Getting into shape: epidermal morphogenesis in *Caenorhabditis elegans* embryos

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Summary

The change in shape of the *C. elegans* embryo from an ovoid ball of cells into a worm-shaped larva is driven by three events within the cells of the hypodermis (epidermis): (1) intercalation of two rows of dorsal cells, (2) enclosure of the ventral surface by hypodermis, and (3) elongation of the embryo. While the behavior of the hypodermal cells involved in each of these processes differs dramatically, it is clear that F-actin and microtubules have essential functions in each of these processes, whereas contraction of actomyosin structures appears to be involved specifically in elongation. Molecular analysis of these processes is revealing components specific to *C. elegans* as well as components found in other systems. Since *C. elegans* hypodermal cells demonstrate dramatically different behaviors during intercalation, enclosure and elongation, the study of cytoskeletal dynamics in these processes may reveal both unique and conserved activities during distinct epithelial morphogenetic movements. *BioEssays* 23:12–23, 2001. © 2001 John Wiley & Sons, Inc.

Introduction

The generation of cell, tissue and organ shape, generally referred to as morphogenesis, is essential for the growth and development of multicellular animals. During morphogenesis, cells modulate the behavior of structural molecules to effect dramatic changes in cell shape, cell–cell connections and cellular arrangement.[1–3] Tissue and organ shape give an organism its characteristic appearance, which, in turn, directly influences how an organism interacts with its environment.

Hypodermal morphogenesis in *C. elegans* is dramatic in the speed and extent of changes in embryo shape. In just 5 hours, the embryo changes from ovoid to vermiform shape.[4–7] The cells that effect this change are relatively few in number and have stereotypical behaviors. The hypodermis of *C. elegans* originates as six rows of cells positioned on the dorsal surface, toward the posterior of the embryo. Shortly after their birth, the two rows of dorsalmost hypodermal cells interdigitate to form a single row of cells in a movement known as dorsal intercalation. After intercalation begins, the two ventralmost rows of cells extend their free edges and migrate over the surface of the embryo, eventually meeting and forming cellular junctions with contralateral neighbors along the ventral midline in a process known as ventral enclosure.[6] Following enclosure, circumferential actin filaments contract, providing the force for the dramatic shape change known as elongation.[5]

Recent analysis of these three basic processes of cell rearrangement and migration, ventral enclosure, dorsal intercalation, and elongation, in the embryonic hypodermis (epidermis) of *C. elegans* indicate a role in morphogenetic events both for well-characterized molecules and new unexpected molecules. In this review, we describe what is known about these processes at the cell biological and molecular levels. Novel findings and descriptions of conserved components are compared with what is known regarding epithelial morphogenesis in other systems.

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Viewing hypodermal cells in vivo

Hypodermal cells are clearly identified by immunostaining with various antibodies, including the antibody MH27, which recognizes an antigen encoded by *jam-1* (junction associated molecule 1, M. Köppen, and J.H., unpublished observations).[8,9] JAM-1 is localized to the adherens junctions of all hypodermal cells and is a useful marker for monitoring the behavior of hypodermal cells during morphogenesis (M. Köppen, and J.H., unpublished observations).[5,10–14] JAM-1-GFP expression can be used to take a "snapshot" of the migrating hypodermis (Fig. 1).

Dorsal intercalation

Intercalating dorsal cells arise as two rows along the anteroposterior axis consisting of ten epithelial cells each. The two rows of dorsal cells are flanked laterally by a row of "seam" hypodermal cells. During the first stages of intercalation, dorsal cells become wedge shaped with their pointed tips oriented toward the dorsal midline (Fig. 2). Expression of JAM-1-GFP shows that the triangular-shaped tips of dorsal cells interdigitate between their contralateral neighbors, eventually elongating such that their extending edges contact the medial edges of the opposing seam cells (Fig. 3). The nuclei of intercalating cells migrate behind the protrusive tips, and eventually reside in a dorsolateral position near the long-

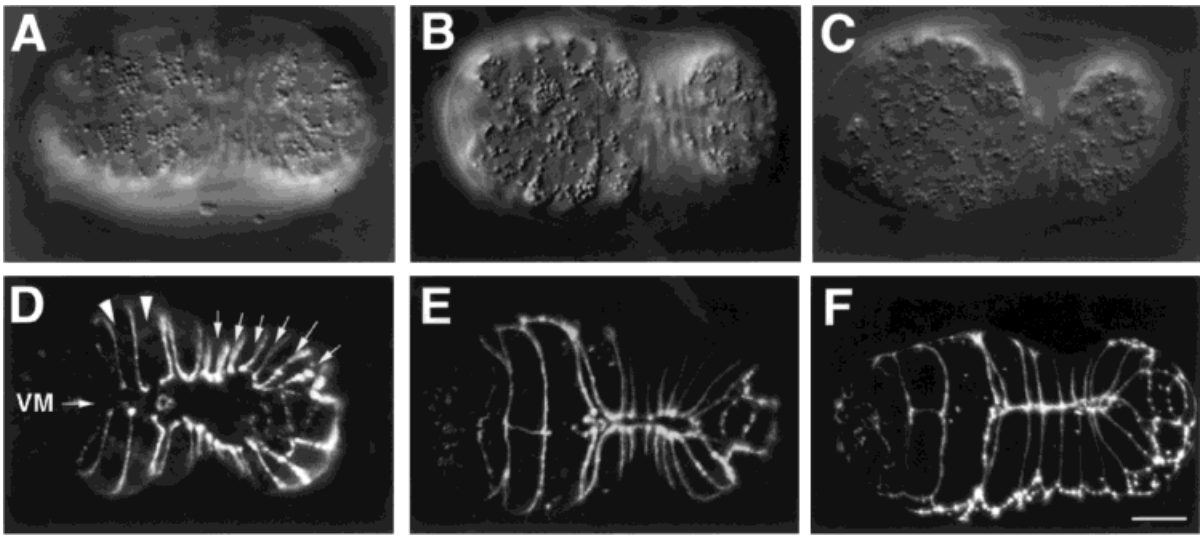

Figure 1. Ventral enclosure is observed using DIC microscopy **A–C:** or by the expression of JAM-1-GFP **D–F:**, which localizes to the mature adherens junctions of all hypodermal cells. A/D, B/E and C/F represent three different embryos. Ventral views of embryos positioned with anterior to the left and posterior to the right are shown. The three time points span the final stage of enclosure and developmentally are less than 5 minutes apart. Anterior leading edge cells on the left side of the embryo are indicated by arrowheads and ventral pocket cells are indicated by small arrows. The ventral surface is enclosed when the hypodermis meets and forms adherens junctions (or fuses) with contralateral cells at the ventral midline (VM). Scale bar, 10μm.

itudinal junction between the dorsal cell and the seam cell.[7] Migration of dorsal nuclei is not required for successful intercalation.[15]

Dorsal cell membranes were monitored by reconstructing TEM thin sections of dorsal cells and by using a transcriptional reporter expressing GFP in subsets of dorsal hypodermal cells[7] and (P. Heid and J.H., unpublished observations). Membrane protrusions were observed to extend basal to and in advance of the intact adherens junctions, intercalating between contralateral pairs of cells from the opposite row.[7] Remarkably, these protrusions extend far beyond the position of the adherens junction (Fig. 2D). No obvious concentration of actin is observed at the leading edge of these protrusions; it remains unknown what molecules are required for their extension and what, if any, force they exert. Immediately following intercalation, a large subset of dorsal hypodermal cells fuse to create a large multinucleate syncytium (several other smaller syncytia are also formed).

