeability barrier function following the knockdown of desmosomal proteins. Simultaneous knockdown of desmoglein-2 and desmocollin-2 by RNAi in cultured Sertoli cells having an assembled barrier—one which mimicked the BTB *in vivo*—resulted in a marked decrease in tight junction barrier function when assessed by transepithelial electrical resistance (TER) measurements (Lie et al., 2010). This was mediated in part by an acceleration in the endocytosis of CAR, as well as the reduction and mislocalization of ZO-1 and Src, demonstrating junctional interplay between the tight junction, ectoplasmic specialization, and desmosome-like junction at the BTB. This might provide an efficient mechanism for transient junction disassembly to permit the movement of spermatocytes across the BTB during spermatogenesis. Germ cells in transit may release factors to stimulate the proteolytic cleavage of desmosomal cadherins in Sertoli and/or germ cells without affecting other junction complexes. Desmosomal cadherins are specifically targeted by the action of multiple proteases such as MMPs and ADAMs during junction remodeling (Bech-Serra et al., 2006; Klessner et al., 2009). Downregulation of desmosomal cadherins would then increase the rate of CAR turnover at the BTB, facilitating the formation of Sertoli–germ cell CAR–CAR interactions to substitute for the Sertoli cell tight junction barrier, which is transiently compromised likely due to Src-mediated dissociation of occludin–ZO-1 interactions (Lie et al., 2010; Fig. 5.4).

5.4. Interplay of tight, adherens, desmosome-like, and gap junctions

Physical and functional coupling of desmosome-like and gap junctions have been detected at the BTB, which are crucial for mediating crosstalk with tight junctions and basal ectoplasmic specializations during junction

assembly and maintenance. Simultaneous knockdown of plakophilin-2 and connexin-43 (a gap junction protein known to bind plakophilin-2) resulted in compelling changes, including the reduction in CAR and N-cadherin cell surface protein levels and the mislocalization of occludin and ZO-1 from the cell surface, which perturbed barrier function in Sertoli cells *in vitro* (Li et al., 2009; Fig. 5.4). These effects were not produced by the knockdown of either plakophilin-2 or connexin-43 alone. This is in line with another report in which plakophilin-2 was silenced in ventricular myocytes and epicardial cells. In this case, connexin-43 was shown to be affected, resulting in a loss of gap junction function (Oxford et al., 2007). While the central paradigm appears to support functional overlap between tight junctions, ectoplasmic specializations, and desmosome-like and gap junctions, additional studies are needed to elucidate the functional significance of their coexistence at the BTB and the underlying mechanisms of their interactions.

6. Concluding Remarks

In this chapter, we have discussed recent findings relating to tight junctions, ectoplasmic specializations, and desmosome-like and gap junctions in the seminiferous epithelium of the mammalian testis. While we have a good appreciation of the molecular components of these junctions, additional functional studies are needed to better understand their regulation during spermatogenesis, including BTB restructuring and germ cell movement because many questions remain unanswered. What role does the basal ectoplasmic specialization have in the maintenance of BTB integrity during spermatogenesis? How do preleptotene spermatocytes contribute to BTB restructuring? What other tight junction proteins are expressed by preleptotene spermatocytes? How is crosstalk mediated between desmosome-like and gap junctions in the seminiferous epithelium? What is the role of Src within the occludin–ZO-1–FAK multiprotein complex at the BTB? Finally, what specific signaling events are involved in the disassembly of Sertoli–germ cell desmosome-like junctions at stage VII of the seminiferous epithelial cycle, followed immediately by the assembly of apical ectoplasmic specialization at stage VIII? Herein, we have also provided a model of crosstalk between different junctions at the BTB with an emphasis on the role desmosome-like junctions, which should serve as a framework for future studies.

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CHAPTER SIX

Abl Family of Tyrosine Kinases and Microbial Pathogenesis

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Abstract

Abl nonreceptor tyrosine kinases are activated by multiple stimuli and regulate cytoskeletal reorganization, cell proliferation, survival, and stress responses. Several downstream pathways have direct impact on physiological processes, including development and maintenance of the nervous and immune systems and epithelial morphogenesis. Recent studies also indicated that numerous viral and bacterial pathogens highjack Abl signaling for different purposes. Abl kinases are activated to reorganize the host actin cytoskeleton and promote the direct tyrosine phosphorylation of viral surface proteins and injected bacterial type-III and type-IV effector molecules. However, Abl kinases also play other roles in infectious processes of bacteria, viruses, and prions. These activities have crucial impact on microbial invasion and release from host cells, actin-based motility, pedestal formation, as well as cell–cell dissociation involved in epithelial barrier disruption and other responses. Thus, Abl kinases exhibit important functions in pathological signaling during microbial infections. Here, we discuss the different signaling pathways activated by pathogens and highlight possible therapeutic intervention strategies.

Key words: Tyrosine phosphorylation, Abl kinase, Actin cytoskeleton, Molecular pathogenesis.

1. Introduction

1.1. Abl tyrosine kinases as central regulators of signal processes in health and disease

The nonreceptor tyrosine kinases c-Abl (Abl-1) and its paralog Arg (Abl-related gene, *Abl-2*) belong to the family of nonreceptor tyrosine kinases in vertebrates. Originally, the c-Abl proto-oncoprotein was identified as the cellular homolog of the retroviral v-Abl encoded by the Abelson murine leukemia virus (A-MuLV) (Abelson and Rabstein, 1970), which exhibits oncogenic potential in mice (Zou and Calame, 1999). In humans, the oncogene breakpoint-cluster region (BCR)-ABL is the causative factor in the development of chronic myelogenous leukemia (CML). The activity of BCR-ABL is deregulated by a translocation in the t(9;22) Philadelphia chromosome that results in the fusion of the BCR gene to the ABL gene. Many insights into the strict regulation of the Abl family kinases were obtained from studies on BCR-ABL, which have been extensively discussed in other reviews (Advani and Pendergast, 2002; Burke and Carroll, 2010; Hazlehurst et al., 2009; Van Etten, 2007) and will not be discussed here. Cellular Abl and Arg kinases are less understood, but it became clear that they play multiple roles in cellular processes including the regulation

and deregulation of proliferation, survival, response to oxidative stress, DNA damage, actin-cytoskeletal reorganization, and motility (Bradley and Koleske, 2009; Hantschel and Superti-Furga, 2004; Hernandez et al., 2004).

Abl and Arg share extensive sequence and structural similarity, which show a similar organization to the members of the large group of Src family kinases (SFKs) c-Src, Lyn, and Fyn (Fig. 6.1). They are composed of an Src homology 3 (SH3) domain, an SH2 domain, and the kinase (SH1) domain (Sirvent et al., 2008). The related Tec family consists of five kinases (Bmx, Btk, Itk, Tec, and Txk), which additionally contain a conserved amino-terminal pleckstrin homology (PH) domain and a Btk-motif (BH) domain, which is unique for Tec kinases and followed by a proline-rich region (PRR) with PxxP motifs (Fig. 6.1B; Bradshaw, 2010). Compared to SFKs, Abl family kinases express a large carboxy-terminal part containing three conserved PxxP motifs, binding domains both for filamentous (F)-actin and DNA. While the Arg kinase also has a microtubule-binding domain, Abl has nuclear localization signals and a nuclear export sequence (Fig. 6.1A; Bradshaw, 2010; Sirvent et al., 2008).

