

# Chapter 8

## Roles for Actin Dynamics in Cell Movements During Development

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**Abstract** Actin-dependent cellular movements and rearrangements are crucial for development. Studies *in vitro* have contributed much to the knowledge of actin biology. However, interesting environmental influences common in developing systems can differentially regulate actin dynamics and organization. In this chapter, we highlight several selected examples of directed cell migration during morphogenesis, in which actin dynamics have been observed directly in live-imaging studies. We discuss similarities and differences between collective cell and single cell migration during development, and we compare what has been learned from *in vivo* studies in developmental systems with *in vitro* studies of single cells.

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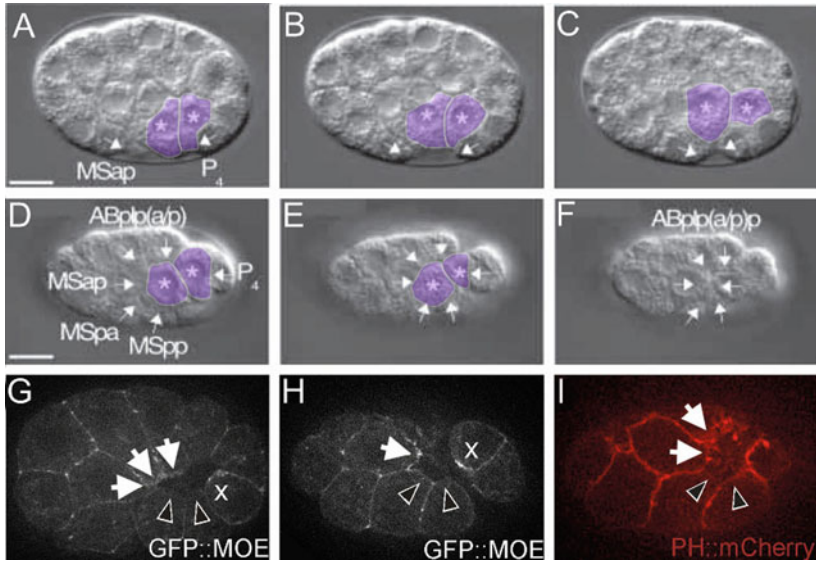
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Actin is integral to the dynamic cellular movements and rearrangements that occur during morphogenesis, a process critical for an organism to develop its final shape. Actin filaments have structural roles in addition to roles in producing forces that can drive cell movements. There are many types of cell movements that occur during morphogenesis, including ingression (single cell migration out of an epithelium, often from the surface to the interior of the embryo), epiboly (spreading and thinning of an epithelial sheet, often to enclose the interior layers of an embryo), invagination (inward folding of a cell sheet into an embryo), involution (inward rolling of an epithelial sheet across an opening), and delamination (separation of two sheets of cells or separation of a cell from a sheet). All of these cell movements involve remodeling of the actin cytoskeleton.

Studies *in vitro* have contributed much to the knowledge of actin biology, from the discovery of actin in muscle extracts to the observation of the delicate architecture of actin networks at the leading edge of a cell (Svitkina and Borisy, 1999). During development, there are significant variations in extracellular milieu, for example a variety of intercellular signals as well as forces exerted by cells in moving tissues, that can differentially regulate actin dynamics and organization. In this chapter, we will highlight several examples of actin-based cell migrations in morphogenesis during development. These models of cell migration are commonly used as paradigms for understanding actin dynamics while taking into account the microenvironment of the cell. Morphogenetic processes often require multiple, redundant actin-based mechanisms. Dissecting the respective contribution of each mechanism is essential to understanding the forces that drive a morphogenetic process.

Cell movements require cell shape changes that are dependent on remodeling of the cytoskeleton. One example of a simple change in cell shape is apical constriction, a process in which cells narrow their apical surfaces, generally by contraction of an apical actomyosin network (Sawyer et al., 2010). Apical constriction can drive cell movements during the processes of ingression or invagination (Lee and Harland, 2007; Harris et al., 2009). For example, in *C. elegans*, the endodermal precursor cells Ea and Ep (referred to collectively here as Ea/p), are born on the surface of the embryo. The Ea/p cells apically constrict, driving their movement to the embryonic interior, and this movement marks the initiation of gastrulation (Lee and Goldstein, 2003; Lee et al., 2006) (Fig. 8.1a–f). Pharmacological inhibition of actin polymerization or depletion of actin regulators, such as the Arp2/3 complex, results in cell internalization defects, supporting a role for actin architecture and/or dynamics in gastrulation (Severson et al., 2002; Lee and Goldstein, 2003). As Ea/p cells internalise, neighbouring cells fill in a gap that is left behind. Observations of F-actin dynamics *in vivo*, using an F-actin-binding domain of moesin fused to GFP (Edwards et al., 1997), have revealed that specific neighbouring cells form



**Fig. 8.1** *C. elegans* endodermal precursor cells internalise by apical constriction. Active cell migration might also contribute to the associated cell movements. Gastrulation stage embryos are shown with Ea/p cells colored purple and marked by asterisks, and the names of neighbouring cells are indicated. (a–c) A lateral view. Ea/p cells shorten their apical surfaces through actomyosin contraction, moving toward the embryonic interior, and neighbouring cells fill in the gap (arrows). (d–f) A ventral view. A ring of six cells fill in the gap (arrows) that is left behind by internalizing Ea/p cells. (g, h) A ventral view of embryos expressing the F-actin marker GFP::MOE. F-actin is enriched in mesodermal precursors specifically where they meet Ea/p (white arrows), and not at the other neighbouring cell boundaries (black arrowheads). The germline cell, P<sub>4</sub>, also has actin accumulation (X), which is not dynamic nor in an extension. (i) A ventral view of an embryo expressing PH domain::mCherry to visualise cell membranes. Membrane protrusions form only where mesodermal descendants contact Ea/p cells. Adapted from Lee and Goldstein (2003). Adapted from Roh-Johnson and Goldstein (2009)

dynamic, Arp2/3-dependent, F-actin-enriched extensions at their borders with Ea/p cells (Roh-Johnson and Goldstein, 2009). Interestingly, the neighbours that form these extensions comprise one side of a closing ring of cells – three of the six cells that form the ring. The role that these extensions play in gastrulation is not well understood. It is possible that the extensions are specializations for cell crawling or cell rolling, or that they participate in sealing the ring upon closure (Roh-Johnson and Goldstein, 2009; Fig. 8.1 g–i). Endoderm internalization in *C. elegans* involves very few cells, with only two cells internalizing and a ring of just six cells closing the gap left, yet it provides one of many examples in which multiple types of cell movements participate together in morphogenesis. The role that actin plays in these developmental processes is under active exploration.