Regulation of dorsal intercalation

Several lines of evidence suggest that an individual dorsal cell may be able to effect the cell-shape changes required for intercalation in a cell-autonomous fashion. (1) The entire dorsal sheet is not required for intercalation. For example, when posterior dorsal cells are prevented from forming through the ablation of their founder blastomere (known as C) anterior (AB blastomere-derived) dorsal cells still intercalate perfectly well. (2) Neighboring seam cells are not required for intercalation; they may be ablated with no effect on intercalation.[7] One possibility is that specific seam cells produce attractant(s) for the migration of specific dorsal cells. While the remaining seam cells in ablation experiments may still provide guidance signals for intercalation, it is clear that not all seam cells are necessary for the process. (3) Apparent *die-1* (for dorsal intercalation and elongation-defective) mosaic animals show intercalation defects consistent with a loss of *die-1* activity only in the affected cells (P. Heid and J.H., unpublished observations; see below).

A comprehensive mutational analysis of dorsal intercalation has not been carried out. Serendipitously, a single mutation, *die-1*, that disrupts intercalation was isolated. *die-1* mutants have a completely penetrant elongation defect, suggesting that successful intercalation is a precondition for elongation. *die-1* has a role late in intercalation since the change of dorsal cells to a wedge shape, one of the earliest markers of dorsal intercalation, occurs in *die-1* animals and intercalation of some AB-derived anterior dorsal hypodermal specific cells persists in *die-1* embryos. Thus *die-1* may have a more restricted role in directing the rearrangement of specific dorsal cells, and other genes must be required during earlier stages of intercalation (Fig. 2A). One possible gene is *apr-1* (APC related protein), since a null mutation in *apr-1* results in a zygotic intercalation defect in which AB- and C- derived dorsal hypodermal cells often fail to intercalate.[16] Alternatively, a

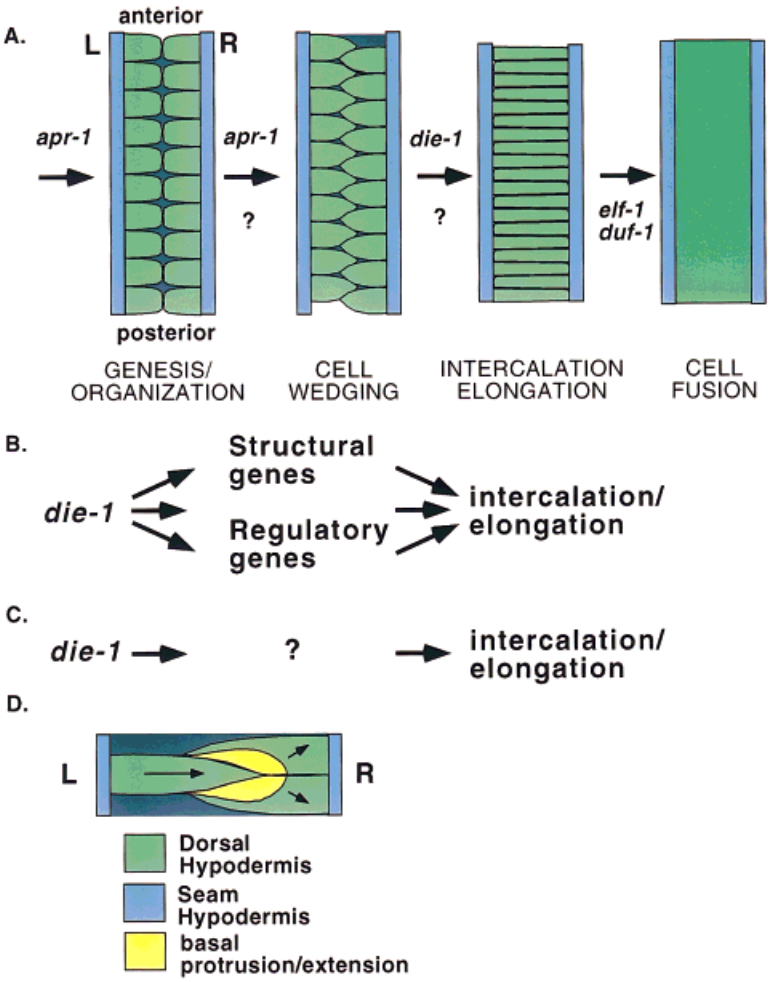

Figure 2. Schematic of dorsal intercalation. **A:** The cellular and genetic pathway for intercalation. After their birth and organization on the dorsal, posterior region of the embryo, dorsal cells (green) are bordered on their lateral edges by a row of seam cells (blue). The cellular borders are monitored using the adherens junction protein JAM-1-GFP (black). During intercalation, dorsal cells become wedge shaped under the control of yet-to-be identified genes (represented by question marks). Next, intercalation of wedge-shaped cells proceeds under the control of *die-1* (primarily in the posterior region) and possibly other genes. Finally, following intercalation, the dorsal cells fuse into a multicellular syncytium. Cellular fusion requires the activity of *elf-1* (for embryonic lack of fusion; W. Mohler and J. White personal communication) and *duf-1* (for dorsal hypodermis unfused; T. Gattegno and B. Podbilewicz, personal communication). **B,C:** Possible roles of *die-1* during intercalation. *die-1* encodes a putative zinc-finger transcription factor. *die-1* might act as a general regulator to control the transcription of multiple structural and regulatory genes during intercalation (**B**), or act as the first gene in a regulatory pathway (**C**). **D:** Schematic showing cell boundaries of dorsal cells during intercalation. Arrows indicate the direction of dorsal cell elongation from left to right. A basal protrusion (yellow) is extended well beyond the advancing, mature adherens junction (shown as JAM-1-GFP expression in black). The cells elongating from right to left also extend these basal protrusions, but for clarity are not shown. The role of the basal protrusions is unknown. The initial interaction of the basal protrusions with seam cells may establish a rudimentary junction, which may serve as a substrate for the subsequent formation of mature junctions, or the interaction may allow cell signaling required to maintain or promote further cell-shape changes and migrations in the dorsal hypodermis. Since *die-1* animals are not defective in cell wedging, but rather in the extension of mature cellular junctions to the contralateral seam cells, it is possible that *die-1* animals are defective in the extension of basal protrusions or the interaction of the basal protrusion with the seam cells, or both. Recent work from our laboratory suggests the latter is more likely (our unpublished observations).

defect in the production or initial organization of dorsal cells may contribute to the dorsal intercalation defect in *apr-1* mutants.[16] How APR-1 may function to regulate the behavior of hypodermal cells is discussed below.

