Morphogenesis at single-cell resolution: studying changes in the shape of the embryo in the tradition of Hörstadius

Jeff Hardin*†‡, William B. Raich† and Jeffrey S. Simske*
*Department of Zoology and †Program in Cellular and Molecular Biosciences, University of Wisconsin, 1117 W. Johnson St., Madison, WI 53706 U.S.A.

Introduction – the legacy of Hörstadius and the study of morphogenesis

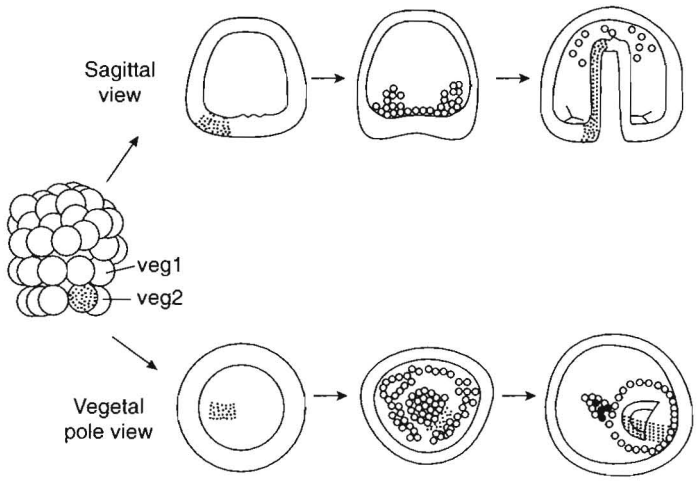
One of the great legacies that Sven Hörstadius left to developmental biology was the notion that specific founder cells in the early embryo generate the major tissue territories of the embryo. These territories comprise groups of cells that engage in two important events: (i) they can engage in inductive interactions, i.e. one tissue can signal another, resulting in changes in differentiation, and (ii) they can engage in region-specific morphogenetic behaviours resulting in changes in the shape of the embryo. Hörstadius focused on the potencies of founder cells in the early echinoderm embryo and how signals in the early embryo regulate the fates of these cells. Although Hörstadius did not focus on morphogenetic movements *per se*, his work has important implications for our understanding of the subsequent dramatic reshaping of the embryo that occurs following these early specification events.

To see how this is so, consider what Hörstadius' careful fate maps of the 64-cell echinoderm embryo imply for mechanisms of archenteron invagination during sea urchin gastrulation (Figure 1). By staining single veg2 blastomeres Hörstadius (1936) showed that the archenteron is initially formed from veg2 progeny in the vegetal plate, a flat sheet of cells that later invaginates to form the primitive gut (Figure 1). His studies also showed that widespread involution of material over the lip of the blastopore does not occur during sea urchin gastrulation. Thus invagination of the archenteron is driven by movements within or near the vegetal plate. More recent studies have refined Hörstadius' results by clarifying the contributions that clones derived from the veg2 founder cells make to the archenteron. These recent studies indicate that there is intermixing of veg1 and veg2 derived cells during late gastrulation (Logan and McClay, 1997; Cameron and Davidson, 1997).

The fate mapping studies performed by Hörstadius also provide clues for possible mechanisms by which elongation of the archenteron occurs. Hörstadius found that veg2 cells give rise to sectors of labelled cells in the

‡To whom correspondence should be addressed.

Figure 1



Fate mapping of veg2 descendants in the sea urchin embryo

Hörstadius labelled individual veg2 cells of the 64-cell embryo with the vital dye Nile Blue. In the blastula through early gastrula stages, veg2-derived clones come to lie within the vegetal plate (sagittal view). During gastrulation, veg2-derived material contributes sectors of material to the archenteron. The sectors narrow circumferentially and lengthen along the animal–vegetal axis (surface view). Adapted from Hörstadius, S. (1936) Willhelm Roux' Archiv. Entwicklungsmech. Org. ***135****, 1–39.*

archenteron; during gastrulation these sectors appear to become longer and narrower (Figure 1). Recent studies have shown that such narrowing is evidence of a type of directed cell rearrangement known as convergent extension within the archenteron (Ettensohn, 1985; Hardin and Cheng, 1986; Hardin, 1989; reviewed in Hardin, 1996).

The work of Hörstadius indicates that any thorough understanding of animal development must begin at the level of single cells. Hörstadius' careful analysis of the interactions that occur in the experimentally manipulated embryo paved the way for modern molecular analyses of patterning and differentiation in the early echinoderm embryo, and in turn served as a model for the study of many other organisms. We have sought a similar, single-cell analysis of morphogenetic movements, since it is the behaviours of individual cells within a tissue that ultimately result in the overall changes in the shape of the entire embryo. Such behaviours include the extension of motile protrusions, changes in cell shape, and the dynamic alteration of cell–cell contacts. A single-cell analysis of morphogenesis is similar to the work of Hörstadius, because it provides a context for interpretation of the disruption of the function of specific gene products that affect morphogenetic processes.

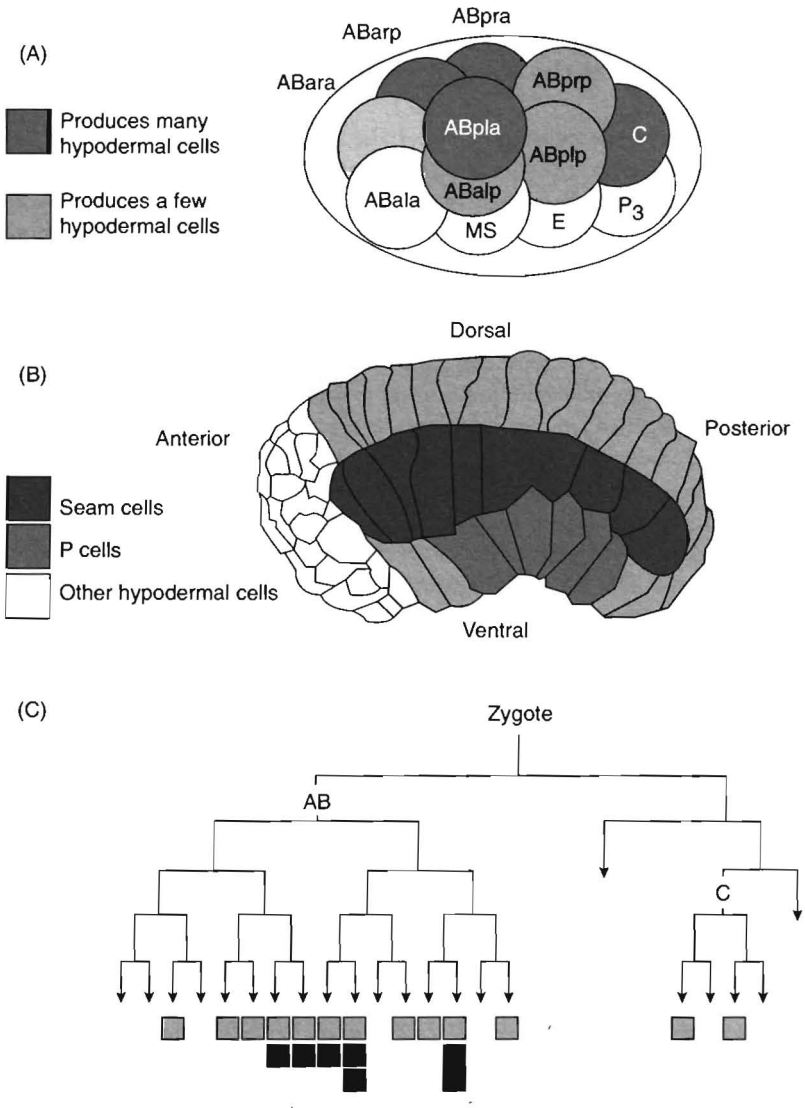
Ventral enclosure of the hypodermis in *Caenorhabditis elegans*: a process that permits analysis of epithelial morphogenesis at the level of single cells

In many ways, the lineage analyses undertaken by Hörstadius have reached their logical fulfilment in the nematode worm, *C. elegans*. The stereotyped development of *C. elegans* is even more reproducible than that of echinoderms, and the complete embryonic and post-embryonic lineages have been determined (Sulston and Horvitz, 1977; Sulston et al., 1983). Such information is extremely useful for pursuing a region-specific analysis of morphogenesis. In addition, in *C. elegans* the availability of robust genetic and molecular tools allows the relatively rapid characterization of genes required for specific morphogenetic events. We have used the embryonic epidermis, or hypodermis, of the *C. elegans* embryo to analyse morphogenetic movements at the level of single, identified cells. We have also begun to analyse the molecules that affect the cellular behaviours underlying specific epithelial morphogenetic events within the hypodermis.