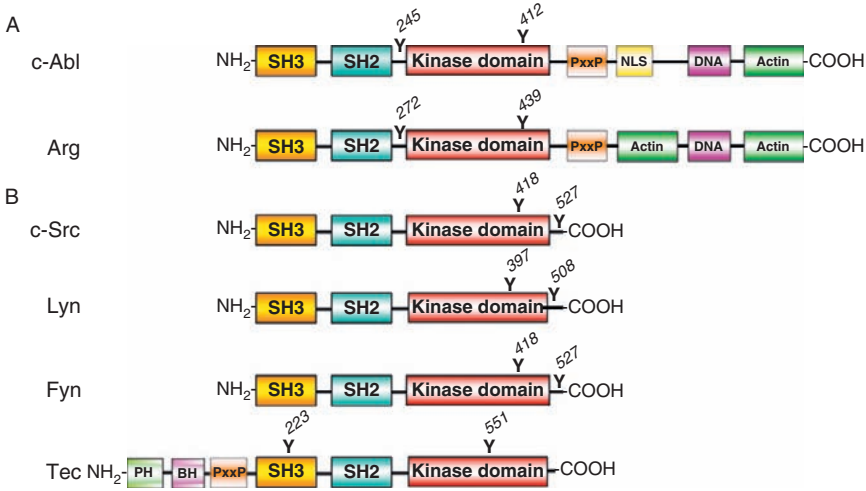

Figure 6.1 Domain structure of Abl, Src, and Tec family tyrosine kinases which are known to phosphorylate several viral and bacterial effector proteins during infection. (A) Structure of the Abl kinase family members c-Abl and Arg with their conserved SH2, SH3, kinase domains, proline-rich regions containing PxxP motifs, nuclear localization signals (NLS), DNA-binding motifs (DNA), and actin-binding repeats (Actin). (B) The SFK member c-Src, Lyn, and Fyn exhibit sequence and structural similarities compared to Abl kinases. Unique for Tec kinases, they contain a conserved amino-terminal PH domain, Btk-motif (BH) domain, and a proline-rich stretch. Phosphorylatable tyrosine (Y) residues are also indicated.

The activity of c-Abl has to be tightly controlled in healthy cells to prevent oncogenic signaling. Both Abl and Arg are expressed as the two alternatively spliced isoforms, type 1a and 1b, which differ in a consensus motif for amino-terminal myristoylation. The myristoyl group binds to a hydrophobic pocket present in the kinase domain and the SH2 domain interacts with the carboxy-terminal lobe of the kinase domain, which promotes a closed, inactive Abl conformation (Hantschel et al., 2003; Nagar et al., 2003). This mode of autoinhibition is relieved by binding of SH3 or SH2 ligands leading to conformational opening of the Abl structure and catalytic activation of the kinase. Phosphorylation of tyrosine residue 245 (Y-245) in the linker and of tyrosine residue 412 (Y-412) in the activation loop stabilizes Abl in an open and active conformation (Hantschel et al., 2003; Nagar et al., 2003). Although the function of Y-245 and Y-412 has been thoroughly analyzed and mutagenesis has confirmed that these phosphorylation sites are required for full c-Abl activation, the involved upstream kinases have not been clearly established. c-Abl may be autophosphorylated in *trans* (Brasher and Van Etten, 2000; Dorey et al., 2001), but SFKs are also candidates that may directly phosphorylate Abl (Dorey et al., 2001; Plattner et al., 1999). Functional interactions between Abl and several receptors [e.g., cadherins, neurotrophin receptors, Robo (Roundabout) receptors] activate Abl kinases, which might involve SFKs (Hantschel et al., 2003; Hernandez et al., 2004; Woodring et al., 2003). Activation of the platelet-derived growth factor receptor (PDGFR) is quite well studied, which stimulates c-Abl and Arg activity in quiescent fibroblasts (Plattner et al., 1999; Srinivasan et al., 2009). Mechanistically, c-Abl recruits a complex composed of activated SFKs (Stanglmaier et al., 2003) and PDGFRs forming a ternary complex which allow Src-induced c-Abl phosphorylation and activation (Srinivasan et al., 2009). c-Abl is also stimulated by members of the epidermal growth factor receptor (EGFR) family (Jones et al., 2006; Plattner et al., 1999), which appears to be independent of SFKs, but may involve an interaction of activated EGFR with the Abl-SH2-binding site that induces the kinase activity (Jones et al., 2006; Zhu et al., 1993). Another mechanism may be initiated by the recruitment of Abl kinases to focal adhesions after the binding of integrin $\alpha 5\beta 1$ to fibronectin (Lewis and Schwartz, 1998; Lewis et al., 1996). Correspondingly, adaptor proteins such as CT10 regulator of kinase I (CrkI), Abl-interacting protein-1 (Abi-1), and noncatalytic region of tyrosine kinase adaptor protein (Nck) or phospholipase C-gamma1 (PLC-$\gamma 1$) were described to activate Abl by functional interactions (Feller, 2001; Hantschel et al., 2003), indicating a complex network of multiple signal transduction pathways leading to the activation of Abl kinases.

The subcellular distribution of Abl in the nucleus, cytoplasm, mitochondria, endoplasmic reticulum, or cell cortex is manifold and reflects the wide range of cellular functions in DNA synthesis or receptor trafficking. A large

body of literature describes the influence of c-Abl in the rearrangement of the actin cytoskeleton. In $abl^{-/-}/arg^{-/-}$ knockout mouse embryos, the lack of Abl kinases results in specific defects of the actin cytoskeleton (Koleske et al., 1998). *In vitro*, Abl kinases regulate F-actin organization, which is required for cell adhesion, migration, and neurogenesis via the formation of lamellipodia, filopodia, and neurite extension (Woodring et al., 2003). How c-Abl regulates the actin latticework is only partially understood, but early work demonstrated a negative role of c-Abl in cell motility. This is supported by the finding that Abl and Arg directly bind to the SH3 domain of Crk adapter molecules and promote tyrosine phosphorylation of CrkII at Y-221 (Feller et al., 1994; Shishido et al., 2001). Phosphorylation of CrkII then leads to the disruption of Abl–Crk–p130CAS (Crk-associated substrate) or Abl–Crk–paxillin complexes (Feller et al., 1994; Hernandez et al., 2004; Woodring et al., 2003). However, other studies provided evidence for a positive role of Abl in cell invasion, in which the small Rho GTPase Rac1 plays a critical role. In an insect cell model, Drosophila Abl kinase (dAbl) acts downstream of Src leading to the activation of Rac in cells, which became invasive and migrate into the area of the posterior compartment (Singh et al., 2010). It has also been suggested that c-Abl phosphorylates the Rac-specific Ras-GEF (guanine nucleotide exchange factor) son of sevenless homolog 1 (SOS-1) (Sini et al., 2004), but it can also phosphorylate Rac effectors, such as members of the WAVE (Wiskott–Aldrich syndrome protein verprolin homologous) complex (Sossey-Alaoui et al., 2007; Stuart et al., 2006; Westphal et al., 2000) and the Abl-interactor proteins Abi1/2 (Dai and Pendergast, 1995; Shi et al., 1995). Via stimulation of actin-related protein 2/3 (Arp2/3)/WAVE complex, a major cellular actin nucleator, the formation of F-actin structures can be regulated.

1.2. Abl kinases are key elements in microbial infections

Microbial pathogenesis is determined by a cocktail of pathogenic factors (either presented on the surface of microbes, secreted into the medium, or injected into the host cytoplasm) which are able to interact with host-cell molecules to trigger specific responses. Bacterial and viral pathogens are prime examples and frequently highjack the actin cytoskeleton to facilitate their pathogenicity either through promotion of their own colonization, entry, or by modulation of specific cellular host responses for microbial benefit. Many bacteria express specialized secretion systems to translocate certain pathogenic factors (also called effectors) directly into the cytoplasm of host cells (Backert and Meyer, 2006; Backert et al., 2008; Enninga and Rosenshine, 2009). Interestingly, those factors often behave as signaling molecules and drastically interfere with the complex signal transduction network and remodel host cell functions (Backert and Selbach, 2005; Cossart and Sansonetti, 2004; Sekirov et al., 2010). Abl kinases were

identified as important key elements in the pathogenesis of several bacteria (*Shigella flexneri*, enteropathogenic *Escherichia coli*, *Helicobacter pylori*, *Anaplasma phagocytophilum*, *Pseudomonas aeruginosa*, *Salmonella enterica*, *Chlamydia trachomatis*), viruses (enteroviruses, vaccinia and variola viruses, and HIV), and prions; the number of different infection models is steadily increasing.