We will highlight several selected examples of directed cell migration during morphogenesis, from movement of a sheet and/or groups of cells to single cell

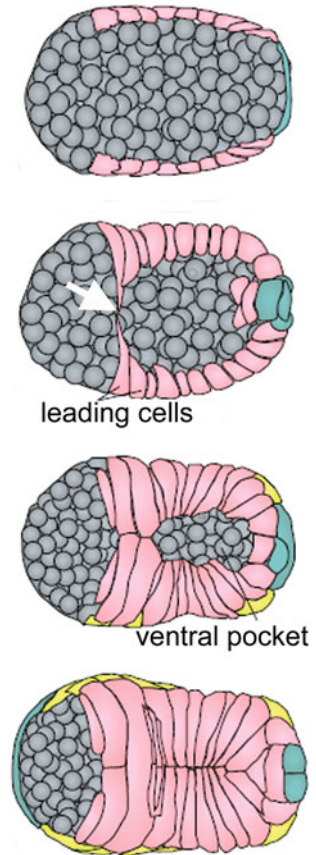
migration. We discuss similarities and differences between concerted cell movements and single cell migration during development, and we compare what has been learned in vivo in developmental systems with in vitro studies of single cells. We focus on examples in which actin dynamics have been observed directly in live-imaging studies, and we discuss key signalling pathways that regulate actin dynamics in actively migrating cells during morphogenesis.

## 8.1 Movements of Cell Sheets During Morphogenesis

### 8.1.1 *C. elegans* Ventral Enclosure – Closing Both Ends

Cells can move as a sheet in dramatic rearrangements of the germ layers of an animal. In *C. elegans*, epidermal cells are born on the dorsal side of the animal as two rows of cells (Chisholm and Hardin, 2005). These cells intercalate (referred to as dorsal intercalation), forming a single row on the dorsal midline. After dorsal intercalation, the epidermal sheet undergoes epiboly, spreading and fully enclosing the animal as the two edges of the sheet meet on the ventral side. Ventral enclosure occurs in two phases (Williams-Masson et al., 1997) (Fig. 8.2). In the first phase, two anterior pairs of cells, termed the “leading cells”, extend long, actin-rich protrusions, making contact with each other on the ventral side. In the second phase, the cells posterior to the leading cells, termed the “pocket cells”, close the remaining gap. Both the leading cells and the pocket cells are important for ventral enclosure, as perturbing either cell population by laser irradiation of individual cells results in ventral enclosure defects (Williams-Masson et al., 1997). Both the leading cells and the pocket cells form F-actin-rich structures. Live imaging of adhesion complexes shows protrusions similar to filopodia, as well as broad lamellae, from the leading cells (Raich et al., 1999). Phalloidin staining reveals that the protrusions from the leading cells are enriched with F-actin (Williams-Masson et al., 1997; Sawa et al., 2003). In addition to proposed roles for filopodia in cell motility during ventral enclosure, these actin-rich fingers may play a role in facilitating strong cell-cell adhesion after cell contact is established (Raich et al., 1999). In a process termed “filopodial priming”,  $\alpha$ -catenin is rapidly recruited to sites where contralateral filopodial tips first make contact. This loading of a cell adhesion complex member into the tips may facilitate rapid cell-cell adhesion as the epithelium seals on the ventral side. The ventral pocket cells accumulate a continuous belt of F-actin along the edge of each cell facing the pocket. The formation of this F-actin belt suggests that a purse-string mechanism may be driving the closure of the ventral pocket, a mechanism analogous to pulling closed a drawstring bag, except that each cell’s portion of the drawstring acts as a contractile unit (Williams-Masson et al., 1997). This observation leads to a model where the leading cells that seal at the midline produce a tension that pulls the ventral pocket cells around the embryo toward the ventral side. Once the pocket cells are pulled close enough to form a ring, ventral enclosure completes by an actin purse-string mechanism (Fig. 8.2).

**Fig. 8.2** Schematic of *C. elegans* ventral enclosure. Ventral cells are drawn in pink. The first 2 pairs of cells, the leading cells, extend long protrusions and make contact with their contralateral neighbours (*arrow*). After the leading cells make contact, the remaining cells, termed the pocket cells, extend around the embryo and meet along the midline. Figure adapted from Chisholm and Hardin (2005)

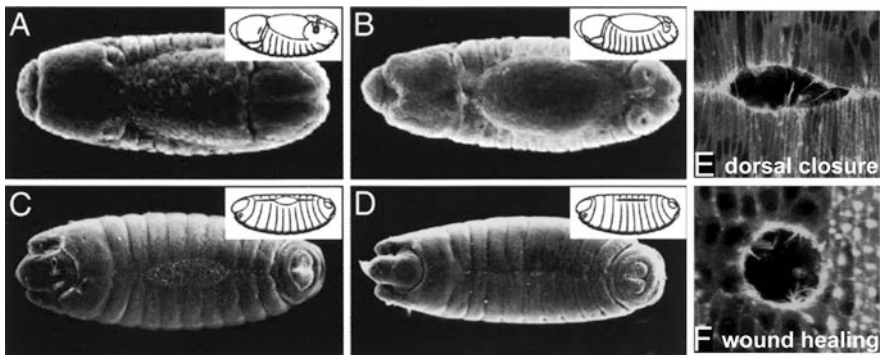


As morphogenetic events involve multiple actin-dependent processes acting in concert, different cells must simultaneously employ different mechanisms for actin regulation. Many actin regulators are involved in ventral enclosure. Several components of the Rac signalling pathway have been implicated in this process. These include homologs of the GTPase Rac, a Rac1-associating protein (Sra), and a Nck-associating protein (HEM2/NAP1/Kette) (Lundquist et al., 2001; Soto et al., 2002; Patel et al., 2008). The ventral enclosure defects observed in Rac signalling mutants may be due to disruption of function of the Arp2/3 complex, a complex that nucleates new actin branches off pre-existing actin filaments. Indeed, the Arp2/3 complex, as well as one of its upstream activators, WASP, have been shown to regulate ventral enclosure (Severson et al., 2002; Sawa et al., 2003). Several of the Rac signalling components as well as Arp2/3 and WASP, have been shown to localise to the leading edge of the leading cells, suggesting a role for these proteins in the protrusive activity (Sawa et al., 2003). Ena/Vasp also regulates ventral enclosure, presumably through its effects on dynamics at the plus end of actin filaments (Withee et al., 2004; Sheffield et al., 2007). Thus, key actin regulators play roles in ventral enclosure

and have predictable roles in ventral enclosure. Little is known yet about the precise effects of these proteins on actin dynamics during ventral enclosure. Improving microscopy techniques for visualization of actin architecture and dynamics may allow for a greater understanding of how these key actin regulators function in this system.