The hypodermis is born as a set of 80 cells in the dorsal region of the embryo, and is derived from two founder cells within the early embryo known as AB and C (Sulston et al., 1983; Figure 2). Soon after birth the hypodermal cells form adherens junctions with their neighbours to form an epithelial sheet of six rows of cells. Shortly thereafter the hypodermal cells undergo changes in shape and position. The two dorsal rows of cells undergo cell rearrangement via intercalation to form a single row of cells straddling the dorsal midline of the embryo in a movement known as dorsal intercalation, as we have described elsewhere (Williams-Masson et al., 1998). After dorsal intercalation is under way, the hypodermis begins to spread ventrally in a process known as ventral enclosure, shown schematically in Figure 3. We have shown that ventral enclosure of the embryo by the hypodermis proceeds in two steps in wild-type embryos (Williams-Masson et al., 1997). Four anterior hypodermal cells, termed leading cells, initiate the movement of the hypodermis to the ventral midline. As the leading cells approach the ventral midline, the remaining unenclosed region of the ventral hypodermis, termed the ventral pocket, closes. As it does so, the ventral margins of the cells lining the pocket narrow markedly, shortening their apical contacts with the pocket. The entire process can be visualized with Nomarski optics, as well as via immunostaining using the MH27 antibody, which recognizes a component of adherens junctions known as JAM-1 (junction-associated molecule-1; Figure 4). JAM-1 localizes to adherens junctions; the functional role of the JAM-1 protein is the subject of other work in our laboratory, and will not be explored here. Once enclosure is complete, embryos mounted on agar pads turn on their side and begin to elongate (Figure 4).

The morphogenesis of the hypodermis has several distinct advantages for uniting a cellular and molecular analysis of the spreading and movement of epithelial sheets. First, the number of cells participating in the morphogenetic movements within the hypodermis is small. For example, in the case of ventral enclosure, roughly 20 cells are involved, and each cell can be unambiguously identified and followed during morphogenesis. Second, hypodermal morphogenesis is highly stereotyped; the same cells engage in the same changes in position

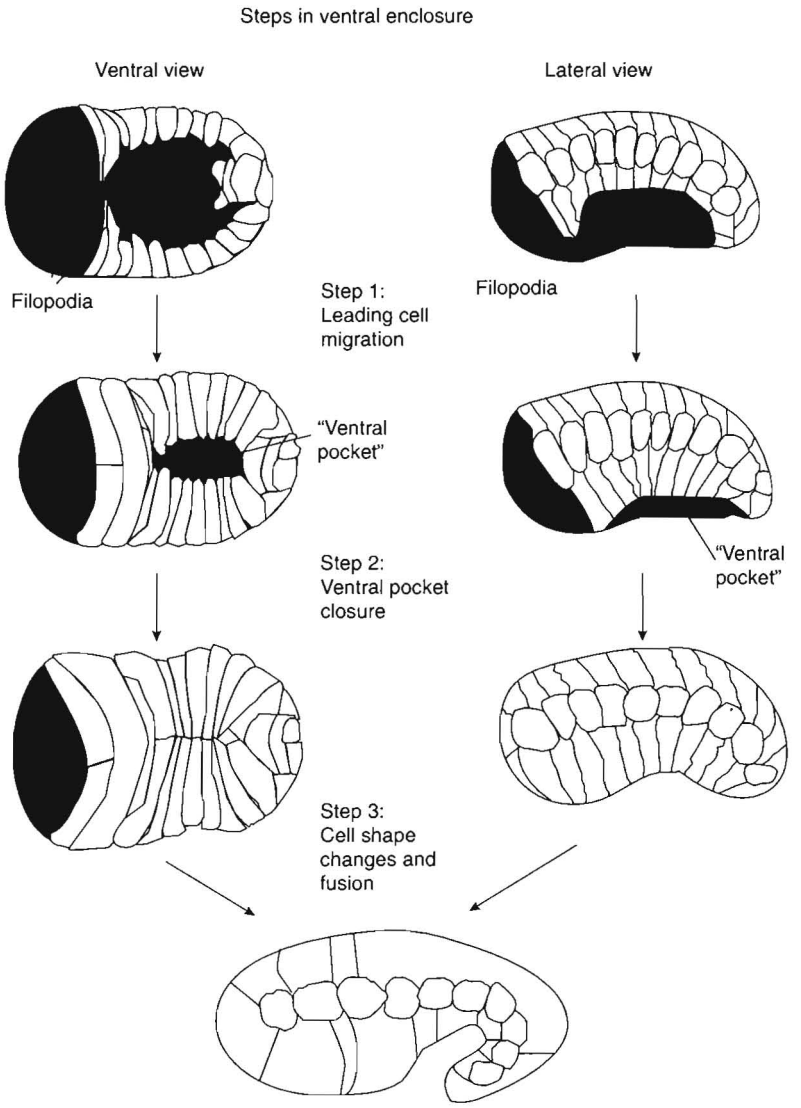
Figure 2



Cell lineages contributing to the hypodermis in *C. elegans*

(A) Schematic representation of the early C. elegans embryo. Blastomeres that contribute to the hypodermis are shown in dark grey (those that produce many hypodermal cells), or light grey (those that contribute few cells to the hypodermis). (B) Schematic representation of the embryo near the completion of ventral enclosure, traced from a scanning electron micrograph (Priess and Hirsh, 1986). The three major types of hypodermal cells are shown using different stippling patterns. Lateral view – the dorsal hypodermal cells have intercalated into a single row at this stage. (C) Schematic representation of the embryonic cell lineage, adapted from Sulston et al. (1983). Descendants of the AB and C blastomeres contribute to the hypodermis. The boxes at the bottom of the lineage diagram represent the three types of hypodermal cells shown in (B). The presence of a box means that the indicated branch of the lineage produces at least one of that type of hypodermal cell; it is not meant to indicate quantities of such cells produced by each branch of the lineage.

Figure 3

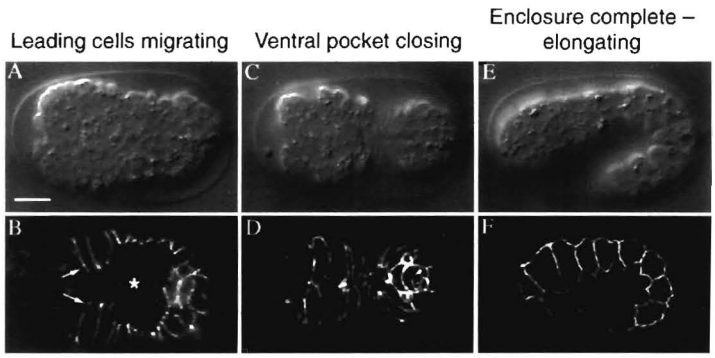


Steps in ventral enclosure of the hypodermis in the *C. elegans* embryo, shown schematically in ventral up (left) and left lateral (right) views

Anterior is to the left in all cases.

and shape from embryo to embryo, allowing experimental and genetic perturbations of embryos to be interpreted with a high degree of precision. Third, the hypodermis is optically transparent, making it convenient for *in vivo* analysis using Nomarski microscopy, green fluorescent protein (GFP) reporters, and multiphoton excitation microscopy (see below).