Here, we review the recent progress in this research field. We highlight the findings that Abl kinases and SFKs are key elements in the efficient establishment of microbial infections via reorganization of the actin cytoskeleton or through the direct phosphorylation of individual injected effector proteins on tyrosine residues (e.g., *Helicobacter* CagA, EPEC Tir, *Anaplasma* AnkA, or *Chlamydia* Tarp) implying the possibility that pharmacological inhibition of Abl kinases might be useful strategies for intervention of microbial infections in future.

2. Functional Role of Abl Kinases in Bacterial Infections

2.1. Importance of Abl kinases during *Shigella flexneri* infections

Shigella is an invasive pathogen that causes shigellosis, a specific diarrheal disease (Nhieu et al., 2005). Upon infection of epithelial cells in the gut system, *S. flexneri* utilizes a so-called type III secretion system (T3SS) which mediates the injection of effector molecules (Ipa and Ipg proteins, VirA, and others) into the host cell cytoplasm (Fig. 6.2A). A crucial step in *Shigella* pathogenesis is the T3SS-dependent invasion of the colonic mucosa by a classical trigger mechanism (Cossart and Sansonetti, 2004; Ogawa et al., 2008). At the site of bacterial docking to target cells, Ipa proteins induce extensive actin-cytoskeletal rearrangements which subsequently provoke the engulfment and entry of *Shigella* (Fig. 6.2A). Host cell entry of *Shigella* requires Abl tyrosine kinases. *Shigella* invasion is abrogated in Abl and Arg double deficient mouse fibroblasts ($abl^{-/-}/arg^{-/-}$) and almost no invasion was observed when wild-type cells were pretreated with the Abl inhibitor STI-571 (imatinib mesylate, better known as Gleevec), which is used to treat human CML (Burton et al., 2003). Although STI-571 can also block the activity of other tyrosine kinases (e.g., c-Kit and PDGFR, and at higher concentrations SFKs, EGFR, and Her/Neu), the experiments using $abl^{-/-}/arg^{-/-}$ knockout fibroblasts indicate that both Abl and Arg exhibit crucial roles in *Shigella* infections. However, the identity of the *Shigella* factor(s) activating Abl is still unknown.

One of the best-characterized cellular targets of Abl tyrosine kinases is the family of Crk adaptor proteins, which are important for modulating signaling cascades that regulate cell adhesion, migration, and immune

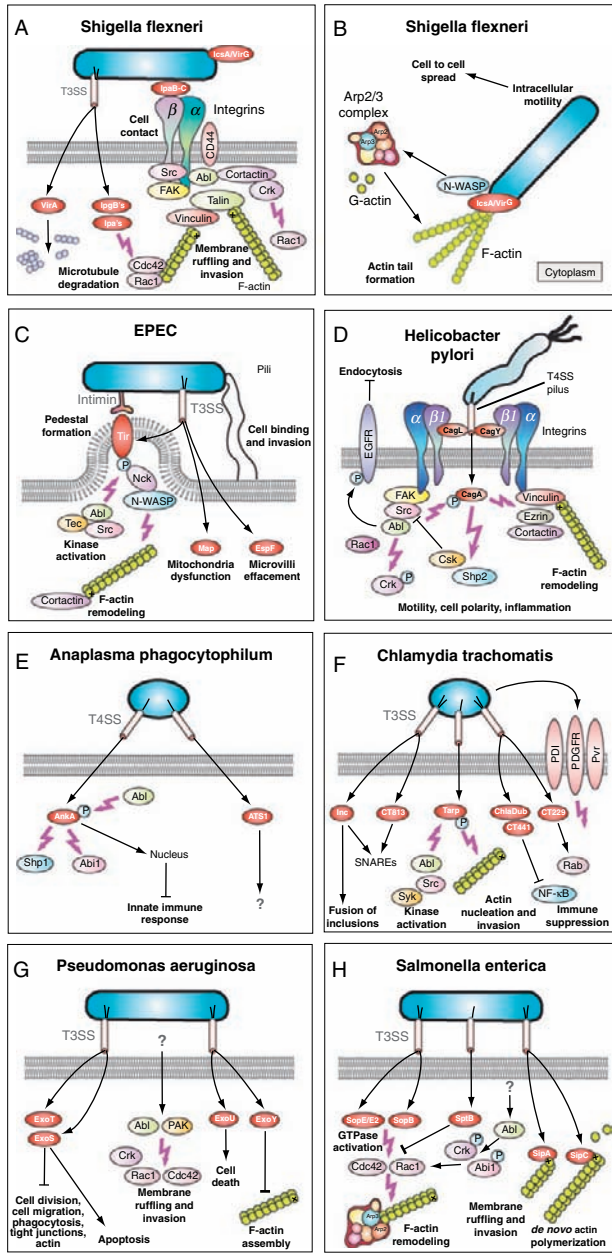

Figure 6.2 Role of Abl tyrosine kinases in bacterial pathogenesis. Abl kinases are key elements in the interaction with attached *Shigella flexneri* (A) and intracellular *Shigella flexneri* (B), enteropathogenic *E. coli* (EPEC) (C), *Helicobacter pylori* (D), *Anaplasma phagocytophilium* (E), *Chlamydia trachomatis* (F), *Pseudomonas aeruginosa* (G), and *Salmonella enterica* (H). For more details, see text.

responses (Feller, 2001; Feller et al., 1994; Kobashigawa et al., 2007). Both, CrkII and Abl, are recruited to the binding sites of bacteria and CrkII is tyrosine-phosphorylated at Y-221 by c-Abl during *S. flexneri* entry. *Shigella* invasion probably also involves the activation of Rho family GTPases (Rac, Cdc42) and downstream proteins (WAVE/N-WASP) that regulate Arp2/3-dependent actin remodeling (Mounier et al., 1999; Stradal and Scita, 2006). Both Rac and Cdc42 are not activated in $abl^{-/-}/arg^{-/-}$ cells which contribute to inhibition of *Shigella* invasion (Burton et al., 2003). Interestingly, the stimulation of Abl/Arg and CrkII during *Shigella* host entry occurs simultaneously with SFK activation (Burton et al., 2003), but it remains unknown whether SFKs activate Abl kinases or if SFKs participate in parallel signaling pathways. In either case, SFK activity clearly amplifies actin polymerization via cortactin phosphorylation thereby promoting bacterial engulfment (Dehio et al., 1995). Cortactin, an F-actin binding protein implicated in Arp2/3-dependent actin nucleation (Selbach and Backert, 2005), is tyrosine-phosphorylated by Src (Dehio et al., 1995) and Abl (Boyle et al., 2007). It has been shown that phosphorylated cortactin binds the SH2 domain in CrkII, which is required for actin rearrangements during *Shigella* invasion (Bougneres et al., 2004).

Once the *Shigella* entered the host cytoplasm as described above, Abl kinases and N-WASP (but not Rac or Cdc42) are necessary for bacteria-induced actin-comet tail formation and intracellular movement of *Shigella* (Burton et al., 2005; Fig. 6.2B). In a first step, the bacterial surface protein IcsA/VirG binds and activates N-WASP leading to actin-comet tail formation (Lommel et al., 2001; Nhieu et al., 2005; Ogawa et al., 2008). However, in $abl^{-/-}/arg^{-/-}$ knockout fibroblast cells, the number of *Shigella*-dependent actin tails is significantly reduced and the actin-comets are shorter (Burton et al., 2005). Abl also directly phosphorylates N-WASP; the expression of nonphosphorylatable N-WASP mutant impairs comet tail elongation, while phospho-mimicking forms of N-WASP rescue the defective comet tail formation in $abl^{-/-}/arg^{-/-}$ knockout fibroblasts (Burton et al., 2005). Thus, Abl kinases are required for efficient *Shigella* actin-tail formation, maximal intracellular motility, and cell-to-cell spread.