### 8.1.2 *Drosophila* Dorsal Closure – Multiple Actin-based Forces Contribute to a Single Morphogenetic Process

The combination of actin-based cell protrusions and actin purse-string mechanisms to drive morphogenesis is not restricted to *C. elegans*. In *Drosophila*, a process known as dorsal closure also requires both actin-rich protrusions and an actin cable. During the final phases of *Drosophila* embryogenesis, there is a large hole in the epidermis on the dorsal side that is covered by a squamous epithelium, the amnioserosal cells (Fig. 8.3a–e). Forces from the migrating epidermal sheet combine with the forces from the contracting amnioserosal cells to drive closure. Amnioserosal cells apically constrict, pulling the leading edge cells toward the ventral midline, and the leading edge of the migrating epidermal sheet forms a supracellular F-actin purse-string that shortens by more than 25% as the hole closes (Kiehart et al., 2000; Hutson et al., 2003). Additionally, the leading edge cells form long filopodial protrusions, approximately 10  $\mu\text{m}$  long. These protrusions are thought to participate in completing dorsal closure by zipping the two edges of the epidermal sheet (Jacinto et al., 2000; Kiehart et al., 2000; Hutson et al., 2003). Zipping occurs with great precision, with cells of the same segmental position meeting on each side of the opening, and



**Fig. 8.3** *Drosophila* dorsal closure occurs through actin-based contributions from multiple tissues. (a–d) SEMs of dorsal closure. The epidermal sheet migrates by actin-based movements, covering the hole that is filled with amnioserosal cells. (e) GFP-actin expressing embryo during dorsal closure. An actin-rich cable and filopodia form at the leading edge. (f) GFP-actin expressing embryo that has been wounded with a laser. As in the embryo in (e), an actin-rich cable and filopodia form along the epithelial front. (a–d) Images from Jacinto et al. (2002)

the closed seam eventually matures into a continuous epithelium. The process of dorsal closure provides an excellent model for teasing apart the forces contributed by multiple tissue types to drive a single morphogenetic process, combining tools of genetics, live microscopic imaging of fluorescently-labelled proteins, and precise laser cuts to assess the directions and relative strengths of forces deriving from each contributing tissue.

Both the actin cable and the filopodia contribute to the migration and sealing of the epidermal sheet during dorsal closure. GFP- labelled moesin or actin shows enrichment continuously along the leading edge of the epidermal sheet (Jacinto et al., 2000; Kiehart et al., 2000; Hutson et al., 2003; Reed et al., 2004). Myosin II also colocalises with actin along the leading edge and is thought to provide the force necessary for the contractile purse string mechanism (Franke et al., 2005). When a laser is used to cut the supracellular actin purse-string, the leading edge recoils from the site of injury, revealing that this cable is under tension (Kiehart et al., 2000). In Rho or myosin II mutants, the F-actin cable disassembles part way through dorsal closure (Harden et al., 1999; Bloor and Kiehart, 2002). Observing GFP-labelled actin in these mutants reveals that the leading edge is less taut, and there is an increase in the number of filopodia, which can often coalesce into broad lamellipodia (Jacinto et al., 2002). Excessive filopodial protrusions were also observed when Rac signalling was depleted (Woolner et al., 2005). Thus, in addition to the role for the actin cable as a purse string, the cable may also have a structural role, maintaining epithelial integrity as well as restraining the formation of excess protrusions.

F-actin rich filopodia can act as sensory processes that investigate the environment (Mattila and Lappalainen, 2008). During dorsal closure, there is evidence that filopodia may sense their contralateral partners. This phenomenon is best visualised when GFP-actin is expressed only in 4 cell wide stripes across the embryo (Jacinto et al., 2000). GFP expressing filopodia on one epithelial front will contact filopodia on the other epithelial front, making contacts with non-GFP expressing filopodia until filopodia reach GFP-expressing filopodia. Once filopodia find their contralateral partner, they appear to draw the epithelial sheets together and align the GFP-expressing stripes (Jacinto et al., 2000). There are two pieces of evidence that suggest that filopodia tug one another (Jacinto et al., 2000). First, the rate of epithelial front movement is slower prior to filopodial engagement:  $0.11 \pm 0.02 \mu\text{m}/\text{min}$  (average  $\pm$  SD) before filopodial contact, and  $0.24 \pm 0.07 \mu\text{m}/\text{min}$  after contact. Second, the actin cable can appear kinked toward the sites of filopodial contact, suggesting that a force is being exerted on the actin cable. These filopodial tethers also pull the epithelial sheet into alignment with their correct neighbours (Millard and Martin, 2008). Depleting filopodia by dominant-negative Cdc42 expression or by blocking Jun N-terminal kinase signalling reveals that dorsal closure can still proceed, but the epithelial sheet is misaligned during sealing (Jacinto et al., 2000). Similar to what is observed during *C. elegans* ventral enclosure, the filopodia during dorsal closure are speculated to participate in  $\alpha$ -catenin-based filopodial priming (Jacinto et al., 2000). During *Drosophila* dorsal closure, rather than forming nascent adhesion complexes when the two tips of filopodia meet as in ventral enclosure,

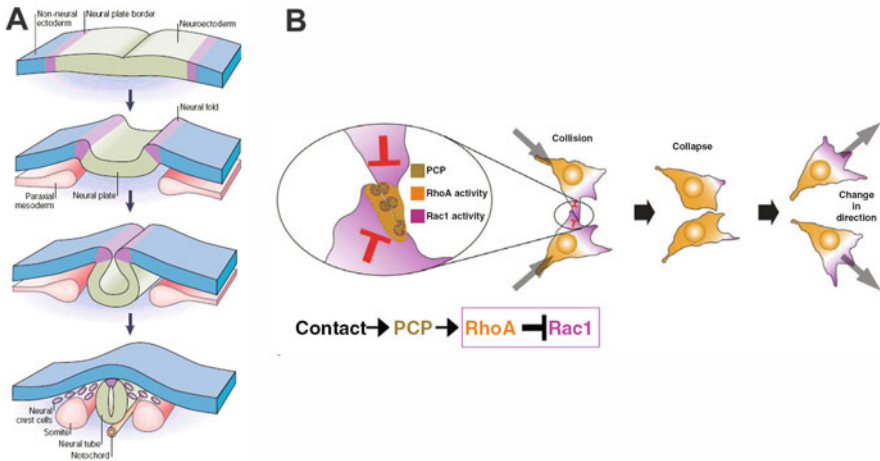
filopodia interdigitate during dorsal closure, and adhesion occurs along the two epithelial fronts.