Figure 4



Stages in ventral enclosure of the hypodermis in *C. elegans*

(A)–(D) are ventral up, with anterior to the left; (E) and (F) are lateral views, anterior to the left. Top row, Nomarski optics. Bottom row, immunostaining using the monoclonal antibody MH27, which recognizes JAM-1 a component of the adherens junction. Leading cells initiate enclosure (A, B; arrows); the open ventral pocket is visible (B, asterisk). The ventral pocket then closes (C and D), and the embryo turns onto its side and begins to elongate (E and F). Bar = 10 μm.

The hypodermis is particularly well suited to studying three types of epithelial morphogenetic movements. Firstly, dorsal intercalation is a modified example of a type of directed cell rearrangement known as convergent extension (see Williams-Masson et al., 1998, for a more detailed investigation of the cellular mechanisms of dorsal intercalation). Convergent extension occurs in many animal embryos when epithelial tissues must undergo dramatic lengthening (for reviews, see Keller and Hardin, 1987; Fristrom, 1988). Secondly, ventral enclosure involves the spreading, or epiboly, of an epithelial monolayer, another common event in early animal embryos (for a review of relevant examples, see Williams-Masson et al., 1997). Third, the meeting of the free edges of the hypodermis at the ventral midline is an example of the sealing of an epithelial sheet, an event that occurs in many types of embryonic tissues, such as the epidermis during vertebrate neurulation (Schoenwolf, 1991), the palatal shelves in amniotes (Ferguson, 1988), and the dorsal epidermis of *Drosophila* (reviewed in Knust, 1997). In the rest of this chapter, we will focus on the mechanisms underlying ventral enclosure of the hypodermis.

Integrated four-dimensional microscopy and multiphoton excitation microscopy: essential tools for analysing hypodermal morphogenesis

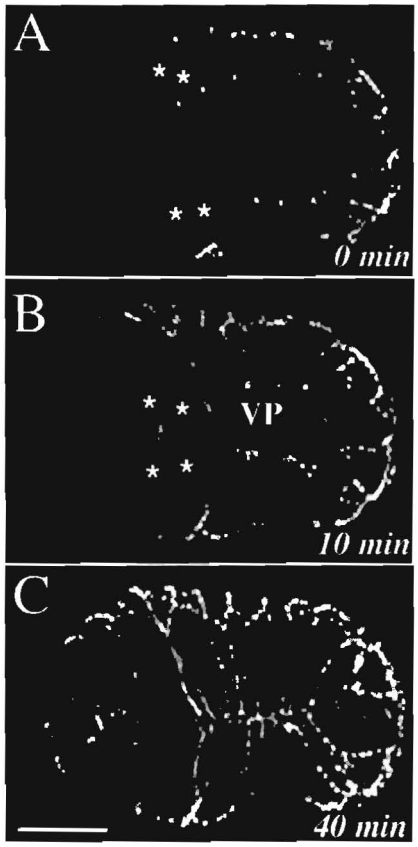
Although the hypodermis is simply organized, the processes of dorsal intercalation and ventral enclosure nevertheless occur in three dimensions over time. The cells in the hypodermis rapidly change position, making analysis of their movements challenging. In order to document and analyse the movements of the hypodermis, we have constructed a digital 'four-dimensional' (4D) Nomarski

microscopy system. Acquired images are compressed and compiled into a QuickTime® movie and played back using custom software written in our laboratory (see George et al., 1998; William-Masson et al., 1997, 1998, for examples of the use of this system in *C. elegans*). In addition, we have added a laser ablation system to the same apparatus, permitting immediate 4D recording of embryos following experimental perturbation. Since the time scale of some of the phenomena we examine is of the order of 30 s, such an integrated system provides a number of advantages over separate systems for ablation and imaging.

In order to assess embryonic phenotypes quickly, it is also advantageous to assess the pattern and position of epithelial cells within the hypodermis without resorting to immunostaining or other techniques requiring processing of fixed specimens. This is particularly true for movements such as ventral enclosure; since enclosure failure results in catastrophic defects in hypodermal morphogenesis, examination of terminal phenotypes can be difficult and misleading, or at least less informative than being able to analyse the defects as they occur *in vivo*. In order to analyse junctional dynamics in living embryos, in collaboration with Anthony Radice (New York Blood Center) we have cloned the gene encoding the JAM-1 protein, and generated translational fusions of the JAM-1 coding region and a mutant variant of the GFP (for details regarding the construct, see Mohler et al., 1998). Embryos carrying integrated copies of the JAM-1-GFP reporter show remarkably bright, stable expression of GFP-tagged protein in precisely the same location as JAM-1 protein recognized via immunostaining (Figure 5). Using strains carrying integrated *jam-1::gfp*, we can rapidly screen for epithelial defects in living embryos identified as homozygous mutants with defective morphogenesis in our mutant screens (see below). For example, we can observe rearrangements, disappearance, and reappearance of adherens-junctional connections between epithelial cells during morphogenetic movements *in vivo* (Mohler et al., 1998). In addition, since some of the defects we have characterized result in ruptures of the embryo (see below), *in vivo* analysis of adherens-junction defects allows us to pinpoint precisely where aberrant junctional connections occur before the catastrophic failure of morphogenesis typical in enclosure-defective mutants.

In order to make effective use of the JAM-1-GFP marker, we have used multiphoton excitation laser scanning microscopy to generate projected, three-dimensional reconstructions of the adherens junctional patterns of living embryos (Figure 5). A major problem with using standard laser scanning confocal microscopy for imaging JAM-1-GFP is that prolonged imaging of embryos results in phototoxicity and inevitable injury to the embryos. These problems can be minimized using two-photon excitation microscopy (Denk and Svoboda, 1997; Potter, 1996). For the mutant variants of GFP most commonly used in our laboratory, S65T and the triple mutant S65A/V68L/S72A, incident light with a wavelength of 900 nm is delivered to the specimen via a high-powered pulsed laser at the focal plane of the objective lens. No excitation of the fluorochrome occurs at this wavelength, and hence no bleaching will occur in the bulk of the sample, eliminating unnecessary phototoxicity. Since fluorochrome excitation only occurs near the plane of focus, images are obtained that are comparable in quality to those obtained using conventional confocal microscopy.

Figure 5



Visualization of adherens junctions in a living *C. elegans* embryo during hypodermal enclosure using multiphoton excitation scanning microscopy

The embryo carries an integrated array containing a translational fusion of the jam-1 gene and a mutated GFP (see Mohler et al., 1998, for details). All views are ventral, with anterior to the left. (A) Early enclosure; asterisks indicate the leading cells. (B) Mid-enclosure. The leading cells have met at the ventral midline. The ventral pocket is clearly visible. (C) Post enclosure. Bar = 10 μm.

