The Zinc Finger Protein DIE-1 is Required for Late Events
during Epithelial Cell Rearrangement in C. elegans

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\textbf{Running Title:} Control of cell rearrangement in \textit{C. elegans}
SUMMARY

The mechanism by which epithelial cells undergo directed rearrangement is central to morphogenesis, yet the regulation of these movements remains poorly understood. We have investigated epithelial cell rearrangement (intercalation) in the dorsal hypodermis, or embryonic epidermis, of the *C. elegans* embryo by analyzing the *die-1* mutant, which fails to undergo normal intercalation. Dorsal hypodermal cells of *die-1(w34)* homozygous embryos initiate but fail to complete the process of intercalation. Despite abnormal intercalation, the subsequent morphogenetic movements that enclose the embryo with epithelial cells and the process of dorsal cell fusion still occur. However, elongation of the embryo into a worm-like shape is disrupted in *die-1* embryos, suggesting that intercalation may be necessary for subsequent elongation of the embryo. Actin filaments are not properly organized within the dorsal hypodermis of *die-1* embryos, consistent with intercalation being a necessary prerequisite for elongation. The *die-1* gene encodes a C2H2 zinc finger protein containing four fingers, which likely acts as a transcriptional regulator. Immunostaining with DIE-1 polyclonal antibodies and expression of a *die-1::gfp* translational fusion protein indicate that DIE-1 is present in the nuclei of hypodermal, muscle, gut, and pharyngeal cells; its distribution suggests that DIE-1 acts in each of these tissues to regulate morphogenetic movements. In addition, Mosaic analysis indicates that DIE-1 acts cell autonomously in the dorsal hypodermis, consistent with this view. Our analysis of *die-1* shows that the dynamic events of epithelial cell rearrangement are under transcriptional control, and shows that early and later phases of epithelial cell rearrangement are genetically distinct.
INTRODUCTION

Cell rearrangements are known to play a critical role during morphogenesis in both vertebrate and invertebrate systems. There are two main cell types that undergo rearrangement: deep (non-epithelial) cells and epithelial cells. Radial intercalation of deep cells occurs during epiboly of the animal cap (Keller, 1980; Warga and Kimmel, 1990) and during convergent extension of presumptive axial mesoderm in Xenopus and zebrafish (Keller and Tibbetts 1989; Wilson and Keller 1991; Warga and Kimmel 1990), and neural tissue in Xenopus (Ellul et al., 2000). Similarly, in Drosophila, terminal filament formation involves rearrangement of mesenchymal cells (Godt and Laski 1995). Several molecular pathways have been implicated in deep cell rearrangement. The transcription factor BRIC-A-BRAC, a BTB domain transcription factor, is necessary for terminal filament morphogenesis in Drosophila ovaries (Godt and Laski 1995). Likewise, spadetail, which encodes a T-box transcription factor, is required for convergence movements of ventrolateral deep cells toward the dorsal side of the embryo in zebrafish (Griffin et al. 1998). Paraxial protocadherin (papc) family members also appear to be required in subsets of deep mesodermal cells for proper dorsal convergence movements (Kim et al., 1998; Yamamoto et al. 1998). papc has been implicated as a proximal downstream target of spadetail, suggesting that spadetail may regulate expression of genes directly involved in cell rearrangement. Recently, it has been shown that Wnt-11 and disheveled family members mediate planar polarity signals in both zebrafish (Heisenberg et al., 2000) and Xenopus (Tada and Smith, 2000; Wallingford et al., 2000) that are required for proper convergence of paraxial mesoderm towards the dorsal midline.

In contrast, less is known about epithelial cell rearrangement, despite its widespread occurrence. Examples of epithelial cell rearrangement include movements at the margin of the enveloping layer in teleosts (Keller and Trinkaus, 1987), shaping of the neural plate in avian embryos (Schoenwolf and Alvarez 1989), elongation of the archenteron during sea urchin gastrulation (Ettensohn 1985; Hardin 1989), intercalation of intestinal cells in C. elegans (Leung et al., 1999), and germband extension in Drosophila (Irvine and Wieschaus 1994). In particular, little is known about regulation of cell rearrangement in epithelia. In Drosophila, gap genes appear to be at least indirectly required for coordination of germ band extension movements (Irvine and Wieschaus, 1994), although it is not known at what level they exert their influence.