2.2. Complex kinase requirements for pedestal formation by pathogenic *Escherichia coli*

Enteropathogenic *Escherichia coli* (EPEC) is a leading cause of human infantile diarrhea linked to the intimate attachment of the bacteria to the host intestinal epithelium. EPEC expresses several pathogenicity-associated factors including pili, the surface protein intimin, and a T3SS which translocates the effectors Tir, Map, EspF (and others) into host cells (Fig. 6.2C). Infection with this pathogen triggers attaching and effacing (A/E) lesions that are characterized by the breakdown of brush-border microvilli and

polymerization of actin in pedestal structures beneath adherent bacteria (Campellone and Leong, 2003; Caron et al., 2006; Dean et al., 2006). The molecular mechanisms of pedestal formation are of significant scientific interest and depend on the T3SS-mediated delivery of Tir into the plasma membrane, which is phosphorylated upon interaction with intimin (Campellone and Leong, 2003; Caron et al., 2006; Dean et al., 2006; Fig. 6.2C). Tyrosine phosphorylation of Tir at Y-474 (Tir^{PY474}) is essential for formation of actin pedestals and can be blocked by tyrosine kinase inhibition or expression of a Tir Y474S mutant (Dean et al., 2006). The role of the second phosphorylation site in Tir (Y-454) is not fully understood; however, cells expressing a Y454F mutant still induce profound pedestal formation (Campellone et al., 2006). In addition, the carboxy-terminal regions in Tir encompass two more tyrosines, Y-483 and Y-511, which control pedestal morphology (Smith et al., 2010). However, if Y-483 and Y-511 in Tir are indeed phosphorylated is not yet clear. Other recent studies revealed that Tir interacts with the SH3 domains of Tec and Abl kinases (but not SFKs) through PxxP motifs in a PRR located at the amino-terminus of Tir. Following binding, it appears that Tec and Abl kinases initially phosphorylate Tir at Y-474 and Tir^{PY474} interacts with SH2 domains of kinases (Bommarius et al., 2007). These results suggest a model that initial phosphorylation of Tir on Y-474 causes recruitment of redundant tyrosine kinases by PRR–SH3 interactions and phospho-Y474–SH2 interactions, which in turn can phosphorylate other Tir molecules and host proteins that induce the assembly of pedestals. However, it remains unclear whether Abl/Tec kinases target the same phosphorylation sites, Y-474, Y-454, Y-483, and/or Y-511. Significantly, Tir^{PY474} then recruits Nck, N-WASP, and Arp2/3 to promote actin polymerization and pedestal formation (Gruenheid et al., 2001; Kalman et al., 1999; Fig. 6.2C). Tir^{PY454} can bind phosphatidylinositol-3-kinase (PI3K) (Selbach et al., 2009), and Y-483 and Y-511 are essential for recruiting SHIP-2, a host inositol phosphatase (Smith et al., 2010), which may have an impact on pedestal production. Finally, Abl, Arg, and Tec colocalize with EPEC-induced pedestals, where they are activated and function in a redundant fashion (Bommarius et al., 2007; Swimm et al., 2004). Interestingly, the Abl substrates and interaction partners CrkII, p130Cas, cortactin, and Grb2 are also predominant proteins in actin pedestals. This recruitment is suggestive of their involvement in dynamic changes of the actin-cytoskeletal structures as initiated by the attached bacteria. More complexity in pathogen-triggered signal transduction arises from reports demonstrating that Tir can also participate in pedestal formation through binding the focal adhesion and cytoskeletal proteins α-actinin, talin, vinculin, and 14-3-3tau in a phosphorylation-independent manner (Goosney et al., 2000; Huang et al., 2002; Patel et al., 2006).

Interestingly, the two closely related pathogens of EPEC, *Citrobacter rodentium* and enterohaemorrhagic *Escherichia coli* (EHEC), also express Tir as a crucial virulence determinant (Campellone and Leong, 2003; Caron et al., 2006; Dean et al., 2006). *C. rodentium* infection induces colonic hyperplasia in mice and serves as a relevant model system in which to study EPEC infections in man. However, *C. rodentium* Tir Y471F mutant strains colonized mice and caused A/E-lesions in a manner similar to strains expressing wild-type Tir. Thus, while Tir plays an important role during *C. rodentium* infection, phosphorylation at Y-471 appears to be dispensable. In contrast to EPEC and *Citrobacter*, EHEC Tir lacks Y-474 or Y-471 and cannot be tyrosine-phosphorylated (Campellone and Leong, 2003; Caron et al., 2006; Dean et al., 2006). Interestingly, pedestals induced by EHEC infection require translocation of another EHEC-specific effector protein (called TccP or EspFU) that interacts with Tir, IRSp53, and N-WASP to promote Nck-independent actin assembly (Campellone and Leong, 2003; Caron et al., 2006; Vingadassalom et al., 2009; Weiss et al., 2009). More studies are necessary to investigate if SFK, Tec, and/or Abl kinases have functions during *C. rodentium* and EHEC infections.

2.3. Importance of Abl and Arg kinases in signal transduction pathways initiated by *Helicobacter pylori*

H. pylori is a human-specific pathogen that persists in the stomach and causes a variety of gastric diseases, such as chronic inflammation, ulceration, and cancer (Parsonnet et al., 1991). Virulent *H. pylori* strains harbor the so-called *cag* (cytotoxin-associated genes) pathogenicity island, which encodes a type IV secretion system (T4SS) to inject the effector protein CagA into gastric epithelial cells using an integrin β1-dependent pathway (Jimenez-Soto et al., 2009; Kwok et al., 2007; Fig. 6.2D). Epidemiological studies have indicated a strong correlation between the presence of CagA and disease development. The finding that *H. pylori* rapidly induces migration (scattering), elongation, and invasive growth of cultured gastric epithelial cells *in vitro* makes CagA an attractive model system to study molecular mechanisms contributing to cancer development.

Upon injection, CagA becomes tyrosine-phosphorylated ($CagA^{PY}$) in its Glu-Pro-Ile-Tyr-Ala (EPIYA) repeats by host cell kinases. The early observations that CagA can be phosphorylated by c-Src *in vitro* and that the SFK members Lyn and c-Src can phosphorylate CagA *in vivo* highlighted the importance of SFKs in *H. pylori* infections (Selbach et al., 2002; Stein et al., 2002). However, $CagA^{PY}$ rapidly induces the inactivation of c-Src by directly binding to the Src kinase and activating its negative regulator carboxy-terminal Src kinase (Csk) (Selbach et al., 2003; Tsutsumi et al., 2003). The inactivation of c-Src correlated directly with tyrosine-dephosphorylation of the actin-binding proteins cortactin, ezrin, and

vinculin and AGS cell elongation (Moese et al., 2007; Selbach et al., 2003, 2004). However, although c-Src is rapidly inactivated (Selbach et al., 2003; Tsutsumi et al., 2003), CagA phosphorylation is not fully abrogated in cells lacking three major SFKs (fibroblasts from c-*src*$^{-/-}$, c-*yes*$^{-/-}$, and c-*fyn*$^{-/-}$ triple knockout mouse embryos, so-called SYF cells) (Selbach et al., 2002). Consequently, it was proposed that CagA may be also phosphorylated by other tyrosine kinases to ensure constant phosphorylation of CagA in sustained *H. pylori* infections. Indeed, it was then shown that the c-Abl kinase is also activated by *H. pylori*. Abl-specific pharmacological inhibitors SKI-DV2-43 or STI-571 and knockdown of c-Abl/Abl-related gene Arg by small hairpin and interfering RNAs (shRNA and siRNA) reduced CagA phosphorylation and efficiently inhibited the AGS cell elongation phenotype (Poppe et al., 2007; Tammer et al., 2007). During infection, c-Abl is rapidly activated by *H. pylori* as monitored by autophosphorylation at Y-412 (Tammer et al., 2007), but not phosphorylation at Y-245 (Poppe et al., 2007). Immunoprecipitated Abl from infections with *H. pylori* was also shown to be active in *in vitro* kinase assays, and phosphorylated recombinant CrkII at Y-221 (Poppe et al., 2007; Tammer et al., 2007). Similar results were obtained during infection. The phosphorylation of CrkII at Y-221 was also induced *in vivo*, which correlated with induced c-Abl activity over time, and which could be inhibited by Gleevec but not the Src inhibitor PP2 (Poppe et al., 2007; Tammer et al., 2007). In addition, both recombinant c-Abl and c-Src phosphorylated wild-type CagA of strain 26695 *in vitro*, but not CagA mutated in all three EPIYA motifs (CagA-Y899/918/972F), suggesting that both c-Src and c-Abl may phosphorylate the same phosphorylation sites in CagA (Tammer et al., 2007). It was further shown that sustained activity of c-Abl is required to maintain CagA in the phosphorylated state during the course of 6 h infection (Poppe *et al.*, 2007; Tammer et al., 2007). Thus, a model can be proposed in which *H. pylori* has evolved a mechanism to use at least two tyrosine kinase families, Abl and Src, for CagA phosphorylation during infection with *H. pylori*.