Ventral enclosure requires the activity of two populations of cells

Using 4D techniques and phalloidin staining, we have analysed the cellular basis of hypodermal enclosure in wild-type embryos (Williams-Masson et al., 1997). The initial migration of the hypodermis is led by the quartet of leading cells, which exhibit actin-rich filopodia at their medial tips (Figure 6). As the quartet of leading cells approaches the ventral midline, the remainder of the leading edge of the hypodermis becomes visible as a group of cells lining the unenclosed ventral pocket. The pocket cells exhibit a localization of actin microfilaments along their free edges, although the density of actin staining varies greatly from embryo to

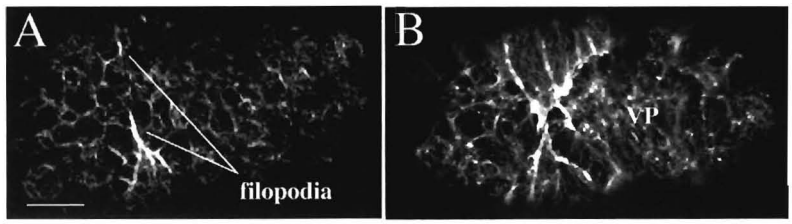


Figure 6

Confocal images of embryos fixed during ventral enclosure and stained with rhodamine phalloidin to visualize actin microfilaments

Ventral views; anterior is to the left. (A) An embryo fixed during early enclosure as the leading cells extend filopodia toward the ventral midline. A filopodium is visible at the tip of each leading cell. (B) An embryo fixed as the leading cells made contact at the ventral midline. The ventral pocket is clearly visible. Bar = 10 μm.

embryo relative to general cortical staining (Figure 6; Williams-Masson et al., 1997). The completion of ventral enclosure requires that contralateral pairs of cells establish junctional connections along the ventral midline. The leading cells are the first cells to establish such junctional connections, followed by the pocket cells (Figure 5C).

The two populations of ventral hypodermal cells — the leading cells and the pocket cells — appear to engage in distinct morphogenetic behaviours during enclosure, based on laser ablation studies. Disabling various ventral hypodermal cells by irradiating them with a laser microbeam during enclosure has reproducible and specific effects on enclosure (Williams-Masson et al., 1997). When leading cells are bilaterally inactivated just after they have crested the equator of the embryo, enclosure fails, and the hypodermis retracts dorsally. If leading cells are inactivated unilaterally, the corresponding unirradiated, contralateral leading cells can migrate past the midline to form junctional connections with its inactivated partner. When the leading cells are inactivated later, when the margin of the hypodermis has progressed significantly past the equator of the embryo, little immediate effect on enclosure is observed. However, the irradiation impedes the ability of leading cells to make permanent connections along the ventral midline, resulting in rupture or 'oozing' of internal cells through breaches in the hypodermis, presumably due to internal pressures generated during elongation. When three or more contiguous pocket cells are ablated during this later period, a rapid, bilateral retraction of the entire ventral margin of the hypodermis occurs, suggesting that the hypodermis is under tension during enclosure (Williams-Masson et al., 1997).

Based upon these experimental results and the pattern of F-actin observed via phalloidin staining, we proposed that enclosure requires the successive activities of two groups of cells within the ventral hypodermis (Williams-Masson et al., 1997): (i) filopodial migration of the leading cells, which serves to pull the hypodermis past the equator of the embryo onto the ventral surface of the embryo, and (ii) constriction of the pocket cells. Once the first

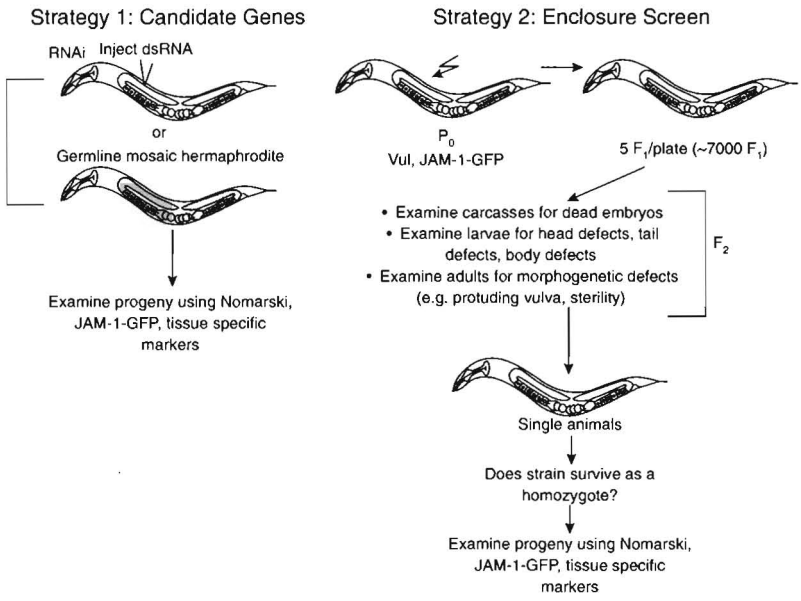
phase has occurred, our laser ablation results suggest that the leading cells are largely dispensable for the further progression of enclosure, with the exception that they must make successful junctional connections at the ventral midline to prevent the rupture of the embryo when it begins to elongate into a vermiform shape.

Ventral enclosure bears some similarities to the process of dorsal closure of the epidermis in *Drosophila*. During dorsal closure, non-muscle myosin localizes to the dorsal margins of the epidermis and is required for the closure process. Dorsal closure is presumed to occur via the constriction of an actomyosin 'purse string' which spans the dorsal margins of epidermal cells at the free edges of the pocket (Young et al., 1993). More recently, components of the signal transduction pathways that trigger dorsal closure have been identified (see Knust, 1997; Noselli, 1998 for recent reviews). By analogy, such a purse string model could account for ventral enclosure of the hypodermis in *C. elegans*. In this model, a ventral actomyosin ring spanning the cells of the pocket and localized at their free ventral edges would contract, pulling together the edges of the hypodermal sheet at the ventral midline. Although such a model is consistent with our analysis of wild-type embryos, it awaits confirmation via genetic analysis of enclosure-defective mutants. There is currently no molecular evidence for a role for a non-muscle myosin or signal transduction molecules similar to those required for dorsal closure in *Drosophila* during ventral pocket closure.

Strategies for identifying genes required for ventral enclosure

An analysis of wild-type enclosure is both necessary and valuable for a thorough understanding of the mechanisms of enclosure. However, such an analysis alone would fall short of the potential of *C. elegans* for providing insight at the molecular level regarding the enclosure process. We have used two major strategies for identifying genes required for various aspects of the process of ventral enclosure (Figure 7). The first strategy involves examining the role of candidate genes in ventral enclosure. Such a strategy is particularly effective in *C. elegans*, since the genome has been sequenced (*C. elegans* Sequencing Consortium, 1998), and the correspondence between the physical and genetic maps is excellent. Requirements for candidate genes can be assessed by one of two techniques. Firstly, rapid assessment of candidate genes is possible using the technique of RNA-mediated inteference, or RNAi (Guo and Kemphues, 1995; Fire et al., 1998). In RNAi, single- or double-stranded RNA specific to the transcript of choice is injected into L4 larvae or young hermaphrodites. The result is specific depletion of both maternal and zygotic pools of mRNA corresponding to the gene of interest. In addition to RNAi, which removes both maternal and zygotic mRNA, such genes can be tested by the generation of germline mosaic hermaphrodites. In this technique, germline mosaic animals are identified from a homozygous mutant (null) strain carrying a wildtype transgene on an extrachromosomal array. Animals that produce exclusively non-viable progeny are assumed to have spontaneously lost the array from the lineages that produce the germline. In some cases standard zygotic loss-of-function mutants for the locus in question

Figure 7



Strategies for identifying molecules that are required for ventral enclosure

Left panel: candidate gene strategies include RNA-mediated interference (RNAi) and examination of offspring from germline mosaic animals. Not shown are techniques for generating deletions in specific genes of interest by deleting screening or transposable element insertion (Plasterk, 1995; Dernburg et al., 1998). For descriptions of the techniques, see the text. Right panel: forward genetic screening. An F_2 screen for enclosure-defective mutants is performed in a vulvaless (Vul) genetic background (lin-7) that carries an integrated JAM-1-GFP reporter (jcIs1), allowing for rapid identification of animals that produce non-viable offspring. Dead embryos are examined via Nomarski microscopy and JAM-1-GFP expression for enclosure defects. Potential enclosure-defective mutants are also assayed for their penetrance and their ability to be propagated as homozygotes.

may not exhibit enclosure phenotypes, but a role for the gene can be uncovered by removing maternally provided mRNA in this way. This is true for the HMP-1 α-catenin and the HMP-2 β-catenin (Costa et al., 1998; see next section). In other cases, deprivation of maternal mRNA results in an arrest phenotype well before the onset of ventral enclosure. This does not mean that the gene plays no role during ventral enclosure, but merely that the gene is required for developmental events prior to enclosure. Such is the case for the ZEN-4 kinesin-related protein (Raich et al., 1998; see below). Another promising reverse-genetic technique, not used in our laboratory, is PCR-based screening for deletions or transposable element insertions into the gene of interest (Plasterk, 1995; Jansen et al., 1997).