The embryonic hypodermis, or epidermis, of the nematode Caenorhabditis elegans is a convenient model system for the study of epithelial cell rearrangement, because individual epithelial cells can be unambiguously identified from embryo to embryo. The hypodermis forms as
a patch of 78 cells on the dorsal and lateral surfaces of the posterior region of the embryo. The first morphogenetic movement within this tissue involves the intercalation of twenty cells along the dorsal midline that eventually form the dorsal hypodermis. Specific cell migrations within the future ventral hypodermis begin as the process of intercalation is completing and are necessary for the process of enclosure (Williams-Masson et al. 1997). Enclosure is followed by the elongation of the embryo into a worm-like shape (Priess and Hirsh, 1986). Once enclosure is complete and elongation is underway, many cells of the dorsal and ventral hypodermis fuse into large multinucleate syncytia (Podbilewicz and White 1994; Mohler et al., 1998). The major events of epithelial morphogenesis in C. elegans are summarized in Figure 1.

Previous studies of dorsal intercalation in C. elegans have shown that dorsal hypodermal cells are initially organized in two rows on the dorsal surface of the embryo (Sulston et al. 1983; Williams-Masson et al. 1998). Cells within each dorsal row rearrange by extending wedge-shaped protrusions between cells in the contralateral dorsal row; the cell tips then migrate until they meet the opposing row of lateral hypodermal cells. The end result of these movements is the formation of a single row of twenty dorsal hypodermal cells. Treatment of wild-type embryos with nocodazole or cytochalasin D prevents intercalation (Williams-Masson et al. 1998), suggesting that intercalation is dependent on both actin filaments and microtubules.

In order to determine what role dorsal intercalation plays in the subsequent morphogenesis of the organism and to understand how intercalation movements are regulated at the molecular level, we have undertaken a genetic analysis of the process. In this paper we analyze die-1 (dorsal intercalation and elongation defective), a gene required for cell rearrangement in the dorsal hypodermis of C. elegans. We show that the dorsal hypodermal cells of die-1 mutant embryos initiate, but cannot complete, intercalation. We also show that mutant embryos enclose with hypodermis, but fail to elongate. die-1 encodes a putative zinc finger protein expressed in cells that display abnormal morphogenesis in the mutant. Mosaic analysis indicates that DIE-1 is specifically required in dorsal hypodermal cells for intercalation to occur. Our results indicate that DIE-1 is a key regulator of directed epithelial cell rearrangement in C. elegans.

MATERIALS AND METHODS

C. elegans strains

The Bristol strain N2 was used as wildtype (Brenner 1974). Nematodes were grown at 20°C in all experiments and were cultured as described by Brenner (1974). To map and balance die-1, the
following mutations and deficiencies were obtained from the *C. elegans* Genetic Stock Center: LG I, bli-4(e937); LG II, rol-6(e187); unc-4(e120); let-23(mn23); let-31(mn31); let-242(mn90); let-243(mn226); let-244(mn97); let-268(mn189); vab-9(e1744); mnC1; mnDf12; mnDf14; mnDf28; mnDf61; mnDf62; mnDf63; mnDf66; mnDf68; mnDf71; mnDf83; mnDf89; LG III, vab-7(e1562); LG IV, unc-31(e928); him-8(e1489); LG V, dpy-11(e224) him-5(e1467). wDf5 (linkage group II) was isolated in a screen for zygotic lethal mutations (Ferguson et al., 1996; our unpublished data).

For additional phenotypic analysis die-1, elf-1(oj55), LG II, jclsl (jam-1::gfp), linkage group IV (Mohler et al. 1998), and ojEx3 were used. jclsl is an integrated version of jam-1::gfp, and was crossed into the die-1 background by standard genetic techniques. The elf-1 mutant is zygotic recessive viable, and lacks cell fusion during embryogenesis (W.A.M., unpublished observations). Males heterozygous for die-1 were mated into homozygous elf-1 hermaphrodites, which were also homozygous for jclsl. To generate double mutants, recombinants were identified by singling elf-1 homozygous F2 progeny; subsequent matings were performed to obtain elf-1; jclsl; die-1/+ hermaphrodites. ojEx3 was produced by injection of plasmid pKK1, which carries a GFP transcriptional reporter for the lbp-1 gene (Plenefisch et al., 2000). This reporter is expressed at high levels in dorsal hypodermal cells.