The findings discussed above suggest that *H. pylori* specifically controls the activity of SFKs and Abl in a time-dependent fashion (Schneider et al., 2008; Fig. 6.2D). While c-Src is only activated during the initial stages of infection (0.5–2 h) and then is rapidly inactivated, c-Abl is constitutively stimulated by *H. pylori* with strongly enhanced activities at late infection time points (2–8 h), supporting a model for the stepwise phosphorylation of CagA by Src followed by Abl family kinases (Poppe et al., 2007; Selbach et al., 2003; Tammer et al., 2007). Activated Abl partially localizes to focal adhesions in infected cells, implying that CagA^{PY} and Abl kinases are involved in deregulating focal adhesion turnover (Poppe et al., 2007). Furthermore, the importance of Abl for downstream signaling leading to the elongation phenotype was also investigated by a series of experiments. For example, overexpression of kinase-dead Abl (K290M) and a

phosphorylation-deficient CrkII mutant (CrkII Y221F) in AGS cells inhibited the *H. pylori*-induced actin-cytoskeletal rearrangements (Tammer et al., 2007). Interestingly, $CagA^{PY}$ formed a physical complex with phosphorylated c-Abl and activated CrkII or Crk-L in infected AGS cells *in vivo* (Poppe et al., 2007; Tammer et al., 2007). The formation of this complex may be achieved by initial interaction between $CagA^{PY}$ and the SH2 domain of the various Crk isoforms (Suzuki et al., 2005), and thus was completely dependent on a functional T4SS (Tammer et al., 2007). Experiments using isogenic mutants indicated that Abl activation is largely mediated by injected CagA and another T4SS factor. Thus, it is tempting to speculate that this unknown T4SS factor is the missing component inducing the small GTPase Rac1 and strong cell motility response by wild-type *H. pylori* (Brandt et al., 2007, 2009; Churin et al., 2001, 2003). Elucidation of the nature of this T4SS factor will be a challenging aim for future research. Finally, it was demonstrated that *H. pylori*-activated c-Abl can phosphorylate EGFR at Y-1173 (Bauer et al., 2009). In this way, *H. pylori* can block EGFR endocytosis and receptor degradation upon prolonged infection of gastric epithelial cells (Fig. 6.2D). Taken together, the results discussed above implicate that Abl kinases represent crucial mediators of infection and are novel promising molecular targets for therapeutic intervention in *H. pylori*-related gastric diseases.

2.4. Role of Abl during infection with *Anaplasma phagocytophilum*

A. phagocytophilum, the agent of human granulocytic anaplasmosis, is transmitted between mammals by blood-sucking ticks and can replicate inside mammalian white blood cells (Rikihisa, 2010). An early report showed that *Anaplasma*'s AnkA protein is transported into the host cell nucleus during infection (Park et al., 2004), and it was proposed that AnkA maybe an injected effector of the *Anaplasma* T4SS (Backert and Meyer, 2006). Like *H. pylori* CagA, AnkA undergoes tyrosine phosphorylation during *A. phagocytophilum* infection (IJdo et al., 2007; Lin et al., 2007). Interestingly, the involved tyrosine residues are located in the carboxy-terminus of AnkA (IJdo et al., 2007) and exhibit sequence homology to the EPIYA motifs of CagA. A genetic manipulation system to inactivate genes in *Anaplasma* is not yet available. Thus, reporter assays were used in the *Agrobacterium tumefaciens* model, a paradigm of T4SS-containing pathogens, to demonstrate that the carboxy-terminus of AnkA is secreted by the agrobacterial T4SS, implying that AnkA is a real T4SS substrate (Lin et al., 2007). Yeast two-hybrid analysis showed that AnkA binds to Abi-1 (Fig. 6.2E), thus suggesting a potential mechanism for AnkA recruitment to Abl (Lin et al., 2007). However, AnkA is also phosphorylated by c-Src *in vitro* and in cultured mammalian cells (IJdo et al., 2007). AnkA and Abl

are critical for *Anaplasma* infection, which is inhibited upon delivery of AnkA antibodies into the host cell cytoplasm, Abl knockdown with RNAi or the application of STI-571 (Lin et al., 2007). In addition, immunoprecipitation studies demonstrated AnkA binding to the host cell phosphatase SHP-1 (Fig. 6.2E), which may be important for establishing a persistent infection (IJdo et al., 2007). Phosphorylation of EPIYA motifs and the presence of SH2 domains in SHP-1 were necessary for the AnkA-SHP-1 binding. Finally, AnkA of the *A. phagocytophilum* Webster strain was reported to localize within nucleus of infected HL-60 cells and bind to the internucleosomal region of chromosomes as well as transcriptional regulatory regions of the CYBB locus, which suppresses the innate immune response in host cells (Garcia-Garcia et al., 2009; Park et al., 2004). These data indicate that AnkA is a crucial virulence factor in *Anaplasma* and the first probable T4SS substrate of obligate intracellular α-proteobacteria.

2.5. Tyrosine kinases in *Chlamydia trachomatis* Tarp phosphorylation

C. trachomatis is an obligate intracellular pathogen that can infect the human eye and the genital tract and cause trachoma and sexually transmitted diseases (Betts et al., 2009; Heuer et al., 2009), and genome sequencing revealed that *C. trachomatis* harbors a T3SS (Stephens et al., 1998). It was found that several *C. trachomatis* proteins can be injected by heterologous T3SSs of pathogens like *Shigella* and *Yersinia* (Fields et al., 2003; Subtil et al., 2001; Fig. 6.2F). Like in EPEC and *H. pylori*, early studies indicated that *C. trachomatis* infection stimulates cellular tyrosine phosphorylation events (Birkelund et al., 1994; Fawaz et al., 1997). These tyrosine-phosphorylated proteins were also thought to be derived from the host cells, despite strain-specific differences in their molecular weight. It is perhaps not surprising that one of these phosphorylated proteins was shown to be of chlamydial origin, the translocated actin-recruiting phosphoprotein (Tarp) (Clifton et al., 2004). Live cell imaging suggests that Tarp is injected and tyrosine-phosphorylated within seconds after host attachment of *C. trachomatis*. Shortly after $Tarp^{PY}$ appears, actin polymerization is observed at the attachment site, indicating that $Tarp^{PY}$ could stimulate actin-driven invasion (Fig. 6.2F). Indeed, a Tarp–GFP fusion protein is also phosphorylated and stimulates actin polymerization. It is noteworthy that Tarp is not the only protein phosphorylated during chlamydial infections. Several pathobiotype-specific proteins of 65–90 kDa are also phosphorylated (Virok et al., 2005). It seems likely that at least some of these proteins are also translocated effectors awaiting identification. To elucidate the mechanisms involved in early events of *C. trachomatis* infection, a large-scale unbiased RNAi screen in *Drosophila melanogaster* S2 cells (Elwell et al., 2008). This allowed identification of candidate host factors in a simple nonredundant, genetically

tractable system. From a library of 7216 double-stranded RNAs (dsRNA), the authors identified approximately 226 host genes, including two tyrosine kinases, Abl and PDGF- and VEGF-receptor related (Pvr), a homolog of the PDGFR family. The potential role of these two kinases in *C. trachomatis* binding and internalization into mammalian cells was also examined. Both kinases are phosphorylated upon infection and recruited to the site of bacterial attachment, but their roles in the infectious process are distinct (Elwell et al., 2008). Evidence was provided that PDGFRβ may function as a receptor, as inhibition of PDGFRβ by RNAi or by PDGFRβ neutralizing antibodies significantly reduces bacterial binding, whereas depletion of Abl kinase has no effect on binding. Bacterial internalization can occur through activation of PDGFRβ or through independent activation of Abl kinase, culminating in phosphorylation of the Rac guanine nucleotide exchange factor (GEF), Vav2, and two actin nucleators, WAVE2 and Cortactin (Elwell et al., 2008) and also involves the protein disulfide isomerase PDI (Abromaitis and Stephens, 2009).