The second major strategy for identifying genes involved in ventral enclosure is forward genetics. Several types of mutations have emerged from screens for enclosure-defective mutants. Firstly, preliminary evidence suggests that at least some maternal effect genes may be required for ventral enclosure (J.S. Simske, unpublished work). Secondly, we and others have identified zygotic lethal

mutations that invariably result in the death of the embryo due to enclosure failure, or perhaps failure of the next major morphogenetic event, the elongation of the embryo, due to prior abnormalities in the enclosure process. Such completely penetrant mutations have been dubbed *zen* (zygotic lethal, enclosure defective; Ferguson et al., 1996). The *zen* mutations represent a major class of mutations that affect ventral enclosure. However, there are many reasons to expect that not all loci affecting a process as complex as morphogenesis will be completely penetrant. Thus a third class of incompletely penetrant, enclosure-defective mutants may exist. For example, if partially redundant processes operate during enclosure, genetic ablation of only one of these processes might not be expected to disrupt enclosure with complete efficiency. Instead, the reliability of enclosure might be reduced, resulting in a reproducible percentage of enclosure failures among homozygotes, but with other homozygotes proceeding to other, later stages of development. In some cases, such mutants might not behave as strict zygotic (or larval) lethals, i.e. a small percentage of homozygotes might be viable and achieve reproductive maturity.

That such incompletely penetrant mutants can be isolated was originally suggested by one of the earliest classes of mutants characterized in *C. elegans*, in which homozygotes are 'variably abnormal', or *vab*. For example, mutations in *vab-1*, which encodes a member of the Eph family of receptor protein tyrosine kinases (Eph RPTKs; George et al., 1997), and *vab-2*, which encodes a member of the Ephrin family of Eph RPTK ligands (Chin-Sang et al., 1999), cause variable defects in hypodermal morphogenesis, including viable adult homozygotes with notched heads and other hypodermal defects. We and our collaborators have shown that a subpopulation of homozygous *vab-1* embryos show terminal enclosure defects (George et al., 1998). However, current evidence does not favour a direct role for VAB-1 and VAB-2 during enclosure. Both VAB-1 and VAB-2 proteins are expressed strongly by neuroblasts, which underlie the ventral hypodermis during enclosure, suggesting that VAB-1 and VAB-2 expression in the hypodermis is not required for enclosure. In addition, enclosure defects in *vab-1 (e2027)* homozygotes are strongly correlated with the persistence of the ventral gastrulation cleft, a transient opening between neuroblasts along the ventral midline that normally closes well before ventral enclosure begins. Taken together, these results suggest that the enclosure defects in *vab-1* homozygotes may be due to improper adhesion between neuroblasts, resulting in steric interference with the migration of ventral hypodermal cells. Our analysis suggests that the interpretation of mutant phenotypes that involve morphogenetic defects must be made with caution, since terminal failures in morphogenesis may result indirectly from defects in prior developmental events. However, there is no reason *a priori* to think that incompletely penetrant mutations cannot be identified that play a more direct role in the enclosure process.

In order to identify other molecules that are more directly required for ventral enclosure, we have conducted an F_2 screen for enclosure-defective mutants. Briefly, mutagenized P_0 worms carrying the integrated JAM-1-GFP translational fusion are scored for F_1 progeny which produce dead embryos, and which may produce larvae with body shape defects or adult progeny with various types of potential morphogenetic defects (Figure 7). The sibling F_2 organisms are

then screened to confirm that heterozygotes carry a mutant allele which results in highly penetrant enclosure defects in homozygotes. Promising candidates are then examined using 4D Nomarski microscopy, JAM-1-GFP fluorescence, and by assaying for tissue-specific markers of differentiation. By a simple extension of this screen, F_2s could be screened for F_3s defective in enclosure to identify maternally acting genes.

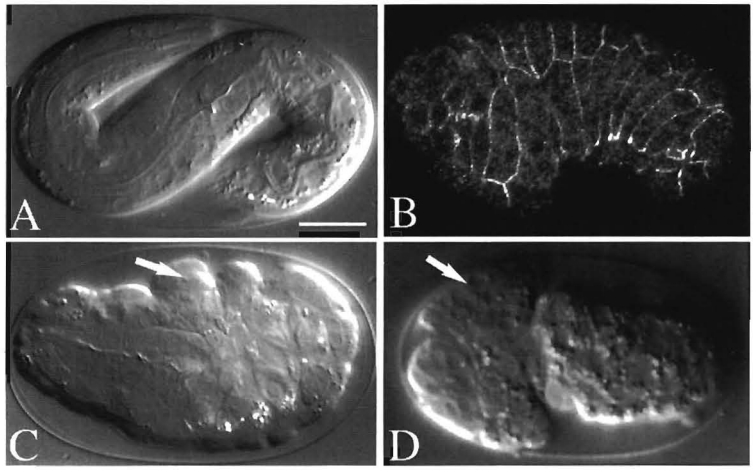
Our laboratory has used a combination of the techniques described in the preceding paragraphs to identify molecules that are required for the successful completion of ventral enclosure. In the following sections, we describe several classes of mutations known to affect ventral enclosure that we have analysed using RNAi, germline mosaics, or forward genetic screens. These studies have provided new insights into the molecular basis of epithelial morphogenesis at the level of single cells.

The cadherin adhesion system is required for specific events during ventral enclosure

One important class of molecules that are involved in epithelial morphogenesis are the cadherins, the well-known class of Ca^{2+}-dependent transmembrane proteins required for epithelial cell adhesion, and the proteins which link them to the actin-based cytoskeleton, α- and β-catenin. In particular, since morphogenetic movements require transmission of forces between the cell surface and the actin-based cytoskeleton, α-catenin, which is thought to link actin microfilaments to the cytoplasmic domain of cadherins via β-catenin/armadillo (reviewed in Huber et al., 1996; Marrs and Nelson, 1996; Barth et al., 1997; Ben-Ze'ev and Geiger, 1998; Adams and Nelson, 1998), would be expected to serve important functions in this regard. We and our collaborators have identified *C. elegans* homologues of E-cadherin, α-catenin, and β-catenin, which are encoded by the genes *hmr-1*, *hmp-1*, and *hmp-2*, respectively. Surprisingly, complete removal of any or all of these proteins from the early embryo via RNAi does not result in general loss of adhesion between blastomeres in the early embryo, in contrast to the situation in *Xenopus* or mammalian embryos (Heasman et al., 1994; Kofron et al., 1997; Ohsugi et al., 1997; Torres et al., 1997). Instead, very specific defects occur during ventral enclosure (Raich et al., 1999). Thus *C. elegans* may be a valuable system in which to analyse the specific requirements for cadherin-mediated adhesion in a morphogenetic event.