The regulation of F-actin dynamics in this system has been investigated by dissecting the phenotypes of mutants of several actin regulators. Filopodia in tissue culture cells are known to be regulated by WASP and Scar proteins through activation of the Arp2/3 complex (Zallen et al., 2002; Pollard and Borisy, 2003). In *Drosophila*, SCAR is the primary activator of Arp2/3 in morphogenesis (Zallen et al., 2002); however, it is unknown whether SCAR plays a role in dorsal closure. There are several upstream activators that do play roles in dorsal closure. Four small GTPases have been shown to be involved in the enrichment of cytoskeletal machinery at the leading edge: Rho1, Rac1, Cdc42 and Ras1. Dominant negative studies suggest that these proteins have overlapping roles in regulating myosin and in actin localization to the actin cable (Harden et al., 1999). Expressing a dominant negative Rac specifically in the epidermis results in defects in myosin and actin localization along the leading edge, whereas dominant negative Cdc42 results in subtle actin and myosin localization defects (Harden et al., 1999). Cdc42 also plays a role in the formation of filopodia (Jacinto et al., 2000). Mutations in Cdc42 abolish filopodia formation, affecting the ability of the leading edge cells to sense their neighbours. Mutants of Abelson kinase (Abl) also exhibit defects in dorsal closure. In embryos expressing a constitutively active Abl kinase, filopodia are absent and replaced with broad lamellae, the actin cable is disorganised, and the cells in the two sheets do not precisely align with one another (Stevens et al., 2008). One known target of Abl is the anti-capper Ena (Gertler et al., 1990). Overexpression of Ena can rescue defects caused by Abl mutations, indicating that the roles of Abl in dorsal closure are mediated by Ena (Gates et al., 2007; Stevens et al., 2008). Furthermore, Ena localises to filopodial tips and affects filopodial dynamics. Ena mutants slow dorsal closure timing and interfere with the ability of cells to match correctly with their neighbours (Gates et al., 2007).

### ***8.1.3 Neural Crest Cell Migration – Delamination and then Cell Contact-Dependent Migratory Behaviours Position Cells***

Neural crest cells are highly migratory, travelling long distances through the embryo, and they are multipotent, giving rise to many tissue types including peripheral neurons, glia, connective tissue, bone, melanocytes, and the outflow tract of the heart (Gammill and Bronner-Fraser, 2003). These “explorers of the embryo” are unique to vertebrates, arising at the border between the neural and non-neural ectoderm during closure of the neural tube (Fig. 8.4a) (Gammill and Bronner-Fraser, 2003). Although the induction and migration patterns of the neural crest have been well studied, the cues that guide cytoskeletal rearrangements important for neural crest cell migration are only beginning to be revealed.

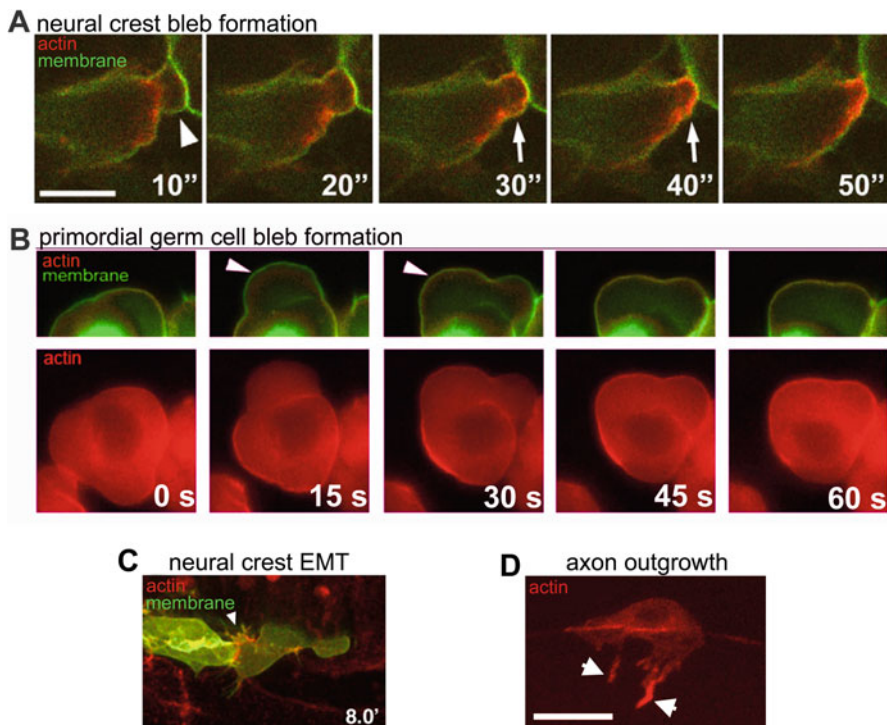
Before neural crest cells begin their migration, they segregate from the neuroepithelium by an epithelial to mesenchymal transition (EMT). During EMT in



**Fig. 8.4** Neural crest cells delaminate from the neural epithelium and then migrate to their final destination. (a) The neural plate border (purple) is located between two cell types, the neuroectoderm (purple) and the non-neuroectoderm (blue). During neurulation, the neural folds elevate and some neural plate cells apically constrict, forming the neural tube. The neural crest cells (grey) delaminate from the neural tube. (b) Contact inhibition of locomotion when two neural crest cells meet, and the signalling pathway regulating contact inhibition

zebrafish embryos, neural crest cells display a sequence of protrusive activities, forming blebs and then filopodial protrusions. Blebbing occurs as delamination begins, followed by the translocation of the cell soma in the direction of the bleb (Berndt et al., 2008). Actin-rich filopodia and lamellipodia then form as neural crest cells exit the neuroepithelium. *In vivo* imaging of actin dynamics confirms that the blebs observed on the neural crest cells are similar to blebs of other cell types, with bleb formation initiated by separation of the membrane from the F-actin network, and with actin filaments accumulating beneath the membrane as the bleb retracts (Fig. 8.5a) (Berndt et al., 2008). Similar bleb dynamics are seen, for example, in mammalian tumour cells (Wolf et al., 2003; Sahai, 2005). When the myosin inhibitor blebbistatin is added to zebrafish embryos, actin accumulation to the bleb is delayed and the blebs fail to retract, but interestingly, lamellipodia and filopodia are not affected. Thus, actomyosin contractility may regulate the dynamics of membrane blebbing in neural crest cells (Berndt et al., 2008).