In addition to the above findings, it was shown that the injected actin-nucleator Tarp is a target of Abl kinase (Elwell et al., 2008) but can also be phosphorylated by SFKs (Jewett et al., 2008). However, like the phosphorylation pattern of injected Tir and CagA as discussed above, Tarp phosphorylation also appears highly complex (Mehlitz et al., 2008). Pharmacological inhibition of SFKs confirmed a role for these kinases in Tarp phosphorylation. Infection of SYF cells showed a dampened, but incompletely blocked, Tarp phosphorylation (Mehlitz et al., 2008). Inhibition of Abl in a SYF background still did not completely block Tarp phosphorylation. Thus, additional kinases were tested and it was found that Syk, but not Btk or Jak2, is a potent kinase of Tarp *in vitro* (Mehlitz et al., 2008). Inhibition of Syk in an SYF background further blocked Tarp phosphorylation. These data reveal a highly promiscuous substrate property of Tarp and set the stage for further functional characterization of Tarp phosphorylation during host cell infection. Further studies using Tarp-derived phospho-peptides, mass spectrometry, and SH2 domain protein microarrays were performed to assess binding between $Tarp^{PY}$ and host cell proteins (Mehlitz et al., 2010; Selbach et al., 2009). Numerous interactions were discovered and the adaptor protein Src homology 2 domain containing transforming protein 1 (SHC1) was among Tarp's strongest interaction partners. Transcriptome analysis of SHC1-dependent gene regulation during infection indicated that SHC1 regulates apoptosis- and growth-related genes (Mehlitz et al., 2010). In addition to Tarp, numerous other effector proteins of *C. trachomatis* have multiple roles in manipulating host cell functions (Fig. 6.2F). Together, these results demonstrate that several bacterial factors and host signaling molecules including Abl, SFKs, PDGFRβ, and probably SYK kinases and others function together to promote efficient uptake and propagation of this obligate intracellular parasite.

2.6. Abl kinase-dependent host cell invasion by *Pseudomonas aeruginosa*

P. aeruginosa is one of the leading causes of nosocomial infections in humans. In patients with preexisting epithelial tissue damage and/or host immunocompromise, *P. aeruginosa* is able to cause severe infections of the respiratory and urinary tract, skin, and eye. In addition, *P. aeruginosa* has a unique ability to cause chronic infections in the lungs of patients with cystic fibrosis, leading to end stage lung disease and death (Engel and Balachandran, 2009). Like *Shigella* or EPEC, *P. aeruginosa* uses a T3SS to directly inject four known effectors into host cells (known as ExoU, ExoY, ExoS, and ExoT). These effector proteins play a role in bacterial invasion (Fig. 6.2G) and are commonly found in combinations of ExoU/ExoT or ExoS/ExoT (Barbieri and Sun, 2004). This provides redundant and failsafe mechanisms for the bacteria to cause mucosal barrier injury, inhibits many arms of the innate immune response, and prevents wound repair (Engel and Balachandran, 2009). Internalization of *P. aeruginosa* by nonphagocytic cells is promoted by rearrangements of the actin cytoskeleton, but the host pathways usurped by this bacterium are not fully understood. In a study by Pielage et al. (2008), siRNA-mediated gene inactivation was used to study 80 genes known to regulate the actin cytoskeleton in Drosophila S2 cells in order to identify host molecules essential for entry of *P. aeruginosa*. This work revealed Abl, Crk, the small GTPases Rac1 and Cdc42, and p21-activated kinase (PAK) as components of a host signaling pathway that leads to internalization of *P. aeruginosa* (Fig. 6.2G). Using a variety of complementary approaches (pharmacological inhibition of Abl, use of $abl^{-/-}/arg^{-/-}$ knockout fibroblasts, and RNAi-mediated depletion of Abl), the role of this pathway in mammalian cells was validated (Pielage et al., 2008). Remarkably, ExoS and ExoT, T3SS-secreted toxins of *P. aeruginosa*, target this pathway by interfering with GTPase function and, in the case of ExoT, by abrogating *P. aeruginosa*-induced Abl-dependent Crk phosphorylation (Pielage et al., 2008). Taken together, this work reveals that *P. aeruginosa* utilizes an Abl signal pathway for entering host cells and reveals unexpected complexity by which the *P. aeruginosa* T3SS modulates this internalization pathway. These results further demonstrate the applicability of using large RNAi screens to identify host signaling cascades usurped by microbial pathogens that may be potential targets for novel therapies directed against treatment of antibiotic-resistant infections.

2.7. Entry into epithelial cells by *Salmonella enterica* is mediated by Abl kinase

Salmonella spp. are classical food-borne pathogens that are able to infect numerous hosts and cause a broad spectrum of diseases in humans and animals, ranging from intestinal inflammation and gastroenteritis up to

systemic infections and typhoid fever (Haraga et al., 2008; Tsolis et al., 2008). *S. enterica* serovar Typhimurium can effectively invade intestinal epithelial cells by inducing a dynamic reorganization of the host actin cytoskeleton using a functional T3SS to inject a series of bacterial effector proteins directly into the host cytosol (Fig. 6.2H). For example, the effector protein SopE promotes GTPase activity of Rac1, which is necessary for the extension of actin-rich membrane protrusions that engulf the attached bacteria, while other effectors like SipA and SipC interact with actin, thus inducing *de novo* polymerization and stabilization of F-actin (Patel and Galan, 2005; Schlumberger and Hardt, 2006). Previous work has shown that host tyrosine kinases (including ACK) are also activated as a consequence of *Salmonella* infection (Murli et al., 2001), but the roles of specific kinases or their substrates in *Salmonella* internalization remained poorly understood. In addition, it was shown that the nonreceptor tyrosine kinase FAK (focal adhesion kinase) is necessary for *Salmonella* invasion, however, this requires the carboxy-terminal PxxP motif of FAK to interact with p130Cas but not its kinase activity (Shi and Casanova, 2006). In a recent study, the roles of c-Abl and the related protein Arg were investigated in the context of serovar Typhimurium infection (Ly and Casanova, 2009). The authors found that bacterial internalization was inhibited by more than 70% in $abl^{-/-}/arg^{-/-}$ knockout fibroblasts and that treatment of wild-type cells with the pharmacological inhibitor of the c-Abl kinase STI-571 reduced serovar Typhimurium invasion efficiency to a similar extent. Bacterial infection led to enhanced phosphorylation of two previously identified c-Abl substrates, CrkII and Abi1, a component of the WAVE2 complex (Fig. 6.2H). Furthermore, overexpression of the nonphosphorylatable form of CrkII (CrkII Y221F) resulted in decreased invasion. Taken together, these findings indicate that c-Abl is activated during *S. enterica* serovar Typhimurium infection and that its phosphorylation of multiple downstream targets is functionally important in bacterial internalization.

3. Functional Role of Abl Kinases in Viral Pathogenesis

During the past decade, it has become clear that numerous viruses utilize Abl kinases for the host–virus interplay. One example is given by the recent observation that Abl kinases are involved in the replication of polyomaviruses (Swimm et al., 2010). For polyomaviruses, sialylated lipids serve as cellular receptors (Tsai et al., 2003). Decrease of the Abl family kinase activity resulted in low levels of cell surface ganglioside receptors for mouse polyomavirus, indicating that Abl family kinases regulate the susceptibility of host cells to polyomavirus infection by modulating gangliosides required

for viral attachment (Swimm et al., 2010). The activity of Abl kinases has also been demonstrated for several other viruses facilitating entry, intracellular movement, or spreading, which are discussed below.