In *hmp-1* homozygotes, the normal number of hypodermal cells form, but the embryos arrest at or prior to the initial stages of elongation, developing a 'humpback' (hmp) morphology, characterized by dorsal humps or bulges in the hypodermis (Figure 8). In wild-type hypodermal cells, actin filament bundles form at the start of elongation and appear to contract to generate the force that elongates the embryo. These bundles are orientated circumferentially around the embryo and are linked to adherens junctions between hypodermal cells (Priess and Hirsh, 1986). In *hmp-1* mutants, circumferential bundles are present, but are detached from the adherens junctions in the dorsal hypodermis, suggesting that the failure to elongate results from the failure to couple the actin contraction to

Figure 8



The role of HMP-1 α-catenin during embryonic morphogenesis

*Anterior is to the left, ventral at the bottom in all panels. (A) Nomarski photomicrograph of a fully elongated wild-type embryo. (B) Immunostaining for HMP-1 protein in a wild-type embryo in the latter stages of enclosure. HMP-1 accumulates at sites of cell–cell contact. (C) Terminal stage hmp-1 mutant embryo with abnormal dorsal bulges (arrow). (D) Embryo from a hmp-1 germline mosaic animal displaying the Hmr phenotype; the posterior of the embryo has enclosed in hypodermis and constricted in circumference while the anterior has not. The anterior neuronal and pharyngeal cells have been extruded to the exterior (arrow). (A)–(C) reproduced from The Journal of Cell Biology, 1998, **141**, 297–308 by copyright permission of The Rockefeller University Press.*

cadherin-based adhesion between hypodermal cells (Costa et al., 1998). *hmp-2* embryos show similar, though somewhat less penetrant, defects during elongation.

In embryos in which both maternal and zygotic pools of functional *hmp-1* mRNA have been removed, either using RNAi or in embryos derived from *hmp-1* germline mosaic mothers, more severe defects during hypodermal morphogenesis are observed. Although enclosure begins normally and ventral hypodermal cells migrate past the equator of the embryo, the leading cells fail to form stable connections at the ventral midline, eventually retracting dorsally (Figure 8). Because enclosure fails in such embryos, the embryo subsequently explodes, spilling its internal contents. As a consequence of retraction of the anterior hypodermis, the rupture occurs preferentially in this region of the embryo. The result is a terminal embryo that vaguely resembles a hammerhead shark, a phenotype known as Hmr. Embryos homozygous for mutations in the gene encoding the HMR-1 cadherin often have an Hmr phenotype similar to offspring from *hmp-1* germline mosaic mothers (Costa et al., 1998). In other cases, the entire hypodermis retracts dorsally, forming a small cap of material on the dorsal side of the embryo (Figure 9). Immunostaining indicates that the HMP-1 α-catenin fails to localize to junctional complexes in both *hmp-2*- and *hmr-1*-

deficient embryos, suggesting that, as in other animals, these proteins form a multiprotein complex (Costa et al., 1998).

Based on these data, how might the cadherin/catenin complex function during ventral enclosure? The primary defect in *hmr-1* homozygotes indicates that cadherin is specifically required for the stabilization of contacts between the tips of the leading cells at the ventral midline. In the absence of the cadherin/catenin complex, these contacts are lost, resulting in retraction of the leading cells, which we presume experience greater mechanical stress than the other cells at the ventral margin of the hypodermis. If this model is correct, then recruitment of cadherin/catenin complexes to the nascent ventral midline junctions might be expected to be one of the earliest events following the contact of leading cell filopodia from the opposite sides of the embryo. Recently, we have used a HMP-1-GFP translational fusion capable of rescuing *hmp-1* homozygotes to analyse the redistribution of α-catenin during ventral enclosure *in vivo*, and the results are consistent with this model (Raich et al., 1999).

Completely penetrant mutations that affect ventral enclosure: the zens

Our results with *hmr-1*, *hmp-1*, and *hmp-2* suggest that seeking additional mutants with completely penetrant defects in hypodermal enclosure (i.e. zygotic lethal enclosure defective or *zen* mutants) will be fruitful for uncovering other molecules involved in epithelial morphogenesis and migration in all animals. Among such *zen* alleles, we sought phenotypes that mimicked our laser ablation results. Thus we sought mutants in which homozygotes (i) arrest at the onset of enclosure or early in enclosure with retraction of the hypodermis onto the dorsal

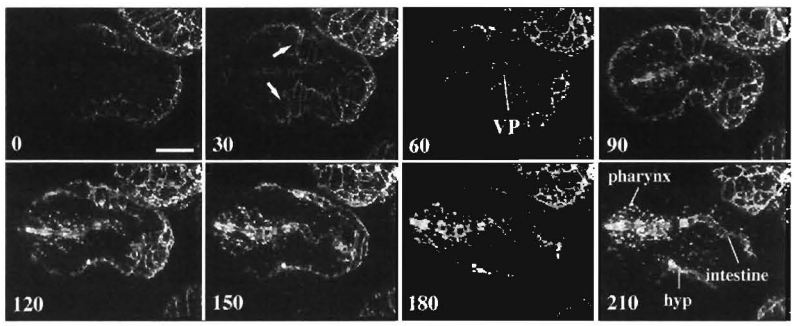


Figure 9

Multiphoton laser scanning microscopy reconstructions of a living *hmr-1* homozygous embryo expressing JAM-1-GFP during ventral enclosure

Ventral view; anterior is to the left. The time in min is shown in each frame. At 30 min, the leading cells (arrows) are retracted away from the ventral midline. At 60 min, the ventral pocket (VP) has closed to its fullest extent. By the end of the sequence (210 min), the hypodermis (hyp) has retracted dorsally, and the pharynx and intestine have been extruded ventrally. Bar = 10 μm.

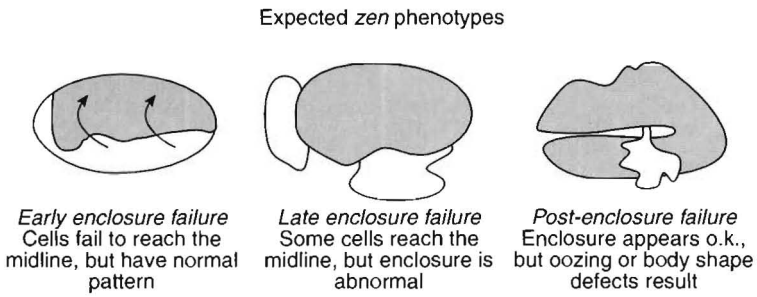
surface of the embryo, (ii) arrest after enclosure is under way, showing defects in either leading cell migration and/or ventral pocket closure with subsequent rupture of the embryo, or (iii) arrest after enclosure appears to occur properly, but rupture or develop body shape defects during elongation due to defects in ventral midline junction formation (Figure 10). Thus far, several enclosure-defective mutants have emerged from this approach. We have established the molecular identity of one of these *zen* mutations, *zen-4*.

The ZEN-4 kinesin-related protein is required for ventral enclosure

zen-4(w35) homozygotes exhibit complete failure of ventral enclosure. Immunostaining with MH27 indicates that the correct number and pattern of hypodermal cells are generated in homozygous *zen-4* embryos. Time-lapse Nomarski movies and MH27 immunostaining of *zen-4* homozygotes demonstrated that the hypodermal cells begin to migrate ventrally, but they cease their migration prior to reaching the ventral midline (Figure 11). As a consequence of the failure in enclosure, elongation of *zen-4* embryos is blocked.

Subsequent mapping and molecular characterization identified the *zen-4* gene. *zen-4* shares the highest identity (31% identity at the amino acid level) with the CHO1 antigen, a kinesin-related protein which has been demonstrated to bundle microtubules *in vitro*, and has been implicated in spindle elongation during anaphase B and cytokinesis in other organisms (Nislow et al., 1992; Kuriyama et al., 1994; Adams et al., 1998). In the *w35* allele, a 5 bp insertion occurs in exon 1 that is predicted to produce a frame shift and a premature stop codon, resulting in a truncated protein of 91 amino acids. The predicted product is truncated upstream of the putative microtubule and nucleotide binding sites, and thus appears to be non-functional (Raich et al., 1998).