How does *die-1* act during intercalation? *die-1* encodes a predicted zinc-finger transcription factor, suggesting *die-1* has a regulatory role during intercalation. Since DIE-1 is probably a transcription factor, there are two general models for how *die-1* may function (Fig.2B,C). *die-1* may act on a single (or a few) downstream regulatory gene which in turn regulates the activity of downstream structural and/or morphogenetic molecules. Alternatively, *die-1* may represent a regulatory branch point, i.e., *die-1* may be required for the transcriptional activation of multiple regulatory and morphogenetic molecules. If the first model is correct, it may be possible to identify downstream targets by a suppressor screen using a weak *die-1* allele. If the second model is correct, a genomics approach may be required to identify targets of *die-1*.[17]

How is elongation disrupted in *die-1* animals? *die-1* may have a critical role in establishing the organization of microfilaments and microtubules in the dorsal hypodermis. In *die-1* animals, actin filaments are formed and become appropriately aligned cell-autonomously, i.e., F-actin cables are aligned in parallel bundles and are closely associated with the apical cortical membrane. Since single dorsal cells frequently do not connect corresponding right- and left-hand seam cells in *die-1* embryos, circumferential actin filaments are discontinuous (P. Heid and J.H., unpublished observa-

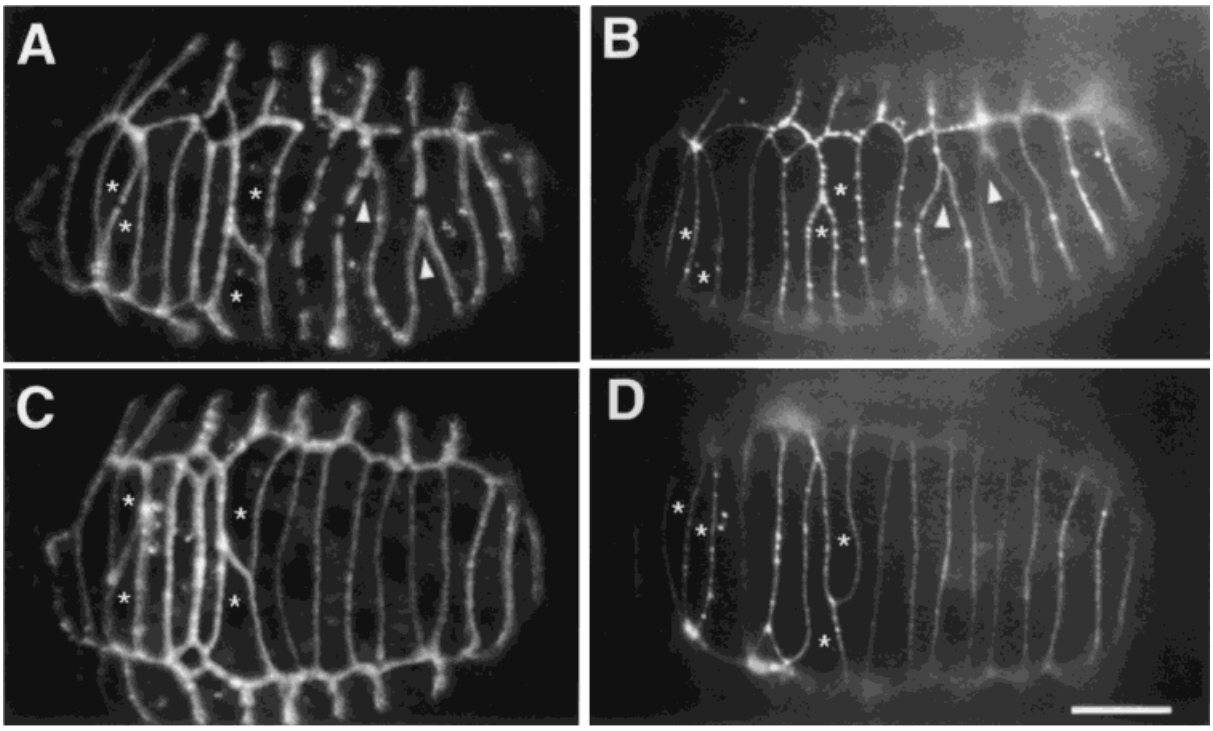

Figure 3. JAM-1-GFP expression during dorsal intercalation. Dorsal views of intercalating embryos are shown with anterior to the left. **A,C:** Two embryos in the late stages of intercalation. **B,D:** The same embryos 10 minutes later. Asterisks indicate the "pointer cells" that intercalate last; the posterior pointer cells are derived from the AB blastomere and abut dorsal hypodermis derived from the C blastomere. Arrowheads indicate dorsal cells derived from the C blastomere that are completing intercalation. The direction of the arrow indicates the extending dorsal cell lateral boundary as determined by the expression of JAM-1-GFP in the mature junction. Notice that C-derived hypodermal cells complete intercalation before the AB-derived pointer cells. Scale bar, 10 μm.

tions). Thus, when actin cables contract as the embryo attempts to elongate, the force of contraction may not be distributed evenly and embryos do not fully elongate. According to this model, intercalation is required to ensure that filaments are properly linked, and anchorage of circumferential filaments to seam cells on opposing sides of dorsal cells is critical for elongation. A second possibility is that *die-1* has a separate function during elongation and thus the failure of *die-1* animals to elongate results from defects in both intercalation and elongation.