**Genetics**

*w34* was isolated from a general, genome-wide screen for ethylmethane sulfonate (EMS)-induced zygotic embryonic lethal mutations (for methods see Ferguson et al., 1996). *w34* was mapped to linkage group II by standard techniques. Recombination between rol-6 and unc-4 placed die-1 very near or to the right of unc-4. Complementation tests against deletion mutants placed die-1 between egl-43 and daf-19.

**Rescue**

*die-1(w34)* was mapped to linkage group II between egl-43 and daf-19, a 0.2 map unit interval corresponding to ~300 kb of DNA. *die-1(w34)* fails to complement the deficiency mnDf71 and complements deficiencies mnDf61 and mnDf83, as well as the lethal mutations let-242(mn90), let-243(mn226), and let-244(mn97). Cosmids spanning the region between egl-43 and daf-19 were obtained from the *C. elegans* Genome Consortium and were tested for transformation rescue by coinjection with the dominant marker rol-6(su1006) (Mello et al. 1991). One pool of three clones
that rescued die-1 to viability was identified, and subsequently the injection of a 10kb subclone of cosmid K10A2 was shown to be sufficient for rescue.

The cDNA yk83h8 was obtained from the C. elegans cDNA Project (Y. Kohara) because it was the longest cDNA that mapped to the minimal die-1 rescuing genomic fragment. yk83h8 was sequenced using ABI PRISM dye terminator cycle sequencing (Perkin Elmer-Cetus, Norwalk, CT). Genomic sequence from cosmid C18D1, available from the C. elegans Genome Consortium, agrees with our cDNA sequence and indicates that the die-1 locus consists of six exons included in the 10kb rescuing fragment.

The genomic region of the die-1 locus was amplified from homozygous w34 embryos by the single-embryo PCR method (Williams et al. 1992). The following sets of primers were used to amplify the genomic region in three fragments that span the predicted coding sequence: GGTGGAAGATTACAAGGAGGATACGGAC, ATCAGGAGCCAGAAGAACAACGAG; AGTCTATCCCTCTCCTCCTCAC, TCTGCTTCTGATTCTGCTGATG; and TTTTCGGTTCTTTCTTAGATTTCG, GAAAAAAGTAGACCTACAAATGGC. In some cases nested internal primers were used to obtain single amplification products. All PCR products were subcloned into either pT7 vector (Novagen, Madison, WI) or PCR-Script (Stratagene, La Jolla, CA). For each amplification product three subclones of two independent samples were sequenced.

**Phenotypic analysis and live fluorescence microscopy**

Phenotypic analysis and image acquisition of Nomarski images were performed as previously described (Raich et al., 1998), with the following modifications: 15-24 optical sections of live embryos were recorded every 30-60 seconds. To monitor GFP expression using jcIs1 and ojEx3, in living embryos, mounted embryos were filmed using multiphoton laser scanning microscopy (MPLSM). Data acquisition and stereo-4D processing were performed as previously described (Raich et al, 1999). In brief, Bio-Rad PIC format files were imported into NIH using customized Stereo-4D Macros (Mohler and White, 1998). Post image processing was conducted on a Power Macintosh G3. Time-point projection stacks were converted in 4D-Turnaround and 4-D Viewer into appended 4-D QuickTime movies and replayed as stereo-4-D animations using a modified version of NIH Image. All macros, the standard version of NIH Image, and 4-D Turnaround/Viewer are available at http://www.bocklabs.wisc.edu/imr/stero4d/stereo4d.html. The modified version of NIH Image is available from JH.
Antibody production and immunostaining

Rabbit polyclonal antibodies recognizing DIE-1 were raised and affinity purified against the following peptide: ALNLTFKNDGTKKEIEE (Quality Controlled Biochemicals, Hopkinton, MA). A modified version of the freeze-crack method (Miller and Shakes 1995) was used to process embryos for immunostaining essentially as previously described (Raich et al., 1998). Affinity-purified DIE-1 antiserum and GFP antiserum (Novagen, Madison, WI) were added in a 1:200 dilution, monoclonal MH27 antibody (kindly provided by Robert Waterston) was added at a 1:500 dilution, and the monoclonal body muscle antibody NE8/4C6.3 (Goh and Bogaert 1991) was added at a 1:20 dilution. All primary antibodies were diluted into a solution of non-fat dry milk and applied to fixed specimens for one hour at 37°C. The samples were washed 2X in PBST for 5 min and incubated in FITC (Sigma Chemical Co., St. Louis, MO) or Texas Red-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA) diluted 1:100 in PBST + 0.5% non-fat dry milk for 1 hour at 37°C. The specimens were rinsed in PBST buffer and sealed in a drop of Slowfade antibleaching solution (Molecular Probes, Eugene, OR).