Figure 10



Expected phenotypes of fully penetrant zygotic lethal, enclosure-defective mutations (zens)

Sites of rupture are merely suggestive; some mutants may show highly region-specific ruptures, while others may not.

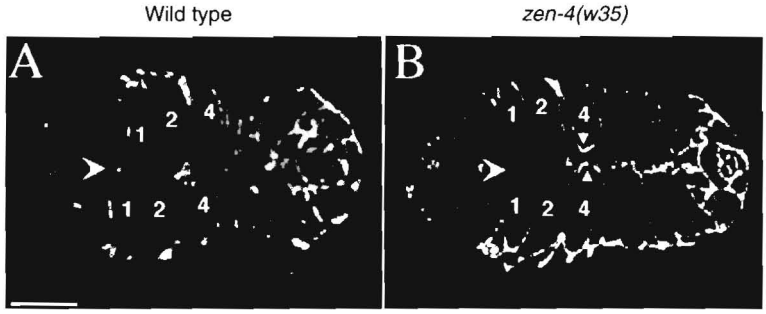


Figure 11

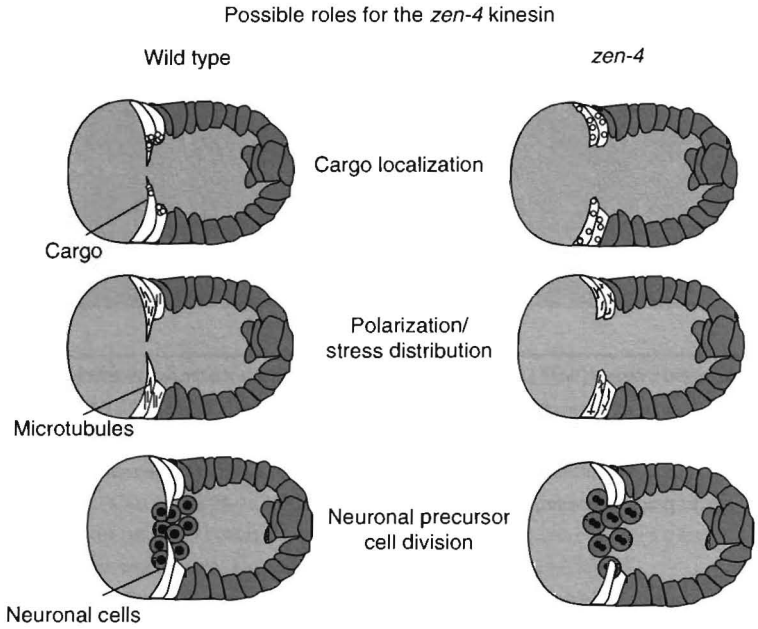
Homozygous *zen-4(w35)* animals initiate but fail to complete enclosure

Confocal sections of wild-type (A) and zen-4 (B) embryos immunostained with MH27. White arrowheads indicate the ventral midline. Note that contralateral pairs of ventral hypodermal cells (labelled 1, 2, 4) have met at the ventral midline in the wild-type animal but have retracted in the zen-4 embryo. Dorsal morphogenesis proceeds normally in zen-4 animals (not shown). Bar = 10 μm.

Using RNAi and germline mosaics, we demonstrated that ZEN-4 is required maternally for the completion of cytokinesis in early blastomeres in *C. elegans* (Raich et al., 1998). Anti-ZEN-4 antibodies brightly stain spindle midbodies, as has been reported for other CHO1/MKLP1 family members in other organisms (Raich et al., 1998). These apparent results indicate that ZEN-4 has functions in mitotic cells, in addition to its function in post-mitotic cells during ventral enclosure.

How might ZEN-4 function during ventral enclosure? Although at this point we can only conjecture, there are several possibilities (Figure 12). First, ZEN-4 may be required to distribute microtubules in the correct orientation or at the correct subcellular sites within ventral hypodermal cells. Since we observe defects in leading cell migration in *zen-4* homozygotes, one possibility is that ZEN-4 is required for rearrangement of the microtubule lattice within leading cells, perhaps to allow them to extend polarized filopodial protrusions. Such a role for microtubules has been suggested in cultured cells, where overexpression of truncated forms of CHO1 results in ectopic formation of dendritic processes (Sharp et al., 1996) and depletion of CHO1 results in defects in dendritic process formation (Yu et al., 1997). Alternatively, ZEN-4 may be required to translocate 'cargo' along microtubules to the ventral edge of migrating hypodermal cells. Such cargo might be required for the assembly of cytoskeletal specializations required for the production or maintenance of protrusions. Although cargo proteins have not been identified for the CHO1/MKLP1 kinesins, such a model would predict that ZEN-4 protein would show preferential localization near the ventral tips of migrating hypodermal cells. A third possibility is that ZEN-4 is required for the divisions of the underlying neuronal cells, and that perturbation of their divisions results in a defective substratum for leading cell migration. We are currently testing these alternatives.

Figure 12



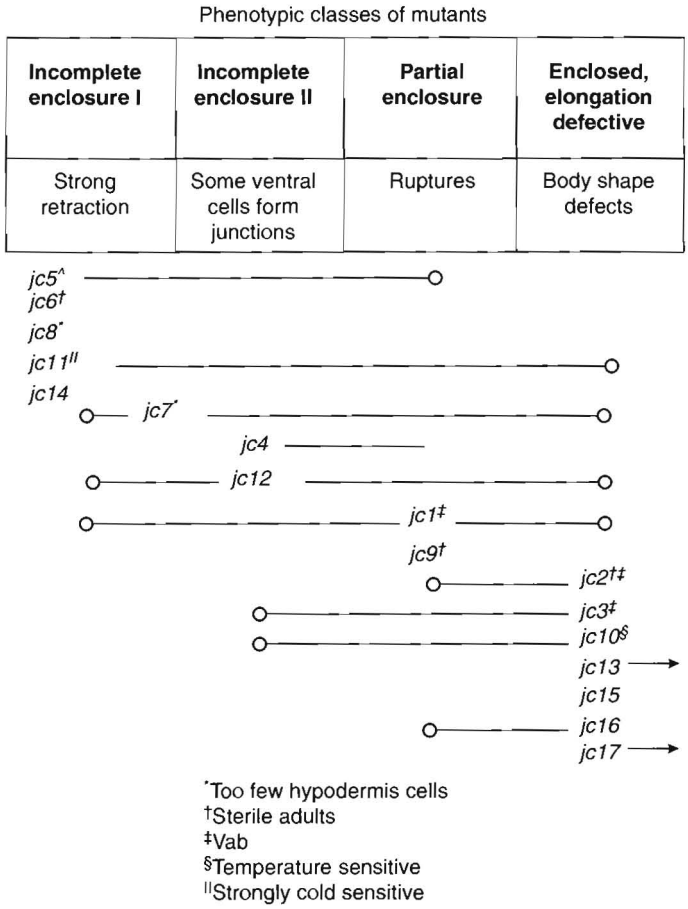
Models for ZEN-4 kinesin function during ventral enclosure

The possible roles for ZEN-4 listed here are not meant to be exhaustive, nor are they mutually exclusive. In the cargo model, ZEN-4 is envisioned as a motor protein shuttling components to the leading edge of migrating ventral hypodermal cells (white dots). In the microtubule polarity/organization model, ZEN-4 is involved in organizing, possibly in an antiparallel fashion, microtubules within leading cells required for their normal function. In the neuronal substrate model, failure of appropriate cell divisions within the underlying neuronal precursor cells results in a compromised substrate for leading cell migration.