Ventral enclosure

It is proposed that enclosure of the ventral surface of the embryo involves a two-step process in which two functionally distinct groups of hypodermal cells meet and form stable adherens junctions or fuse, ultimately sealing the ventral midline (Fig. 4). In the first step, two pairs of anterior, or "leading", cells extend actin-rich filopodia as they elongate toward the ventral midline. The elongation of these cells is essential for any ventral cell pairs to meet and form junctions; ventral enclosure can be completely abrogated by the laser inactivation of these two pairs of leading cells.[6] In the second step, posterior ventral cells become wedge shaped and elongate toward the ventral midline. Once past the equator of the embryo, the progressive change in shape of the posterior cells results in the formation of a "pocket" about the ventral midline (Fig. 1A,B). Actin is concentrated at the distal tips of the posterior cells and has been proposed to close the pocket by an actomyosin "purse-string" mechanism.[6]

Confirmation of a "purse-string" closure of the ventral pocket will require additional experimentation. First, the organization of actin and myosin with respect to the pocket must be determined. According to the purse-string model, actin and myosin would be expected to be organized circumferentially about the pocket.[18] Second, it will be necessary to demonstrate localization of a non-muscle myosin at the leading edges of the posterior cells as well as a requirement for such a non-muscle myosin in the closing of the ventral pocket. *nmy-2*, which encodes a non-muscle myosin heavy chain[19], is a candidate. While it is clear that there is actin present at the free edges of the migrating hypodermis,[6]

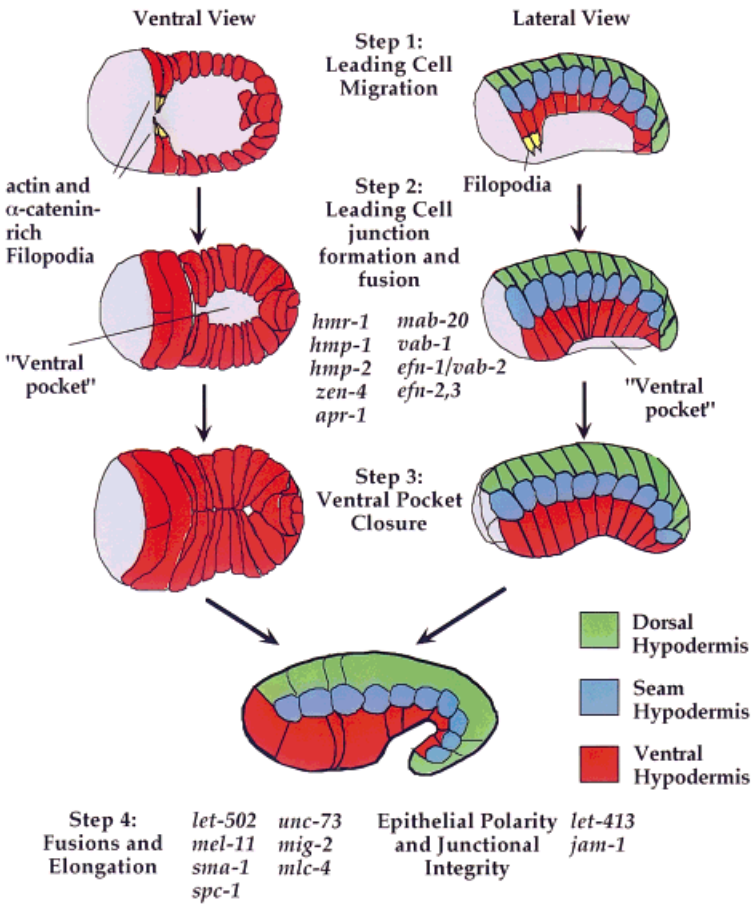

Figure 4. Schematic of ventral enclosure and elongation. The ventral cells that ultimately meet at the midline and seal the embryo are shown in red, the lateral seam cells are shown in blue, and the dorsal cells are shown in green. Ventral enclosure proceeds in three distinct steps. In step 1, actin-rich filopodia (yellow) are extended toward the ventral midline by a pair of anterior leading cells. These cells meet at the midline and form adherens junctions (anterior pair) or fuse (posterior pair). The migration of these leading cells and the rapid formation of cellular junctions requires the function of cadherin, α-catenin, β-catenin, and APC-related genes (*hmr-1*, *hmp-1*, *hmp-2*, and *apr-1*, respectively). The kinesin-like gene *zen-4* is also required for enclosure. EphR and Ephrins are required for the organization of underlying neurons over which the hypodermis migrates and *mab-20* is required for proper seam and ventral hypodermal organization. In step 2, the posterior cells become wedge shaped as they extend to the midline, and their leading tips form the so-called ventral pocket, which closes in step 3. In step 3, the ventral pocket may seal upon contraction of an actomyosin-driven "purse-string" structure, or by the migration of its free edges. After the embryo is sealed with hypodermis, contraction of circumferential F-actin changes the shape of the embryo into an elongated worm. In Step 4, elongation requires SMA-1, a β_H-spectrin that may provide linkage between actin filaments and the apical membrane, as well as a group of genes that likely regulate the activity of non-muscle myosin II (MLC-4) within the seam cells. The shape change in the seam cells may provide significant driving force for elongation. Finally, an elongating embryo requires intact cellular junctions for epithelial integrity. If LET-413 (LAP) is missing, embryos rupture due to mislocalization of junctional molecules such as HMP-1 and JAM-1.

recent data from our laboratory suggest that actin may be localized within short filopodia at the advancing edges of the posterior ventral hypodermal (P) cells. These short filopodia would then extend into and subsequently close the pocket.[14] In order to understand the behaviors of anterior versus posterior ventral cells during enclosure, it is essential to differentiate between such possible mechanisms.

Identification of genes required for ventral enclosure

Various strategies have led to the identification of enclosure-defective mutations. Our laboratory has undertaken a general screen for maternal and zygotic enclosure-defective mutants by scoring for the expression of JAM-1-GFP in arrested embryos. Embryos that suffer incomplete enclosure yet exhibit the wild-type number and organization of hypodermal cells and other major tissues at a gross level are being further characterized. We have also used enclosure mutants identified in a general screen conducted by Joel Rothman and colleagues to identify zygotic lethal mutations (Zels).[20] Zygotic enclosure-defective (Zen) mutants with very specific defects during ventral enclosure comprise a subset of these Zels. Others have examined homozygous deficiency phenotypes to identify putative loci required zygotically for various aspects of hypodermal development including enclosure.[13,21,22] Enclosure-defective animals have also been identified that arise indirectly due to defects in processes other than ventral enclosure (e.g. Ref. (23))

The *C. elegans* cadherin/catenin complex

It was expected that cadherin-based adherens junction proteins would be essential for at least some aspects of enclosure or elongation. Cadherins are single transmembrane cell adhesion molecules that bind to cytosolic β-catenin, which itself binds to α-catenin. α-catenin is thought to interact directly with actin or indirectly through α-actinin. The cadherin/catenin complex localizes to adherens junctions (AJs) and mechanically couples cell adhesion and the cytoskeleton.[24,25]