What signals regulate actin dynamics during EMT? Bmp signalling and Wnt signalling have been implicated in neural crest delamination and migration and have been shown to regulate key actin regulators such as the Rho GTPases (Burstyn-Cohen et al., 2004; De Calisto et al., 2005; Groyzman et al., 2008). BMP4 triggers the downregulation of N-cadherin. N-cadherin normally maintains the neural crest in a premigratory state by two mechanisms: by increasing cell adhesion and by repressing canonical Wnt signalling (Shoval et al., 2007). BMP4 also induces expression of RhoB in the dorsal midline of the neural tube, in a region where



**Fig. 8.5** Cells can migrate by membrane blebs and by actin-dependent protrusions at a leading edge. **(a)** Neural crest bleb formation. The membrane bleb expands past the actin cortex. Then actin accumulates beneath the bleb as the bleb retracts. **(b)** Primordial germ cell bleb formation. The upper panel is the merge of actin in red and membrane in green. The bottom panel is actin only. Much like neural crest bleb formation, the PGC bleb is not enriched with actin near the membrane. Actin then accumulates near the membrane beneath the bleb during retraction. **(c)** Neural crest EMT. Actin-rich protrusions (white arrowhead) form at the leading edge of migrating neural crest cells. **(d)** A *C. elegans* HSN neuron expressing labelled actin. HSN neurons form actin-rich filopodia (white arrows) on the growth cone. **(a, c)** Images from Berndt et al. (2008)

the neural crest forms (Liu and Jessell, 1998). Blocking Rho activity with the C3 exotoxin in chick neural tube explants inhibits neural crest cell delamination and disrupts formation of actin stress fibers (Liu and Jessell, 1998). Pharmacological agents that block Rho kinase (ROCK) or myosin II can also decrease the number of cells undergoing EMT in zebrafish embryos (Berndt et al., 2008). These studies suggest that Rho signalling may positively regulate EMT in the neural crest. However, a recent study has shown that both in explants and in vivo, inhibition of Rho signalling enhanced emigration of the neural crest rather than preventing EMT (Groysman et al., 2008). Blocking Rho signalling with a membrane-permeable C3 enzyme in chick neural tube explants enhances cell emigration from the explants. The membrane-permeable C3 enzyme is effective at much lower concentrations than

the C3 exotoxin used in earlier studies, and it is possible that the increased specificity could account for the differing result (Liu and Jessell, 1998; Groysman et al., 2008). Consistent with the newly proposed role for RhoB in preventing migration, disrupting RhoB activity by another means, with a dominant negative RhoB GTPase construct, results in fewer stress fibers and increased emigration from the neural epithelium (Groysman et al., 2008). Inhibiting ROCK activity with Y27632 also results in a similar effect: more cells emigrate, and vinculin-containing focal contacts are reduced, suggesting that Rho/ROCK is required to maintain F-actin stress fibers in neural crest progenitors before EMT (Groysman et al., 2008). Interestingly, blocking Rho or ROCK activity by either pharmacological experiments or dominant negative constructs also results in the downregulation of N-cadherin *in ovo* in chick, suggesting that Rho/ROCK is also involved in maintaining neural cell adhesion (Groysman et al., 2008). These studies indicate that Rho and ROCK activity have important roles in neural crest cell emigration, but directly conflicting results leave unsettled the issue of whether Rho and ROCK promote or inhibit emigration (Berndt et al., 2008; Groysman et al., 2008).

After the neural crest cells undergo EMT, they follow specific migratory patterns to multiple destinations. In general, neural crest cells from the cranial region migrate in three streams from the rhombomeres to the branchial arches. Neural crest cells from the trunk regions migrate along a medial route, through the somites, or a dorsolateral route, between the ectoderm and somites (Kuriyama and Mayor, 2008). Several cytoskeletal regulators, including N-cofilin, Nedd9, Syndecan-4, and Myosin-X, affect the migratory behaviour of neural crest cells (Gurniak et al., 2005; Matthews et al., 2008; Aquino et al., 2009; Hwang et al., 2009; Nie et al., 2009). *In vivo* imaging of migratory patterns of various populations of the neural crest reveal that these cells can arrange in chain-like formations, with cells contacting each other through filopodia-like processes (Fig. 8.5c) (Teddy and Kulesa, 2004; Young et al., 2004; Kasemeier-Kulesa et al., 2005). Contacts made by these processes to neighbouring cells can vary from short-range contacts (10–20  $\mu\text{m}$ ) to remarkably long-range contacts (up to 100  $\mu\text{m}$ ) (Teddy and Kulesa, 2004). When a neural crest cell becomes separated from a filopodial contact in the stream, the cell appears to move in an undirected manner (Kasemeier-Kulesa et al., 2005). Although direct observation alone cannot resolve the functions of these contacts, it raises the possibility the filopodial extensions may play roles in the collective and directional migration of the neural crest.

Neural crest cells have been shown to display contact inhibition of locomotion *in vivo* (Fig. 8.4b) (Carmona-Fontaine et al., 2008; Goldstein and Hamada, 2009). Contact inhibition of locomotion is a long standing hypothesis by which cell contacts influence the direction of cell movements: at sites where a cell contacts another cell, protrusions involved in cell migration cease forming, and protrusions form at other sites instead. Contact inhibition of locomotion was first observed in fibroblasts *in vitro* (Abercrombie and Heaysman, 1954; Abercrombie et al., 1957). It has since been shown that when two neural crest cells come into contact *in vivo*, their protrusions collapse at the site of contact, and they can change their direction of migration. This behaviour appears to be

regulated by a PCP signalling pathway, as inhibition of Dishevelled (Dsh) or classic PCP genes (Wnt11, *strabismus* or *prickle1*) prevents the collapse of lamellipodia, and these cells fail to significantly change the direction of migration upon contact (Carmona-Fontaine et al., 2008). Furthermore, Dsh is enriched at sites of cell-cell contact (Carmona-Fontaine et al., 2008). Signalling appears to work through RhoA, as RhoA is required for filopodia retraction (Rupp and Kulesa, 2007), and RhoA is active at sites of cell-cell contact (Carmona-Fontaine et al., 2008). Consistent with this, PCP signalling has been shown to activate RhoA, and this activation has an inhibitory effect on Rac activity in neural crest cells (Matthews et al., 2008). It has been proposed the contact inhibition may account for the directional migration of a stream or sheet of neural crest cells, as only the exposed end of a cell at the leading edge can extend protrusions when other sides are in contact with other cells. Other studies have shown neural crest cells with extensions at both the leading and trailing end, making simultaneous contacts in lines of cells (Teddy and Kulesa, 2004; Rupp and Kulesa, 2007). It is possible that some filopodia-like extensions at the trailing ends of cells may be retraction fibers – contacts left behind that are progressively retracted – rather than filopodia extended in this direction. Differences in neural crest cells migratory mechanisms between frog, mouse and chick are also possible. Further studies examining the formation of filopodial-like protrusions at specific times and locations and comparing experimental systems may shed more light on this issue.