3.1. Enteroviruses utilize c-Abl for multiple pathogenic processes

Coxsackieviruses belong to the nonenveloped, linear single-stranded positive-sense ssRNA viruses of the enterovirus family. Among them, Group B coxsackieviruses (CVB) are important pathogens that cause meningitis and myocarditis (Tracy and Gauntt, 2008). The specific interactions of CVB with host cell receptors initiate the infection and viral spread. The current model of pathogenesis involves two types of receptors whose coordinated action enables viral attachment and invasion. Coxsackievirus and adenovirus receptor (CAR) represents the major receptor for CVB that allows tight interaction of the virus. Since CAR is only localized in protected tight junctions, CAR is normally not accessible for CVB. Thus, CVB targets decay-accelerating factor (DAF) which is abundantly presented on the apical surface of host cells and may provide the first contact with the virus (Fig. 6.3A). Attachment of the virus to DAF activates Abl kinases and triggers Rac-dependent actin rearrangements that permit virus movement to the tight junction (Coyne and Bergelson, 2006). Inhibition of Abl using STI-571 or downregulation of Abl expression by siRNA led to an accumulation in apical clusters and blocked the infection. At the tight junctions, CVB interaction to CAR promotes conformational changes in the virus capsid that facilitate virus entry. SFK member Fyn is activated upon DAF binding leading to the phosphorylation of caveolin and transport of the virus into the cell within caveolar vesicles independently of Abl signaling (Coyne and Bergelson, 2006), indicating that coordinated activities of Abl and SFKs promote different steps of viral infection (Fig. 6.3A).

Another enterovirus (enterovirus 71, EV71) utilizes c-Abl to induce apoptosis of neuronal cells, which could be prevented by STI-571 (Chen et al., 2007; Fig. 6.3B). EV71 is a member of the Picornaviridae family which may cause severe neuronal diseases, such as meningitis encephalitis or encephalomyelitis. EV71 induces the expression of cyclooxygenase (COX)-2 as an important neurotoxic factor in CNS injury. It was reported that EV71 induced PDGFR/PI3K/Akt/p42/p44 MAPK signaling pathways to activate COX-2 expression (Fig. 6.3B), which could be attenuated by pretreatment with the c-Src inhibitor PP1 (Tung et al., 2010). Notably, PP1 also blocks Abl kinases; hence, it would be interesting to investigate whether c-Abl contributes to COX-2 induction as well and if Src and Abl have redundant functions. However, the finding that pharmaceutical suppression of Abl kinase activity effectively inhibited the EV71-induced neurotoxicity and neuronal loss indicated that targeting

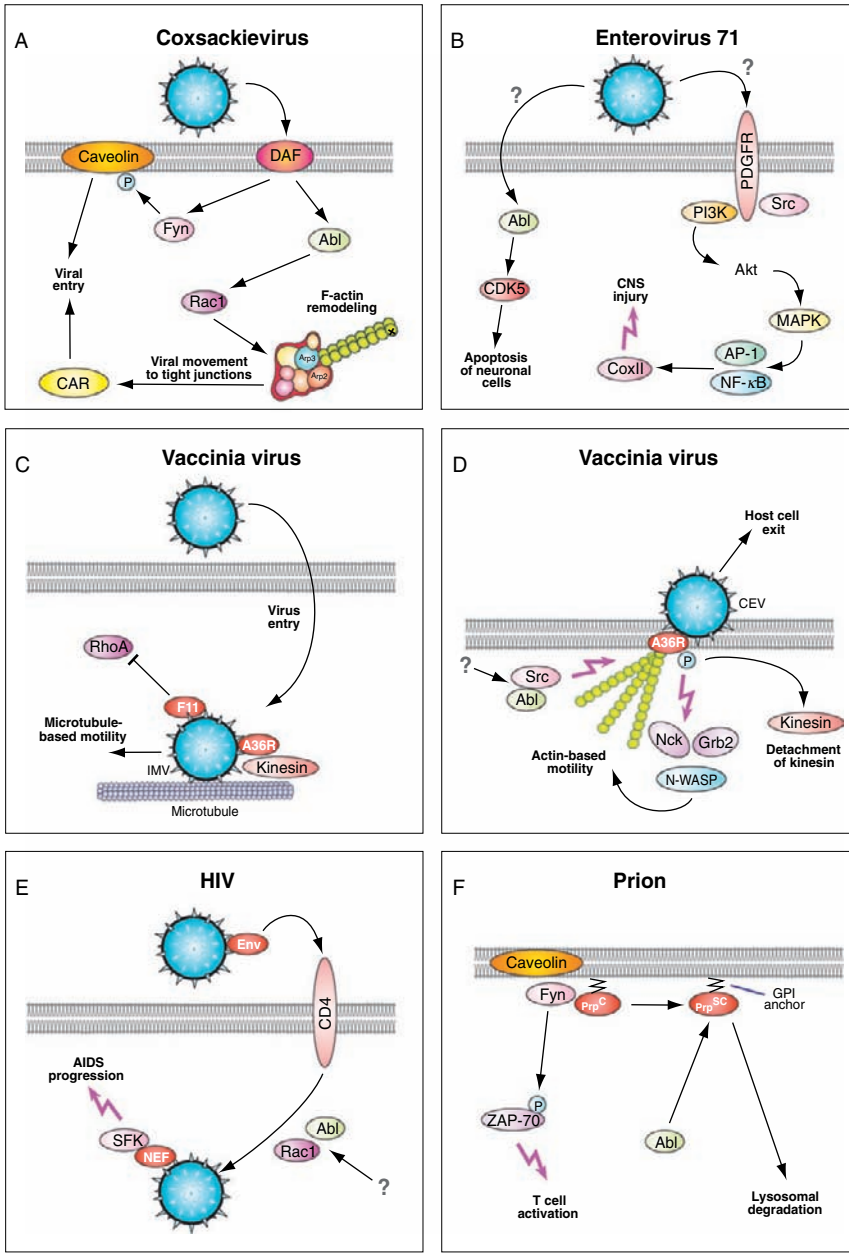

Figure 6.3 Functional role of Abl kinases in viral and prion infections. Abl kinases regulate important host cell responses during infection with coxsackievirus (A), enterovirus 71 (B), vaccinia virus (C, D), HIV (E), and prion proteins (F), which are implicated in viral entry, intracellular movement, toxicity, or propagation. For more details, see text.

Abl kinases may provide new potential therapeutic targets, which is important since specific therapy to treat neurological diseases induced by EV71 is still difficult.

3.2. Functional role of Abl in poxviral intracellular movement and host cell exit

Vaccinia and variola viruses are members of the Poxviridae family, a family of large, double-stranded DNA viruses that replicate in the cytoplasm of host cells and encodes enzymes for transcription and DNA replication (Perdiguero and Esteban, 2009). Vaccinia virus has a complex morphogenic pathway that leads to the formation of distinct infectious virions. The first virion produced is the intracellular mature virus (IMV) which is surrounded by a single membrane. Some IMVs remain within the cell before cytolysis and are transported on kinesin-1-associated microtubules (Fig. 6.3C) to the juxta-nuclear region where they are enveloped by an extra double membrane and form intracellular enveloped virions (IEV). IEV bind to cellular kinesin and are transported via microtubules to the cell periphery, where the outer viral membrane fuses with the plasma membrane becoming cell-associated enveloped virus (CEV) on the cell surface. Actin polymerization occurs at the plasma membrane, forming motile CEV-tipped microvilli. Once detached, CEV forms extracellular enveloped virus (EEV). The viral membrane contains the vaccinia virus protein A36R, which is necessary for actin polymerization and virulence (Fig. 6.3D). The kinase c-Src colocalizes with virions and directly phosphorylates A36R at Y-112 and Y-132 (Wolffe et al., 2001). Src-phosphorylated A36R facilitates the detachment of kinesin (Newsome et al., 2004) and recruits host cell proteins, such as Nck, Grb2, N-WASP, and Arp2/3 to initiate a signaling cascade that leads to the formation of actin tails beneath the CEV (Frischknecht et al., 1999; Moreau et al., 2000; Scaplehorn et al., 2002; Fig. 6.3C). Cell motility of vaccinia also requires another viral protein, F11L, which stimulates cellular projections and cell motility in response to vaccinia infection. F11L binds to activated RhoA, thereby preventing downstream signaling resulting in a loss of actin fibers (Valderrama et al., 2006). Later, it was reported that phosphorylation of A36R (Y-112 and Y-132) occurs by SFKs and Abl collaboratively which are both recruited into vaccinia-induced actin tails (Newsome et al., 2006; Fig. 6.3D). Reeves and colleagues further demonstrated that CEV uses Abl- and SFKs for actin-based motility in a redundant manner (Reeves et al., 2005). Interestingly, CEV release requires Abl, but not Src kinases supported by the finding that application of Gleevec promoted survival in infected mice emphasizing the applicability of Abl kinase inhibitors in viral infections (Reeves et al., 2005).