To analyze the organization of actin filaments within the hypodermis of die-1 mutants, embryos were collected and stained with phalloidin as previously described (Costa et al. 1997). The only modification to this protocol was the use of Alexa 488 phalloidin (Molecular Probes, Eugene, OR) at a concentration of 10 units per ml. Fluorescent images of fixed samples were obtained using a Bio-Rad MRC1024 confocal laser scanning microscope.

die-1::gfp translational fusion

A minimal die-1 rescuing fragment was linearized by digestion with MluI, which cuts uniquely in the first intron of die-1. The GFP intron insertion vector pPD103.75 (courtesy of A. Fire, Carnegie Institution of Washington) was also cut with MluI, which drops out a fragment containing the entire GFP sequence flanked by consensus sequences for intron donor and acceptor splice sites. This fragment was cloned into the linearized die-1 rescuing vector. This construct was co-injected into die-1/mnC1 animals with jam-1::gfp, and pRF4 (rol-6). A strain carrying an extrachromosomal array of these constructs (jcEx23) can rescue die-1 homozygous embryos to viability, showing that DIE-1 remains functional with GFP inserted near its amino terminus. This array was subsequently integrated into the genome by crossing it into an N2 background, treating animals with 3000 rads of gamma-irradiation, and identifying animals that produce 100% rollers in the F2 generation (Mello and Fire, 1995). GFP expression was visualized in living embryos using
standard epifluorescence and MPLSM. Expression was weakly detected in some nuclei but could not be seen consistently or clearly. To enhance the GFP signal embryos were processed via the freeze crack method, stained with rabbit polyclonal anti-GFP antibodies (Novagen), and processed for immunofluorescence as above.

Mosaic analysis

In order to assess the role of DIE-1 in specific cells, animals expressing die-1::gfp in mosaic fashion were identified and analyzed for proper intercalation. Embryos from the rescued strain carrying jcEx23 were fixed and stained with GFP polyclonal antiserum followed by FITC-conjugated goat anti-rabbit secondary antibody and at the same time stained with monoclonal MH27 antibody followed by Texas Red-conjugated goat anti-mouse secondary antibody. This allowed visualization of all epithelial cell boundaries in the red channel; epithelial junctions could also be seen in the green channel in those cells that expressed the array. Mosaics were identified as embryos lacking epithelial staining in some cells in the green channel (see Results). Mosaic embryos were subsequently analyzed for morphogenetic defects.

Northern blot

To determine the number and sizes of transcripts produced from the die-1 locus, total RNA was prepared from wild-type worms as previously described (Burline et al. 1997). PolyA+ RNA was then isolated using the PolyATtract kit (Promega Corporation, Madison, WI). 2 µg of RNA was loaded per lane on a 1.2% agarose (w/v), 16% formamide (v/v) gel. The gel was blotted onto Hybond-N+ nylon filter (Amersham, Buckinghamshire, UK) and RNA was cross-linked to the filter using a UV-Stratalinker 1800 (Stratagene, La Jolla, CA) for 30 seconds. Probe was generated from linearized die-1 cDNA that was gel purified using the Qiaquick Gel Extraction kit (Qiagen Inc., Santa Clarita, CA). 100ng of purified cDNA was labeled using random decamers, 1µl Klenow, 5µl dCTP32, and 2.5µl of a mix that contained dA, dT, and dG at 1mM each. The reaction was incubated for one hour at room temperature. The Northern blot was prehybridized for 30 minutes at 65°C in Denhardt’s reagent (Sambrook et al. 1989). Probe was purified by passage over a spin column and hybridizations were performed at 65°C for 24 hours. Blots were washed 3X 30 min in 0.1X SSPE and exposed to Scientific Imaging Film (Kodak, Rochester, NY).
RESULTS

*die-1 is required for rearrangement of dorsal hypodermal cells and elongation of the embryo*