## 8.2 Single Cell Migration During Morphogenesis

### 8.2.1 Zebrafish Primordial Germ Cell Migration – Single Cells Come Together to Form Cell Clusters and Migrate Together to Their Final Destination

Studies in vitro predominantly examine the migration of single cells. In development, although cells often migrate as sheets or groups of cells (Friedl and Gilmour, 2009), there are also examples of individual cell migration. Primordial germ cell (PGC) migration involves both single and collective cell migration.

In zebrafish, PGCs are specified at four different regions in the embryo. The four populations of PGCs migrate to the site of gonad formation within the first day of development. The fidelity of this process is demonstrated when ectopic PGCs are transplanted randomly in the embryo: Transplanted cells efficiently migrate to the appropriate location (Ciruna et al., 2002). PGCs transition to migration in three stages (Reichman-Fried et al., 2004; Blaser et al., 2005). First, the PGCs appear rounded and morphologically indistinguishable from their somatic neighbours. Second, PGCs extend protrusions in all directions but do not actively migrate. Third, approximately 1 h later, the PGCs become sensitive to directional cues provided by somatic cells secreting the chemokine SDF-1a. The PGCs begin sending out polarised protrusions (Doitsidou et al., 2002; Weidinger et al., 2002; Raz,

2004), and transition into a migratory phase that requires the downregulation of E-cadherin. PGCs migrate as individual cells until they form two clusters on each side of the body axis. At these sites, the PGCs send out small protrusions and remain at the same location for approximately 3 h (Reichman-Fried et al., 2004). They then migrate as a cluster to the site where the gonad will develop. High resolution imaging of GFP driven specifically in the PGCs reveals that the clusters move by individual cell migrations, with a lack of coordinated movement within the cluster, each cell exhibiting variably-directed short-range migrations. Furthermore, close cell-cell contacts are not observed. Consistent with cells in the cluster moving independently, each cell can spend a portion of its time at the front of the cluster. The cells at the front, which may be exposed to the highest levels of SDF-1a, exhibit directed migration toward the cue.

During PGC migration, PGCs cycle between two phases: A “run” phase, when they actively migrate, and a “tumble” phase, when they lose their polarity and stay stationary (Reichman-Fried et al., 2004). The tumble phase has been interpreted as a pause, in which cells may resample the environment, and reorient their polarity, which may allow cells to more readily and precisely reach their target. Phalloidin staining of fixed PGCs shows F-actin enriched in the cell cortex (Blaser et al., 2005). Live imaging of EGFP-actin fusion protein reveals that there is an enrichment of actin at the cell front during directed cell migration. However, when the cells form protrusions, each protrusion extends past the belt of actin and is not itself enriched with actin. Thus, PGCs also form membrane blebs during their migration. During the tumbling phase, the cells lose their polarity yet still continue to form membrane blebs (Fig. 8.5b). Similar to blebs observed on neural crest cells, once the bleb is in its expanded state, F-actin accumulates beneath the bleb, and the bleb retracts. Experimentally disrupting actomyosin contractility by treating embryos with the myosin inhibitor blebbistatin, or expressing dominant negative constructs to prevent the phosphorylation and activation of myosin light chain, leads to loss of membrane bleb formation, and PGC migration is impaired (Blaser et al., 2005). These results are consistent with the hypothesis that a process dependent on actomyosin contraction, perhaps cytoplasmic flow, is required for bleb formation. PGCs differ from neural crest cells in that neural crest cells form blebs only during delamination from the neural epithelium and not during long distance migration.

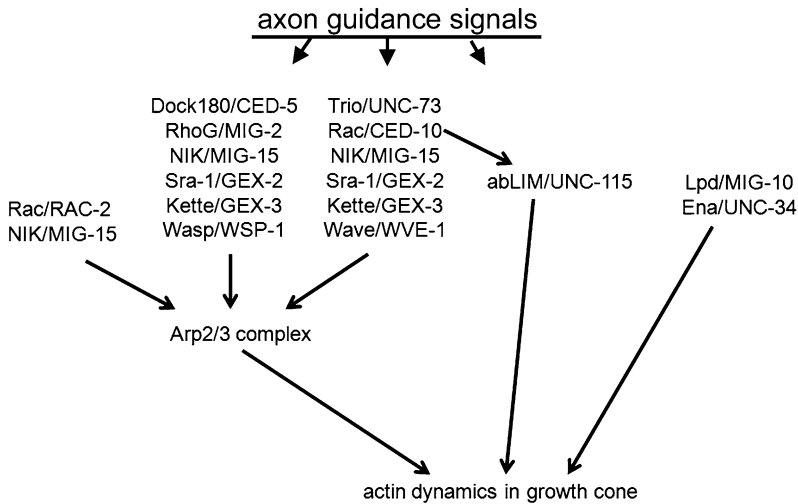
Interestingly, among the proteins that have been found to modulate PGC migration is a viral protein. Nef is a myristoylated HIV-1 protein abundant at early stages of infection, and Nef is known to disrupt cell migration when expressed in fibroblasts. Nef affects migration by interacting with the P21-activated kinase Pak2 and down-regulating the actin filament severing activity of cofilin. Fibroblast cells expressing Nef have disorganised F-actin. Nef can also inhibit SDF-1-induced chemotaxis of T-lymphocytes (Stolp et al., 2009). PGCs expressing Nef also have altered migration patterns (Stolp et al., 2009). Whether Nef blocks the migration of PGCs by similar mechanisms as in other cell types is currently unknown. However, expression of Nef without the Pak2-interacting domain in zebrafish has no effect on PGC migration, suggesting that Nef’s interaction with Pak2 is critical in inhibiting PGC migration.

A central player in many migrating cells is the phosphoinositide 3-kinase (PI<sub>3</sub>K) family of proteins. In *Dictyostelium*, phosphatidylinositol (3,4,5)-triphosphate (PIP<sub>3</sub>) accumulates at the leading edge in response to receptor activation (Kolsch et al., 2008). This accumulation recruits several downstream proteins that regulate actin dynamics. During zebrafish PGC migration, loss of PIP<sub>3</sub> results in slower PGC motility and reduced filopodial-like protrusions (Dumstrei et al., 2004). However, in contrast to *Dictyostelium*, PIP<sub>3</sub> is uniformly localised around the cell periphery in PGCs. Thus, although PIP<sub>3</sub> is required for overall PGC migration, PIP<sub>3</sub> is unlikely play a role in directional PGC migration.

### 8.2.2 *C. elegans* Axon Guidance – Using a Genetic System to Identify Proteins Required for Single Cell Migration In Vivo

Axon outgrowth is a classic example of single cell migration during morphogenesis. Axon outgrowth is led by the guidance of the growth cone. Growth cone guidance in vivo is an excellent paradigm to study how a cell responds to cues in its extracellular environment, and specifically how the cell remodels its actin cytoskeleton to respond appropriately to this cue. Growth cone guidance is studied in a variety of systems. Among these, *C. elegans* is an ideal genetic system to tease apart the signalling pathways that regulate the cytoskeleton in axon guidance because *C. elegans* lends itself readily to genetics and RNAi, and loss of many of the worm's 302 neurons produces behavioural phenotypes in otherwise viable, fertile strains of worms.