3.3. Abl inhibition blocks HIV entry of host cells

An enveloped, single-stranded RNA lentivirus, human immunodeficiency virus (HIV-1) is the causative agent of acquired immunodeficiency syndrome (AIDS). The virus infects and kills cells of the immune system such as $CD4^+$ T helper cells, macrophages, and dendritic cells, resulting in specific damage of the immune system and finally, AIDS. The Nef protein of HIV appears to be a multifunctional factor and plays a pivotal role in viral persistence and pathogenesis *in vivo* (Jere et al., 2010). Importantly, SFKs target Nef target proteins through binding to the SH3 domain of the SFK members Fyn, Hck, Lck, Lyn, and c-Src (Arold et al., 1997, 1998; Choi and Smithgall, 2004; Saksela et al., 1995; Fig. 6.3E). This might lead to the activation of SFKs, since it was shown that Nef induces the activation of Hck through a mechanism that involves displacement of the SH3 domain from a negative regulatory interaction with the catalytic domain (Briggs et al., 1997; Moarefi et al., 1997; Trible et al., 2006). The interference of Nef with SFK appears to be important in AIDS progression (Fig. 6.3E). Downregulation of Hck using antisense oligonucleotides in human macrophages led to an inhibition of HIV replication (Komuro et al., 2003). Remarkably, expression of a Nef mutant lacking the PxxPxR motif necessary for SH3 binding and Hck activation resulted in a delay in inducing an AIDS-like disease *in vivo* (Hanna et al., 2001). There are new data published implying a similar role for Abl kinases. Entry of human immunodeficiency virus type 1 (HIV-1) is initiated by binding of the envelope glycoprotein (Env) to the receptor CD4 (Fig. 6.3E), and one of two coreceptors, CXCR4 or CCR5 (Harmon et al., 2010). It was described that the Abl kinase inhibitors imatinib, nilotinib, and dasatinib block HIV-1 entry and infection by the inhibition of the Rac-controlled actin cytoskeleton rearrangement (Harmon et al., 2010).Taken together, these observations suggest a critical role for the interaction of Nef with nonreceptor tyrosine kinases in AIDS progression indicating that targeting those host kinases can be applied in anti-HIV treatment.

4. Role of Abl Kinases in Transmissible Spongiform Encephalopathies

Transmissible spongiform encephalopathies (TSEs) are fatal neurodegenerative diseases occurring in many different host species including bovine spongiform encephalopathy (BSE), scrapie of sheep, and Creutzfeldt –Jakob disease (CJD) (Prusiner, 1998). There is no effective treatment available once the clinical symptoms have developed. The development of TSEs is associated with the appearance of the prion protein (PrP) leading to

the most accepted "protein-only" hypothesis (Laurent, 1996). According to this hypothesis, TSEs are caused by the self-propagating conversion of the normal host cellular prion protein (PrP^{C}) into the abnormal protease-resistant isoform (PrP^{Sc} or PrP^{res}) in an autocatalytic manner (Prusiner, 1982). Today, PrP is established as a new class of infectious agents; however, the molecular basis in the development of prion-associated diseases is not well understood.

PrP^{C} is variably glycosylated at two N-glycosylation sites and is carboxy-terminally attached to the cell surface by a glycosyl phosphatidylinositol (GPI) anchor. GPI-anchored PrP^{C} was found in lipid rafts, which are composed of highly cholesterol- and glycolipid-enriched membrane domains (Taylor and Hooper, 2006; Fig. 6.3F). Lipid rafts are associated with a large number of signaling molecules such as G-protein-coupled receptors and protein kinases suggesting that signaling transduction pathways might play a role in TSEs. Accordingly, previous publications described a functional role of PrP^{C} as a signaling molecule with major findings indicating that PrP^{C} interacts with and activates SFKs (Fig. 6.3F). Ligation of PrP^{C} with specific antibodies activated Fyn via caveolin in fully differentiated serotonergic or noradrenergic 1C11 neuroblastoma cells (Mouillet-Richard et al., 2000). In T cells, PrP^{C} bound to Fyn and phosphorylated zeta-chain-associated protein kinase 70 (ZAP-70) suggesting that PrP^{C} is part of multiprotein signaling complex within microdomains involved in T cell activation (Mattei et al., 2004). This observation was further expanded on scrapie-infected neuronal cells, in which an increased Src kinase activity was detected that correlated with an increased level of Src protein resulting in the activation of Src-dependent downstream signal transduction pathways (Gyllberg et al., 2006). These first reports point to nonreceptor tyrosine kinases as key elements in the neuropathology of the prion diseases. However, an investigation of the influence of Fyn in $Fyn^{-/-}$ knockout mice argues against a prime function of Fyn in the development of PrP^{Sc}-associated astrogliosis, the accumulation of PrP^{Sc}, or the clinical symptoms. Only a moderately shortened survival time of the $Fyn^{-/-}$ mice was observed suggesting that Fyn kinase may support neuroprotective functions of PrP^{C} (Schwarz et al., 2004).

Interestingly, it was also demonstrated that inhibition of c-Abl strongly activates the lysosomal degradation of preexisting PrP^{Sc} without interfering with the *de novo* formation of PrP^{Sc} (Ertmer et al., 2004). Although a clearance of PrP^{Sc} was not observed in *in vivo* studies, the level of PrP^{Sc} was significantly decreased by STI-571 in scrapie-infected mouse spleens, which also delayed the appearance of PrP^{Sc} in the central nervous system and the onset of clinical disease in mice (Yun et al., 2007). These data indicate that specific interference with cellular signaling pathways could represent a novel strategy in treatment of TSEs.

5. Concluding Remarks

Microbial pathogens utilize remarkable strategies to highjack multiple host cell signaling factors and pathways to facilitate pathogenesis. As one of the key regulators, the nonreceptor tyrosine kinase c-Abl was identified which is frequently activated in response to bacterial, viral, and prion pathogenic effectors leading to a reorganization of the host actin cytoskeleton. Another molecular mechanism was unraveled demonstrating that Abl kinases can also directly phosphorylate bacterial effectors that are injected via specialized secretion systems or presented on the surface of viruses and thus are accessible to host kinases. Those posttranslational modified pathogenic effectors in turn can selectively activate intracellular signal transduction pathways. A recent proteomic screen systematically identified binding partners of all known tyrosine-phosphorylated bacterial effectors by high-resolution mass spectrometry (Selbach et al., 2009). Altogether 39 host interactions were identified, all mediated by SH2 domains, including four of the five already known interaction partners. Interestingly, individual phosphorylation sites recruited a surprisingly high number of cellular interaction partners suggesting that individual phosphorylation sites can interfere with multiple cellular signaling pathways. Future studies will investigate the contribution of each of these signal cascades to pathogenesis. Finally, since highly specific pharmacological inhibitors against Abl kinases are available (Hantschel and Superti-Furga, 2004), which already represent well-tolerated drugs for first-line therapy in CML patients, it is tempting to speculate whether microbial infection can be combated by targeted therapy against Abl kinases.

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