Inactivation of cadherins and catenins in mouse, *Xenopus*, and *Drosophila* disrupts embryonic cell adhesion. Such studies indicate these proteins regulate essential processes such as formation of the trophoblast epithelium in the mouse,[26–29] blastula organization and body axis specification in Xenopus[30,31] and gastrulation and tracheal outgrowth in Drosophila.[32–34] Finally, the adherens junction protein β-catenin also interacts with and participates in Wnt/wingless signal transduction pathways, indicating that there may be considerable cross-talk between the regulation of cadherin-based cell adhesion and cell-fate specification.[35]

The cadherin/catenin complex has been identified in *C. elegans*, and homologues of the adherens junction proteins cadherin, β-catenin and α-catenin (encoded by *hmr-1*, *hmp-2* and *hmp-1*, respectively) are required for enclosure and elongation.[36] Even though the percent identity between *C. elegans* AJ proteins and the AJ proteins from other species is low, these proteins likely function as a classic cadherin/catenin complex. Sequence comparisons show: (1) HMP-1/α-catenin contains β-catenin and actin-binding regions, (2) HMP-2/β-catenin contains the same number of armadillo repeats as *Drosophila* ARMADILLO (as well as α-catenin- and cadherin-binding sites) and (3) HMR-1/cadherin contains extracellular homotypic binding (EC) repeats and an intracellular β-catenin-binding site.[36] In embryos, *hmr-1* is required for the proper localization of HMP-1 and HMP-2 at the adherens junctions, a function analogous to that of classic cadherins.[36]

Surprisingly, mutations in *hmr-1*, *hmp-1* and *hmp-2* result in less severe phenotypes than anticipated. Specifically, by analogy to other developing animals, a general defect in cell adhesion at least as early as gastrulation was expected; however, no phenotypes are observed prior to morphogenesis of the hypodermis. Cell adhesion among migrating sheets of hypodermal cells appears to be completely normal.[36]

One likely explanation for these differences is genetic redundancy. Although there are at least fourteen genes with similarity to cadherins in *C. elegans*, HMR-1/cadherin is the only molecule in the genome that possesses all the structural motifs of classical cadherins.[36,37] Early embryonic adhesion functions therefore may be carried out by cadherin-related proteins or by non-cadherin-based adhesion systems.

Loss of maternal and zygotic *hmr-1* results in a more severe phenotype than a similar loss of *hmp-1* and *hmp-2*. Loss of *hmr-1* often results in a complete failure of enclosure, with the retraction of the entire hypodermal sheet onto the dorsal surface, whereas *hmp-1* and *hmp-2* embryos most often develop anterior enclosure defects collectively known as the Hammerhead phenotype.[14,36] What is responsible for this? One possibility is that the other catenin-like molecules substitute for *hmp-1* and *hmp-2*.

A search of *C. elegans* genomic sequence reveals two genes closely related to β-catenin; *hmp-1* and the gene corresponding to the EST CELK02251. However RNA-mediated inactivation (*RNAi*) of CELK02251 does not increase the severity of *hmp-1* phenotypes.[14,36] This result suggests there may be additional functional homologues of *hmp-1*. Alternatively, the greater severity of *hmr-1* phenotypes relative to *hmp-1* or *hmp-2* could be explained if HMR-1 mediates catenin-independent adhesive functions.

What about β-catenin? There are three β-catenin genes in *C. elegans*: *wrm-1*, *hmp-2* and *bar-1*. Each gene is required at a distinct stage of development: *wrm-1* is required to transduce a signal from the P2 to the EMS blastomere at the 4-cell stage,[38,39] *hmp-2* is involved in embryonic morphogenesis.[36] and *bar-1* is required postembryonically for two sequential cell-fate decisions during vulval development (maintenance of the vulval equivalence group and vulval cell-fate specification[40]).

Two lines of evidence that suggest that *hmp-2* is the only β-catenin required for enclosure. (1) *bar-1* null animals are viable, indicating BAR-1 has no role during embryogenesis. (2) Yeast two-hybrid and/or immunoprecipitation experiments suggest that only HMP-2 can interact with HMR-1, whereas only BAR-1 and WRM-1 (through LIT-1) can interact with POP-1 to mediate transcriptional activation.[41] Thus the three β-catenins may mediate separable functions as proposed by Eisenmann et al.[40] and HMP-2 may simply provide the embryonic morphogenetic function. It should be pointed out, however, that a requirement for *wrm-1* during enclosure has not been tested. Inactivation of zygotic *wrm-1* should answer this question.

What if HMP-2 is the only β-catenin required for enclosure? This possibility raises several interesting questions. Does HMP-2 regulate transcription? If so, what are the molecules, if any, that HMP-2 interacts with to regulate transcription? If HMP-2 does not function as a transcriptional regulator, does another group of molecules substitute for this activity? One possibility is that WRM-1 and perhaps BAR-1 mediate signaling to the nucleus in subsets of hypodermal cells.

HMR-1, HMP-1 and HMP-2 regulate actin-dependent processes

What is the effect of *hmp-1* zygotic loss-of-function mutations on the organization of F-actin? Since cadherin–catenin complexes link cell adhesion to the actin cytoskeleton, a strong prediction is that actin filament organization would be disrupted in *hmp-1* animals. At the end of enclosure, actin is normally concentrated into circumferential bundle filaments (CBFs)[5,6] and this organization is also observed in *hmp-1* animals. In *hmp-1* embryos, however, CBFs become detached from the adherens junctions (AJs).[36] In these embryos, translation of the mechanical force of CBF contraction into elongation of the embryo is presumably disrupted. Remarkably, detachment of actin filaments is observed only in the dorsal hypodermal syncytium, resulting in dorsal folds in the hypodermis, known as the Humpback (Hmp) phenotype.