Growth cone guidance is mediated by filopodial and lamellipodial dynamics that are driven by actin dynamics. Growth cones produce these protrusions, which make contact with substrates and in turn function in propelling the growth cone forward. Many actin regulators control the formation of these actin-based structures (Fig. 8.6). In *C. elegans*, Arp2/3 activation, aBLIM/UNC-115, and Ena/UNC-34 directly regulate actin dynamics (Lundquist et al., 1998; Yu et al., 2002; Struckhoff and Lundquist, 2003; Norris et al., 2009). Ena/UNC-34 also genetically and biochemically interacts with the single *C. elegans* lamellipodin (Lpd) homolog, MIG-10 (Chang et al., 2006). RhoG/MIG-2, Rac/RAC-2, and Rac/CED-10 act redundantly for axon guidance, and the Nck-interacting kinase (NIK) MIG-15 functions in all three Rac signalling pathways (Shakir et al., 2006). Thus, NIK/MIG-15 is a core component of each signalling pathway. The Rho GTPases and their upstream activators act as modulators for specificity. For example, the Rho GTPases RhoG/MIG-2 and Rac/CED-10 are regulated by the guanine nucleotide exchange factors (GEFs) Trio/UNC-73 and DOCK180/CED-5, respectively (Steven et al., 1998; Lundquist et al., 2001; Wu et al., 2002). Furthermore, genetic analysis indicates that RhoG/MIG-2 is in the same pathway as the upstream activator WASP/WSP-1, while Rac/CED-10 is in the same pathway as the upstream activator Wave/WVE-1 (Shakir et al., 2008). Both Rac GTPases converge on Sra-1/GEX-2 and Kette/GEX-3 and regulate Arp2/3 function (Shakir et al., 2008). Thus, taken together, there are three pathways that regulate Arp2/3 (Fig. 8.6). The components of



**Fig. 8.6** Several pathways regulate actin during *C. elegans* axon outgrowth

these pathways again highlight the idea that there are several core components that are used in each pathway to elicit a response (e.g. Sra-1/GEX-2 and Kette/GEX-3), and the specificity of each pathway is then dictated by specific Rho GTPases and upstream regulators. There is also crosstalk between major pathways, as Rac/CED-10 can function through abLIM/UNC-115 (Struckhoff and Lundquist, 2003). The Arp2/3 complex itself has also been shown to have roles in neuronal migration. Recently it was shown that depleting *C. elegans* of Arp2/3 results in defects in mechanosensory neuron migration (Schmidt et al., 2009).

Growth cones respond to signals in their extracellular environment and alter actin dynamics in response. One such signal in *C. elegans* is the Netrin homolog UNC-6. Netrin/UNC-6 is a conserved axon guidance cue. A motor neuron, HSN, responds to Netrin/UNC-6 by asymmetrically localizing the receptor DCC/UNC-40 toward the direction of the signal (Adler et al., 2006). Lpd/MIG-10 also localises asymmetrically in the growth cone in response to Netrin/UNC-6, through the activity of Rac/CED-10 (Chang et al., 2006; Quinn et al., 2008). This asymmetric Lpd/MIG-10 localization is also coincident with asymmetric F-actin accumulation (Quinn et al., 2008). Plasma membrane markers can reveal projections, or neurites, from the cell body of developing HSN neurons (Fig. 8.5d) (Adler et al., 2006). These neurites are F-actin rich, and form toward the Netrin/UNC-6 cue. The HSN neuron has a clear leading edge, and filopodia and lamellipodia grow and retract in the direction of the signal (Adler et al., 2006). Defects caused by increased Netrin/UNC-6 signalling are suppressed in loss-of-function mutations in Rac/CED-10, Ena/UNC-34, and abLIM/UNC-115, suggesting that growth cone outgrowth and turning by Netrin/UNC-6 signals are mediated by these cytoskeletal regulators (Gitai et al., 2003). Interestingly, although filopodia are present on all

growth cones, suggesting that the formation of these F-actin rich structures is likely to be critical for axon guidance, lack of filopodia in *end/unc-34* mutants is consistent with proper HSN guidance (Chang et al., 2006). In these cells, lamellipodia still form. Thus, in vivo, it appears that dynamic filopodia form, but at least in some contexts are dispensable for guidance, and perhaps other cues in the extracellular milieu stimulate alternative migratory mechanisms, most likely via lamellipodia.

The growth of an axon is important for guidance, but the inhibition of outgrowth is also important for precision. Although many proteins function intracellularly to promote axon outgrowth, there are few proteins yet known to negatively regulate this process. CRML-1, the *C. elegans* CARMIL homolog, was identified in *C. elegans* to inhibit axon outgrowth by affecting Trio/UNC-73 activity, although mammalian CARMIL acts to promote glioblastoma migration (Yang et al., 2005; Vanderzalm et al., 2009). CRML-1 and Trio/UNC-73 physically interact, and together control the direction of growth cone migration by altering the levels of a guidance receptor, Robo/SAX-3. Thus, through the inhibition of Rac signalling, CRML-1 can negatively regulate neuronal migration.

## 8.3 Conclusions

### 8.3.1 Collective Cell Migration

This chapter discusses three different modes of collective migration: migration of epithelial sheets, cell clusters, and cell streaming. Interestingly, actin-based cell migrations during morphogenesis often occur through collective cell migration instead of single cell migration (Friedl and Gilmour, 2009). Why do cells in development so often migrate in groups? One hypothesis is that cell clusters can in general generate more force than can single cells (Kolega et al., 1982).

*C. elegans* ventral enclosure and *Drosophila* dorsal closure both involve multiple actin-dependent cell movements. Different force-generating mechanisms are evident during *Drosophila* dorsal closure including actomyosin contraction of the amnioserosal cells, a supracellular purse string at the leading edge, and dynamic filopodia. These are coordinated spatially and temporally, regulating a single morphogenetic process (Hutson et al., 2003). This process is similar to *C. elegans* ventral enclosure, which involves a combination of forces from actively migrating leading cells and an actin purse string-like mechanism in the pocket cells. In both *C. elegans* ventral enclosure and *Drosophila* dorsal closure, filopodia aid in closing a ring. Actin purse-string mechanisms and filopodia formation are involved in both of these epibolic movements, and key molecular components may be conserved. The Arp2/3 complex, a major actin regulator, is required for *C. elegans* ventral enclosure (Sawa et al., 2003), but no role for Arp2/3 has been described for *Drosophila* dorsal closure. While upstream Arp2/3 activators such as Wave may play a role during *Drosophila* dorsal closure, it is possible that other actin nucleators, such as formins or Spire, may play a role in dorsal closure, and that Arp2/3 may be

acting redundantly with these players. Indeed, a formin, Diaphanous, localises to the actin cable, and embryos expressing constitutively active diaphanous exhibit dorsal closure defects, mainly through defects in amnioserosal cell apical constriction (Homem and Peifer, 2008). The differences observed between *C. elegans* ventral enclosure and *Drosophila* dorsal closure suggest that there is some plasticity in mechanisms of regulation through evolution, with different inputs acting on a common outcome.