The first observable defect in *die-1* homozygous embryos is the failure of dorsal hypodermal cells to undergo intercalation (Fig. 2). *w34* embryos produce the normal complement of hypodermal cells and the dorsal cells become wedge-shaped as in wild-type embryos (Fig. 2B). However, many of the cells fail to finish the process of intercalation and remain wedge-shaped until they eventually fuse into syncytia, and their nuclei do not complete their contralateral migration (Fig. 2D). Despite abnormal intercalation, *die-1* embryos still enclose with hypodermis (Fig. 2F). Significantly, the process of elongation is also affected in *die-1* embryos. Wild-type embryos elongate to almost four times the initial length of the embryo; *die-1* embryos never elongate more than twice their initial length (Fig. 2F).

Because it is difficult to visualize hypodermal boundaries clearly by Nomarski microscopy, a *jam-1::gfp* translational fusion (Mohler et al. 1998) was expressed in *die-1* homozygous embryos. This allowed the analysis of hypodermal cell movements in developing *w34* embryos with the use of multiphoton laser scanning microscopy (MPLSM). The intercalation defect is readily apparent in *jam-1::gfp*-expressing *die-1* embryos (Fig. 2D). However, it was observed that some of the dorsal cells reproducibly stretch across the dorsal surface of the embryo, suggesting that intercalation is not completely disrupted in mutant embryos. The cells that were observed to intercalate in mutant embryos were consistently located in the anterior half of the dorsal hypodermis, and correspond to the boundary between anterior dorsal hypodermis, derived from the AB blastomere, and posterior dorsal hypodermis, derived from C (Williams-Masson et al., 1998)s. The posterior dorsal hypodermis was never observed to progress beyond wedging by the time cells fused into syncytia, consistent with results obtained via MH27 immunostaining (data not shown).

**Late steps in intercalation are defective in *die-1* embryos**

Previous studies of fixed material indicated that intercalating dorsal hypodermal cells extend basolateral protrusions immediately beneath the level of the adherens junction as they begin to interdigitate (Williams-Masson et al., 1998). In order to examine these protrusions dynamically in living *die-1* embryos, we used a transcriptional reporter for the *lbp-1* gene (Plenefisch et al., 2000) to drive expression of GFP in posterior dorsal hypodermal cells during intercalation. In addition, we used *elf-1* (*oj55*) to suppress cell fusion so that the position of individual dorsal cells could be scored in terminal embryos. *oj55* mutants are homozygous viable, undergo dorsal intercalation...
normally, and are normal in other respects, but lack cell fusion during embryogenesis (W.A.M., unpublished observations). MPLSM analysis of die-1; elf-1 embryos indicates that dorsal cells appear normal during the early phase of intercalation. Dorsal cells extend dynamic protrusions that appear indistinguishable from those in wild-type embryos, and appear correctly polarized by the time the cells' apical surfaces become wedge-shaped (n = 6 embryos examined for both elf-1 and die-1;elf-1; Fig. 3A,B; F,G). However, the cell bodies of posterior dorsal cells fail to translocate, and their nuclei do not complete their migration (Fig. 3H,I). These results indicate that dorsal hypodermal cells are polarized in die-1 embryos, and that they can extend protrusions that are normally associated with cell rearrangement. However, they posterior dorsal cells are defective in late steps in the rearrangement process.

**die-1 mutant embryos display additional morphogenetic defects**

Multiphoton recordings of die-1 embryos expressing jcIs1 also made it possible to analyze fusion of dorsal hypodermal cells (Fig. 2H). die-1 embryos appear to undergo premature fusion of the dorsal hypodermis, based on timing of fusion relative to the onset of jam-1::gfp expression. It was clear from these measurements that fusion occurs approximately 45 minutes early in die-1 embryos. Similar measurements of the time at which leading cells meet at the ventral midline were made; enclosure occurs at the same relative time in mutant and wild-type embryos (Table 1).

The precocious fusion phenotype raised the possibility that the apparent intercalation defect associated with die-1 embryos results because the cells do not have time to intercalate before they fuse. To exclude this possibility, we produced die-1(w34); jcIs1 embryos that were also homozygous for elf-1(oj55). While elf-1 single mutants undergo normal intercalation, we found that dorsal cells do not fuse in double mutant embryos, and that intercalation still fails to occur (Fig. 2L). The posterior dorsal cells did not intercalate by the time muscle activity was observed (data not shown), more than two hours after intercalation begins in wild-type embryos. This indicates that the process of intercalation is not simply delayed in die-1 embryos, and that precocious fusion is not masking the ability of the dorsal cells to rearrange in the die-1 mutant.