Here, as perhaps is true for enclosure, functional homologues of α-catenin in *C. elegans* may compensate for loss of HMP-1 in the ventral hypodermal cells and differences between hypodermal cell types are only observed in the absence of *hmp-1* activity. Alternatively, detachment may occur in all hypodermal cell types, but may only be observed in dorsal hypodermis due to structural differences between hypodermal cell types.[36]

What then is the exact role for HMR and HMP proteins during enclosure? To address this issue, α-catenin-GFP (HMP-1-GFP) and JAM-1-GFP fusion proteins were monitored in vivo in three dimensions during enclosure using two-photon microscopy, both in wild-type and *hmr-1* mutant animals. Leading cells extend actin and HMP-1-GFP-rich filopodia.[6,14] In wild type, once the filopodia meet at the ventral midline, HMP-1-GFP is rapidly (within 5–10 minutes) deposited into nascent AJs and then along the ventral midline, both anteriorly and posteriorly from the point of first contact. Shortly thereafter, JAM-1 is deposited, which is indicative of a mature *C. elegans* AJ. In *hmr-1* animals, the ability of filopodia to extend and touch is unimpaired, but they fail to form junctions, and the leading cells retract to the dorsal surface.[14] Thus, filopodia require cadherin molecules not for their extension but rather for the rapid formation of nascent junctions at sites where they make contact. Interestingly, the organization of the cytoskeleton during ventral enclosure appears poised for rapid adherens junction formation. Immediately prior to filopodial contact, polarized actin filaments and high levels of cytoplasmic HMP-1 are localized to the filopodia.[14] In naïve cells, especially cells in culture, actin and α-catenin may not be organized in such a way as to facilitate rapid AJ formation.[43] In *hmr-1* animals, the posterior ventral cells (pocket cells) also fail to form junctions in many cases, suggesting that these cells may have a similar requirement for rapid junction formation. Indeed, filopodia are observed extending from posterior cell leading edges, and similar, albeit less dramatic, changes in HMP-1-GFP distribution are observed during AJ formation in these cells.[14]

Disruption of proteins that interact with β-catenin may also result in enclosure phenotypes. For example, APC (adenomatous polyposis coli) proteins directly interact with and regulate β-catenins in other organisms, and defective APC proteins generally disrupt the same processes affected by mutations in β-catenin.[44] *apr-1* encodes the only related protein in *C. elegans*.[38,39] APR-1 is similar to other APC proteins only in the ARD (Armadillo repeat domain[16]; see Ref. 45 for review). APR-1 interactions with WRM-1, HMP-2 and BAR-1, or its interactions with other proteins will likely differ from that in other organisms.

As expected, *apr-1* has a zygotic role during enclosure: mutations in *apr-1* disrupt ventral enclosure and result in phenotypes most similar to those observed in *hmr-1* mutant embryos.[16] *apr-1* also has a maternal role in P2-EMS signaling at the 4-cell stage and a postembryonic function in the specification of the vulval equivalence group.[16,39,46] The identified functions for *apr-1* differ from those of APC proteins from other systems, since *apr-1* acts as a positive effector of Wnt/β-catenin signaling (reviewed in Refs. 16 and 47). *apr-1* has a unique role during embryogenesis: unlike mutations in genes that encode cadherin/catenin complex components, mutations in *apr-1* disrupt both dorsal intercalation and ventral enclosure, suggesting that there may be conserved mechanisms underlying intercalation and enclosure.

The role of Eph/Ephrins and Semaphorins in ventral enclosure

Mutations in *vab-1* and *vab-2* disrupt enclosure. *vab-1* and *vab-2* encode an Eph receptor and ephrin ligand, respectively. VAB-1 is the only EphR in *C. elegans*, whereas there are at least four presumptive GPI-linked ephrin ligands.[48,49] Ephrins have been implicated in direct cell–cell signaling and neuronal pathfinding. This also appears to apply to *vab-1* and *vab-2*, which affect the organization of neuroblasts in the embryo and are expressed in non-overlapping subsets of these cells.[23,48]

There are at least two ways in which *vab-1/vab-2* enclosure defects could arise. (1) The disorganization of neuroblasts could result in the persistence of the gastrulation cleft along the ventral midline. This cleft could serve as a physical barrier, preventing the migration of hypodermal cells toward the midline. (2) Neuroblast disorganization could disrupt the proper spatial presentation of a separate guidance molecule, and hypodermal cell guidance toward the midline would be prevented. Supporting the first model is the observation that, in *vab-1* and *vab-2* animals, the intrinsic behavior of the hypodermis appears normal. Leading cells migrate toward the ventral midline and extend filopodia, while posterior cells constrict at their leading edge in an attempt to form a pocket even in the presence of the most severe gastrulation clefts.[23,48] In support of the latter model is the observation that enclosure can fail even though the gastrulation cleft is sealed.[23,48] Taken together, these data suggest that the interaction between *vab-1* and *vab-2* is not directly involved in regulating hypodermal cell migration, but specifically in the organization of the substrate over which they migrate.

A comprehensive analysis of all the presumptive GPI-linked ephrin ligands in *C. elegans* suggests that neuroblast organization can directly influence enclosure.[49] Specifically, *efn-2* and *efn-2*, *vab-2/efn-1* double mutants affect the relative position of posterior hypodermal cells hyp8 and hyp9 relative to hyp7, and are required for ventral enclosure. EFN-2 and VAB-2/EFN-1 are expressed in neuroblasts during enclosure and by tail hypodermis (hyp8, hyp9, hyp10 and hyp11) following enclosure. Given the late onset of hypodermal expression, these results indicate that the organization of

underlying neuroblasts is required for enclosure of the posterior hypodermis.[49]

Recently, an unexpected molecule, *C. elegans* Semaphorin2a (ceSema2a), encoded by *mab-20* (for male tail abnormal), was shown to be required during ventral enclosure.[50] Semaphorins are a family of transmembrane or secreted proteins that provide guidance cues during neural development.[51] Many Semaphorins provide repulsive cues, and ceSema2a appears to function in such a manner. As expected, *mab-20* mutations affect axon guidance; however, in *mab-20* mutants, inappropriate contacts are also observed among hypodermal cells during migration of hypodermal precursors, ventral enclosure and male tail morphogenesis.[50] These results are intriguing, as it suggests that (negative) regulatory pathways regulate the organization of hypodermal cells at the ventral midline during enclosure. By analogy to neurons, one possible target for *mab-20* repulsive cues during enclosure may be leading cell filopodia, since filopodia may provide a sensory function in growth cone steering. [52] Thus, cell–cell signaling molecules regulate the interaction among underlying neuronal blast cells and hypodermal cells required for proper ventral enclosure and subsequent morphogenesis.