Screening for additional mutations that affect ventral enclosure

We have continued to screen for additional mutations that affect ventral enclosure, using the F_2 lethal screening strategy outlined in Figure 7, including alleles that display incompletely penetrant defects in ventral enclosure. Thus far we have examined ~7000 mutagenized haploid genomes, and we have isolated several alleles that display enclosure defects, based on Nomarski microscopy and examination of JAM-1-GFP patterns. A summary of the range of enclosure and other defects observed for the candidate alleles we have isolated thus far is shown in Figure 13. Included in Figure 13 are alleles for which there are no observed enclosure failures, but in which the subsequent defects in body shape suggest that enclosure may have been aberrant (e.g., *jc13*, *jc15*, *jc17*). At present, we have not determined how many distinct complementation groups these alleles represent. In some alleles (e.g., *jc1*, *jc5*, *jc7*, *jc11*), a range of defects (and hence terminal arrest phenotypes) are observed among dead embryos; in others (e.g., *jc4*, *jc16*), embryonic arrest occurs within a relatively narrow window during morpho-

Figure 13



Classification of representative mutant alleles with possible enclosure abnormalities based on the range of phenotypic defects observed

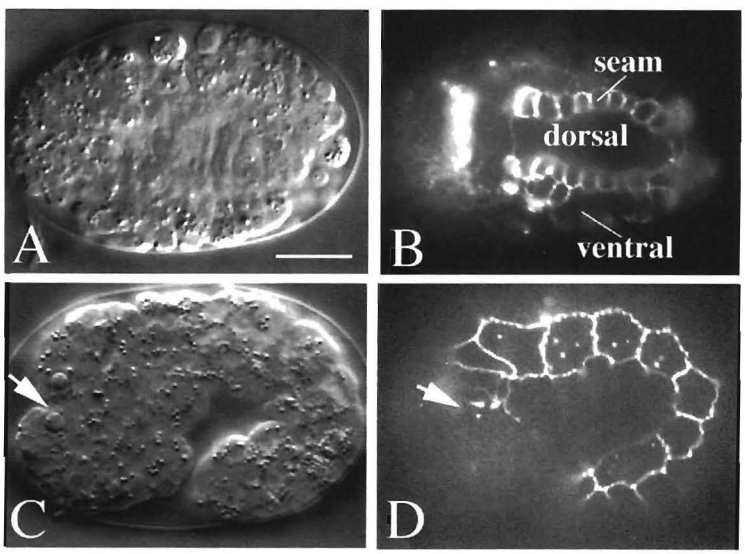
Staging of defects is approximate. Those alleles which only exhibit body shape defects (jc10, jc13, jc15, jc17) may exhibit successful (i.e., the embryo is covered in hypodermis), though somewhat abnormal, ventral enclosure (e.g., the hypodermis is misaligned).

genesis. Several alleles display larval defects in head, tail or body morphology, and can thus be considered to be Vab. Many (but not all) of the alleles can be propagated as homozygotes. Since these enclosure-defective mutants are derived from homozygous mutant adults, the possibility exists that there is a maternal as well as a zygotic contribution to the mutant phenotype. In addition, an appreciable proportion (3 of 17) show mild or strong heat or cold sensitivity (*jc5*, *jc10*, *jc11*). One interpretation of these results is that a morphogenetic process such as ventral enclosure is particularly sensitive to relatively weak or conditional losses of function of some of the proteins required. Another possibility is that several concurrent processes with partial functional redundancy operate during enclosure. Thus either a weak loss of function of a protein required for enclosure

or the loss of a partially redundant process results in stochastic failures among the cells that accomplish enclosure. Furthermore, such stochastic failures could occur at various stages in the enclosure process. The 'reliability' of enclosure would thereby be reduced, leading to the incompletely penetrant defects we have observed. Other interpretations of our results are possible, however. For example, the percentage of mutations which show temperature sensitivity is highly dependent on the process being studied. Some molecular pathways, such as production and assembly of the extracellular matrix, are particularly susceptible to the isolation of temperature-sensitive mutations for reasons that are not immediately apparent (reviewed in Kramer, 1997). In some cases an entire process is itself temperature-sensitive, such as dauer larva formation (reviewed in Johnsen and Baillie, 1997). However, thus far we have not observed any general temperature sensitivity for the process of ventral enclosure.

To illustrate the sorts of enclosure defects we have observed in some of these alleles, Figure 14 shows several types of enclosure and other morphogenetic defects associated with the allele *jc1*. A total of 37% of homozygotes fail to complete ventral enclosure. In some cases (16%) the hypodermis retracts bilaterally onto the dorsal surface of the embryo (Figures 14a and 14b). In the other cases (21%) arrested embryos partially complete enclosure, but rupture in the anterior, posterior, or ventral regions (Figures 14c and 14d). Still others (37%) display elongation defects or late embryonic defects. Finally some *jc1* hatchlings

Figure 14



Enclosure and body shape defects in homozygous *jc1* embryos and larvae

Anterior is to the left in all panels. Bilateral retraction of the hypodermis is visualized by Nomarski microscopy (A) and JAM-1-GFP (dorsal view). The lateral hypodermal cells (seam cells) form two bilateral rows along the anterior–posterior axis (arrows). Head rupture is visualized by Nomarski microscopy (C) and JAM-1-GFP (D). The seam cells are visible on the left side of the embryo (large arrow), but the hypodermis is disorganized in the anterior and fails to enclose the embryo (small arrow). Bar = 10 μm.

display body shape defects of various sorts (Figure 15). Although the molecular identity of the gene mutated in *jc1* is currently unknown, our ability to isolate such mutants suggests that such screens will be useful for identifying additional genes whose functions are required for ventral enclosure. Further genetic characterization of *jc1* and the other alleles listed in Figure 13 is currently under way.

The future: analysing morphogenesis in light of Hörstadius' legacy

Sven Hörstadius sought to understand early development at the level of single cells. His careful attention to experimental analysis and his detailed description of how individual cells contribute to developmental processes showed that such an analysis is possible. Our application of single-cell analysis to morphogenetic movements in *C. elegans*, combined with our ongoing genetic analysis, lies squarely within the tradition Hörstadius began more than half a century ago. The task that lies ahead – a thorough genetic analysis of morphogenetic movements in *C. elegans*, such as ventral enclosure – will undoubtedly be complex. Morphogenesis is an inherently multigenic process; in addition, our work and that of others has established that mutations in single genes have pleiotropic effects on morphogenesis. Nevertheless, by establishing what individual cells normally do in a simply organized embryo such as the *C. elegans* embryo, and then analysing the effects of mutations at the level of single cells, we are beginning to extend our understanding of morphogenesis from the cellular to the molecular level.

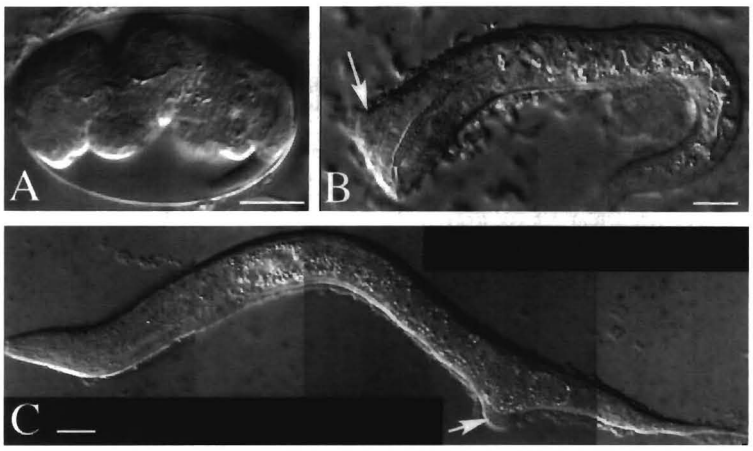


Figure 15

Post-enclosure defects in *jc1* homozygotes

(A) Body shape defects in a jc1 embryo that has successfully completed ventral enclosure. (B) A hatched larva with pronounced head shape defects (arrow). (C) An older larva with a ventral protrusion in the pre-anal region (arrow). Bars = 10 μm.

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