Factors that are required to prevent filopodia formation and migration are also important for dorsal closure and possibly ventral enclosure. During the last stages of dorsal closure, the two epithelial leading edges must recognise each other and cease active migration. It is possible that apposition of the two edges of the migrating epithelium during dorsal closure results in contact inhibition, preventing the overmigration of the leading edges. Contact inhibition in neural crest cells is regulated by PCP/non-canonical Wnt signalling. When two neural crest cells contact each other, Dishevelled becomes localised to the membrane at areas of cell-cell contact, and RhoA becomes active (Carmona-Fontaine et al., 2008). RhoA is thought to direct the collapse of filopodia at the cell contact zones and aid in the change of migratory direction. However, during dorsal closure, when filopodial tips touch, the filopodia do not retract immediately (Jacinto et al., 2000). Rather they appear to contact each other, perhaps tethering the edges of the epithelial sheet and also aligning them. It will be interesting to determine if contact inhibition does occur during *Drosophila* dorsal closure and *C. elegans* ventral enclosure, perhaps via a different mechanism than in neural crest cells.

### 8.3.2 *Single Cell Migration – Amoeboid Versus Mesenchymal Migration*

The mechanism of bleb formation appears to be different between neural crest cells and PGCs. PGCs require local actomyosin contraction, which produces cytoplasmic flow, a flow that may contribute to formation of a membrane bleb. When PGCs are treated with blebbistatin, the membrane blebs do not form (Blaser et al., 2005). Neural crest cells, on the other hand, can still form membrane blebs when treated with blebbistatin, suggesting that actomyosin contraction is not required for bleb formation, but is required for bleb retraction (Berndt et al., 2008).

It is possible that this difference in bleb formation may account for the difference in long distance migration mechanisms between neural crest cells and PGCs. Neural crest cells exhibit blebs during delamination from the neural epithelium. They then adopt characteristics of a mesenchymal cell with a clear leading edge, actively migrating over longer distances. PGCs, on the other hand, form membrane blebs during long-range active migration. Thus, unlike what is seen in many other systems where actin polymerization produces the force to form and extend a pseudopod during migration, zebrafish PGCs have adopted a different form of motility.

Why do PGCs actively migrate by membrane bleb formation rather than actin polymerization-induced protrusions? The fact that cells can convert from amoeboid to mesenchymal forms of movement, and vice versa, suggests that a cell can change its migratory behaviour in response to its environment. It has been shown that bleb-dependent motility occurs as a result of changes in cell contacts or cell-cell adhesion (Shook and Keller, 2003). Rather than making contacts with an underlying substratum, bleb-dependent motility without attachment to a substrate allows cells to squeeze past obstacles and navigate through matrices (Hegerfeldt et al., 2002; Gadea et al., 2007; Tournaviti et al., 2007). This form of motility is similar to amoeboid motility. Cancer cells have also adopted this amoeboid form of motility, bypassing requirements for matrix metalloproteases in migration (Friedl, 2004; Sahai, 2005; Wyckoff et al., 2006). It is possible that PGCs have also adopted this form of motility to bypass a requirement for adhesion-based mechanisms.

### ***8.3.3 What Can We Learn About Actin Dynamics in a Model Developmental System?***

Many actin regulators play conserved roles between cell migrations in morphogenesis and cell migrations in vitro. Components of the Rac signalling pathway, as well as key actin regulators such as Ena, are involved in actin dynamics in diverse systems. There are, however, some clear differences between in vivo and in vitro studies. Notably, filopodia in *C. elegans* growth cones are dispensable for axon outgrowth in vivo. This is markedly different than the proposed function for filopodia during axon outgrowth in vitro (Drees and Gertler, 2008). It is possible that there are other factors that are present in the extracellular milieu of an animal that could be providing a redundant role or providing an alternative mechanism for axon guidance.

The strength of analyzing actin dynamics during morphogenesis is that one can understand the role of actin in its native environment. Morphogenetic processes seldom involve a single actin-based force-generating mechanism. More often, a morphogenetic process requires multiple, redundant actin-based mechanisms. Dissecting the contribution of each actin-dependent process is an important step for which developmental systems provide invaluable models. *Drosophila* dorsal closure, for example, is a powerful system for measuring the force contributions of each actin-dependent mechanism in a single morphogenetic process. Laser cut experiments have revealed that the supracellular purse-string at the leading edge and contraction of the amnioserosa contribute to most of the forces required for dorsal closure (Hutson et al., 2003). The forces provided by the filopodia at the leading edge appear essential only for the late stages of closure. Thus, analyzing actin-dependent forces during morphogenesis allows for the understanding of how cells and tissues coordinate their forces and how these forces are regulated in space and time.

These types of force studies need not be limited to dorsal closure. *C. elegans* is an attractive model for applying laser microsurgery to analyze the contributions of multiple actin-dependent processes during endodermal internalization and ventral enclosure. Similar cell movements and actin-based structures can also be found during wound healing. When *Drosophila* or *Xenopus* embryos are wounded with a needle, the leading edge cells surrounding the wound form a supracellular actin cable as well as filopodia (Wood et al., 2002; Clark et al., 2009; Martin and Parkhurst, 2004) (Fig. 8.3f). The wound heals in part by an actin purse-string mechanism. When the wound size is sufficiently decreased, filopodia can reach across the wound. The filopodia then form tethers with one another and appear to facilitate wound closure. Teasing apart the forces in these processes is an important step to understanding the mechanisms of cell and tissue movements.

Actin dynamics have only recently been analyzed in real time in several model systems. Given the optical clarity and low light scattering in embryos of some model systems like zebrafish and *C. elegans*, actin dynamics can be imaged readily during a number of morphogenetic events even inside embryos. Furthermore, with the development of technology to image cells deep within an animal while minimizing toxic effects of intense laser energy (Condeelis and Segall, 2003), actin dynamics in a host of cells can be imaged in their native environments. The future of this research will likely involve interdisciplinary approaches combining *in vitro* and *in vivo* studies.

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