The kinesin-like protein ZEN-4 is required for enclosure

Zygotic enclosure-defective (Zen) mutants comprise a subset of zygotic embryonic lethal (Zel) mutants identified in a general screen conducted by Joel Rothman and colleagues.[20] One such mutation, *zen-4*, was found to result in ventral enclosure defects. *zen-4* encodes a member of the CHO1/MKLP family of kinesin-like proteins.[53] Consistent with the role of other CHO1/MKLP1 proteins in the organization of antiparallel microtubule bundles required for cell division, loss of ZEN-4 results in disorganized microtubules at the midbody of the mitotic spindle and in cytokinesis defects in the 1-cell zygote.[53,54]

How is enclosure disrupted in *zen-4* animals? Often, *zen-4* animals derived from a heterozygous parent are found to contain a reduced number of hypodermal cells, presumably due to cytokinesis defects in founder cells (our unpublished observations). Such animals may arise due to a gradual loss of maternal ZEN-4 during embryonic cell divisions. If gradual loss of ZEN-4 can affect hypodermal cell production, it is reasonable to predict that other cell lineages, such as those generating substrate cells, are similarly affected, including the neuroblasts over which the hypodermis must migrate. As mentioned above, disruption of the organization of these neuroblasts has been demonstrated to prevent enclosure.[23,48,49] Enclosure may therefore fail due to the insufficient production of hypodermal cells, underlying ''substrate'' cells, or both.

ZEN-4 may also have a postmitotic role in regulating epithelial movement during enclosure. Significantly, it is possible to identify enclosure-defective animals in which all hypodermal cells are generated. It therefore remains possible that ZEN-4 has a purely morphogenetic function during epithelial cell movement. To demonstrate this unambiguously will require inactivation of *zen-4* specifically in the fully formed hypodermis.

How do CHO1/MKLP1 proteins function in postmitotic cells? In cultured neurons and in podocytes, CHO1/MKLP1 proteins are required for neurite and process extension, respectively.[55,56] Transport of MTs within dendrites and the antiparallel (non-uniform) arrangement of MTs required for process extension depends on CHO1/MKLP, suggesting that non-uniform organization of MTs is a conserved function of CHO1/MKLP family proteins in postmitotic cells.[57]

In a similar way, ZEN-4 may bundle microtubules in an antiparallel fashion in the hypodermis. Transposition of antiparallel microtubules is thought to contribute to the forces that drive cytokinesis[58] and, by analogy, may provide the force to elongate epidermal cells.

Regulation of enclosure

While it is clear that normal actin and microtubule dynamics are essential for enclosure, very little is known regarding their complex regulation.[6] Clues as to which proteins may regulate enclosure can be gleaned from other systems in which cellular migrations are intimately linked to, and dependent upon, the extension of filopodia and lamellipodia. For example, the co-regulation of actin and microtubule dynamics are required for the movement of epithelial free edges during wound healing and fibroblast migration.[60,61] Cell-shape changes and migrations of fibroblasts, and the outgrowth of neuronal dendrites and axons are dependent upon the modulation of actin dynamics, which are regulated to a great extent by GTPases of the Rho, Rac and CDC42 families.[62] In neurons and fibroblasts, each of these proteins have distinct roles in the organization of stress fibers, lamellipodia and filopodia, respectively. Such findings suggest that similar proteins may function during the morphogenesis of embryonic epithelia in various organisms. This is the case during dorsal closure in *Drosophila*, where Rho, Rac and CDC42 GTPases interact with PKN (protein kinase N/protein kinase C-related) and JNK (c-Jun amino (N)-terminal kinase) pathways to regulate leading edge and lateral cell stretching required for dorsal closure.[63–66] Not surprisingly, in *C. elegans*, CeRhoA, CeRac1, CDC42Ce and p21-activated kinase (CePAK), a downstream target of CeRac1 and CDC42Ce, have been isolated and CeRac1, CDC42Ce and CePAK localize to adherens junctions during embryogenesis.[67,68] These expression data are consistent with a role for GTPases and p21-activated kinase during enclosure. It is difficult, however, to prove an essential role for these proteins during enclosure due to an earlier requirement during development. For example, *RNAi* of CDC42Ce results in a loss of polarity at the 1-cell

stage, which leads to a gross disorganization of the embryo (A. Kay, C. Hunter, M. Gotta, and J. Ahringer, personal communication). A clear goal of the analysis of ventral enclosure must be a thorough understanding of how these proteins regulate cytoskeletal dynamics during this process.

Elongation

One of the most immediate consequences of successful intercalation and enclosure is that the embryo is primed for the spectacular morphological change of elongation. The progression from a lima-bean-shaped embryo to the worm shape of the larva is dependent on circumferentially organized microfilaments and microtubules. Following enclosure, actin and tubulin polymers are aligned circumferentially within the dorsal and ventral hypodermis and are associated with the apical membrane. Actin filaments are anchored at lateral cell margins, which abut the neighboring seam cells, and are attached to adherens junctions at these sites.[5,36] As elongation proceeds, the length of actin filaments in the seam cells decreases along the dorsoventral axis, and the cells correspondingly elongate along the anteroposterior axis.[5]

As for dorsal intercalation and ventral enclosure, elongation and its associated hypodermal cell-shape changes require the presence of intact actin filaments and microtubules.[5] Treatment with cytochalasin D prevents elongation and treatment with nocodazole results in disorganized elongation. Surprisingly, these effects are reversible; removal of either drug results in a continuation of elongation.[5] The force for elongation is likely generated by actomyosin contraction and distributed by microtubules. Microtubule inhibitors do not entirely disrupt elongation; instead they cause the formation of surface abnormalities, which are likely to be dependent on the microfilament lattice, since these abnormalities do not arise in the presence of cytochalasin D.[5] Interestingly, microfilament organization as assessed by phalloidin staining appears normal following treatment with microtubule inhibitors.[5]

How is the force of constriction translated into elongation, and how is the elongated state maintained? The embryo is covered by an extracellular layer called the embryonic sheath. This sheath appears to have anchorage sites for actin filaments and is apparently required for elongation.[5,69] One possible model, elaborated in[5], is that the surface of the elongating embryo is like a tent: the cells and associated sheath are the fabric, the microfilaments the ropes, and the microtubules the poles and guy wires. In this model, microfilaments provide the force to pull the sheath across the microtubule lattice, which in turn distributes the force evenly.

The linkage of actin to adherens junctions and the embryonic sheath is likely to be critical in converting the force of contraction into elongation. A candidate for linking the actin cytoskeleton to the apical plasma membrane is the protein SMA-1, a homolog of β_H-spectrin (a novel isoform of the β-spectrin family which was originally identified as the product of the *Drosophila* Karst gene[70]). Such attachment would serve to convert circumferential actin contraction into cell-shape change and elongation of the embryo. Consistent with this model, *sma-1* mutants are defective in elongation and SMA-1 is associated with the apical membrane in dorsal and lateral hypodermis (V. Praitis and J. Austin, pers. comm.). Further evidence supporting this model comes from the recent identification of a *C. elegans* α-spectrin homologue, *spc-1*, required for elongation (K. Norman and D. Moerman, pers. comm.). Since some elongation occurs in *sma-1* animals, SMA-1 alone cannot account for the anchorage of filamentous actin to cell membranes.

How is the process of elongation regulated? The best clues so far come from the analysis of the *let-502* (Rho-binding/human myotonic dystrophy kinase) and *mel-11* (smooth muscle myosin phosphatase) genes. *let-502* was identified as a gain-of-function, elongation-defective mutation in a screen for morphogenetic mutants. *mel-11* was identified as a maternal effect suppressor of *let-502*.[71] Based on homology, it seems likely that LET-502 and MEL-11 proteins work to regulate the contraction of non-muscle myosin II. More recently, it has been shown that mutations in Rac-GEF (encoded by the *unc-73* gene) and *mig-2* (a Rac homologue) enhance the *mel-11* phenotype, and that LET-502 and MEL-11 activities are antagonistic during elongation.[72] Supporting these genetic data are the expression patterns of MEL-11 and LET-502: LET-502 is strongly expressed in seam cells, where MEL-11 levels are low.[72] Thus it appears that the function of LET-502 during elongation is to promote the contraction of the seam cells. During smooth muscle contraction in vertebrates, Rho kinases phosphorylate and inactivate myosin phosphatase, allowing the activation of myosin and subsequent muscle contraction. By analogy, MEL-11 activity is likely down-regulated by LET-502. Interestingly, mutations in *mlc-4*, a non-muscle myosin regulatory light chain, have a zygotic elongation defect and a *mlc-4::gfp* gene is expressed in the seam cells.[73] These results suggest that MLC-4 may be a target of LET-502 and MEL-11 regulation. One interpretation of these results is that *C. elegans* non-muscle myosin in the seam cells is associated with circumferential actin in an actomyosin structure analogous to that found in the contractile apparatus at the edge of closing wounds in vertebrate epithelial tissues (reviewed in Ref. 18). Contraction of this actomyosin assembly may be the driving force behind elongation of the seam cells and seam cells may, therefore, provide significant major mechanical force for elongation of the embryo.

Epithelial integrity

During elongation, epithelial integrity must be maintained. Cellular junctions are a hallmark of polarized epithelial cells;

the cellular junctions that link cells must remain intact, otherwise the embryo will rupture as the actomyosin ring contracts. A key regulator of cellular junctions and epithelial polarity in *C. elegans* is the protein LET-413, which encodes a *C. elegans* LAP (for LRR and PDZ) family protein similar to Scribble in Drosophila.[74,75] LET-413 contains 16 leucine-rich repeats (LRRs) and one PDZ domain; LET-413 is expressed basolaterally in *C. elegans* epithelia and is required to maintain epithelial integrity, since *let-413* animals rupture at the two-fold stage.[74] In *let-413* embryos, JAM-1 localization is disrupted and *jam-1*animals have apparent defects in adherens junction sealing, consistent with a role for LET-413 in regulating epithelial integrity[74] (M. Köppen, P. Sims and J.H., unpublished observations).

Perspectives

Although some of the mechanisms underlying elongation of the embryo are becoming clear, questions remain regarding intercalation and enclosure. None are more intriguing than those concerning the function of actin filaments and microtubules and how their early functions might be linked to the process of elongation. For example, as intercalation and enclosure near completion, actin filaments and microtubules become organized circumferentially. Is the circumferential organization of actin filaments and microtubules required for the completion of dorsal intercalation and ventral enclosure, or is circumferential organization a functionally distinct but temporally coincident event?

The processes of intercalation, enclosure and elongation require filamentous actin and microtubules, yet few other shared molecular requirements have been established. With the exception of *apr-1*, mutations that affect ventral enclosure appear to have no effect on dorsal intercalation and mutations in *die-1* appear to have no effect on ventral enclosure. This distinction suggests that the processes of dorsal intercalation and ventral enclosure are dramatically different at all but the most basic levels. We anticipate that the investigation of these seemingly distinct processes will reveal unique strategies for the regulation of actin filament and microtubule dynamics during epithelial morphogenesis.

The discovery of a cadherin–catenin system required for enclosure and elongation, as well as a regulatory pathway associated with non-muscle myosin II during elongation, demonstrates that conserved morphogenetic molecules are used during *C. elegans* hypodermal morphogenesis. The further characterization of the cadherin-catenin system should advance our understanding regarding the relationship between the formation of cellular junctions and the regulation of cytoskeletal dynamics. For example, pre-organization of cytoskeletal and associated proteins within filopodia, which we have referred to as "filopodial priming", may facilitate more rapid epithelial sheet sealing. The polarization of actin with respect to sites of insertion into forming AJs may be rate limiting for the formation of AJs in dynamic systems.[14] The ability of filopodia to establish junctional complexes has also been observed in primary vertebrate tissue culture cells,[76] and together these experiments address the complex relationship between filopodia and junctional dynamics during development.

Molecules that participate in generating mechanical force, especially those associated with actin and microtubules, are likely to be essential for a variety of processes and may be regulated in similar ways in each case. An excellent example of this dual function is found in *Xenopus*, where the spatial organization of microtubules relative to the cytokinetic furrow and relative to actomyosin "purse-string" structures at wound sites is conserved.[59] An exciting possibility is that molecules such as ZEN-4 will have conserved functions in cytokinesis and morphogenesis and will therefore highlight the similarities between seemingly dissimilar processes.

C. elegans is one of several excellent model systems used to study epithelial morphogenesis and a synergy exists between these systems. During intercalation, directed cell rearrangement or cell extension occurs in a planar sheet, serving as a paradigm for other similar systems such as neurulation in chordates, gastrulation in the sea urchin, and germband extension in Drosophila.[77–80] In the latter case, transcription factors have been identified that delimit when and where cell rearrangements are allowed; however, the molecules that effect intrinsic cell behaviors (shape change, movement) have not been well described.[80–82] In this sense, *die-1* represents an inroad into the mechanisms of cell movement within a planar epithelium. During ventral enclosure, hypodermal cells cover the embryo in a manner similar to dorsal closure in *Drosophila*. The components of multiple signalling pathways regulating epithelial movements during dorsal closure have been identified, yet a description of the structural genes upon which these regulatory pathways impinge is just emerging.[83] In *C. elegans*, both structural and regulatory molecules have been identified, and the further study of unexpected genes required for intercalation and enclosure, particularly *die-1*, *mab-20* and *zen- 4* should reveal novel mechanisms of morphogenetic movements in hypodermal tissue. A compelling general observation at present is that the types of molecules identified in different systems are not completely overlapping, suggesting that each system independently contributes to our understanding of epithelial morphogenesis.

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