We were able to identify other hypodermal defects within die-1 embryos. In wild-type embryos the lateral hypodermis consists of two linear rows of 10 cells, one on each side of the embryo (Fig. 4A). die-1 terminal embryos appear to have one or two cells pinched out of each lateral row (n = 30 out of 30 examined via MH27 immunostaining; Fig. 4B). This phenotype was observed only in older embryos, after the intercalation defect is already apparent. The initial pattern
of lateral cells appears to be normal in all die-1(w34) embryos examined. We also observed defects associated with the ventral hypodermis. In wild-type embryos, ventral cells meet at the midline in a pairwise manner. Most of these pairs of cells form new adherens junctions once they reach the midline, while some of the anterior pairs of cells fuse with each other upon contact (Raich et al., 1999). In die-1 embryos we frequently observed incorrect cell fusions (5/6 embryos examined via MPLSM), skewed pairing of cells (5/6 embryos), and failure of some cells to reach the ventral midline (4/6 embryos, see Fig. 4D). Despite the abnormal arrangement of ventral hypodermal cells on the ventral side, mutant embryos consistently enclosed with hypodermis, indicating that intercalation is not a prerequisite for ventral enclosure.

Die-1 embryos also display non-hypodermal defects. In wild-type animals the pharynx develops as a cluster of cells in the interior of the anterior half of the animal. The pharynx eventually elongates, connecting to the surface of the embryo via the buccal cavity (Fig. 4E). 9 of 33 die-1 embryos analyzed using jam-1::gfp were determined to have defects in pharynx attachment (Fig. 4F). To examine body wall muscle, immunostaining experiments were performed with NE8/4C6.3, a body muscle specific antibody (Goh and Bogaert, 1991). Body muscle cells appeared to be present in normal quantities, and were generally organized into four quadrants, as is the case in wildtype. In wild-type embryos, muscle cells organize into tight rows within each quadrant and muscle tissue is never observed to cross between quadrants (Fig. 4G). However, in die-1 mutants, crossing between quadrants was observed (Fig. 4H). Crossing of muscle was primarily confined to the posterior half of the embryo. Finally, in wild-type embryos, the intestinal rudiment is attached to the posterior region of the pharynx at its anterior end and the anus at its posterior (Fig. 4I). In contrast, in die-1 mutants we observed cases in which the intestine appeared severed from either the posterior pharynx or the anus, or both, resulting in a straight intestine, rather than the typical curved rudiment at the comma stage (n = 11 out of 22 embryos examined; similar results were obtained from offspring of die-1 germline mosaic mothers; Fig. 4J). These data are consistent with a role for die-1 in multiple morphogenetic events during embryogenesis; alternatively, intercalation defects could lead to subsequent defects in other tissues.

Actin filaments form, but do not organize properly within the dorsal hypodermis of die-1 embryos.

Actin inhibitors (Priess and Hirsh 1986; Williams-Masson et al. 1998) and loss of the function of the actin-binding protein α-catenin (Costa et al., 1998; Raich et al., 1999) are known to disrupt elongation in C. elegans embryos. To address whether actin organization is disrupted in
die-1 mutants, embryos from die-1 heterozygous hermaphrodites were collected and stained with Alexa phalloidin. Actin forms into parallel, circumferentially oriented arrays within the dorsal hypodermis of wild-type animals (Fig. 5A). As elongation proceeds these arrays become uniformly spaced, thicker, and more distinct (Fig. 5B). Constriction of these filaments is thought to result in the subsequent elongation of the embryo (Priess and Hirsh 1986). In die-1 embryos the dorsal midline region of the dorsal hypodermal syncytium is often devoid of organized actin. In contrast, small regions of organized actin can be seen in lateral regions of the dorsal hypodermis (Fig. 6C). Actin organization often appears relatively normal in the anterior dorsal hypodermis of die-1 embryos (Fig. 5C). This region corresponds to the portion of the embryo in which intercalation is usually least affected, suggesting that the failure of cells to intercalate may be responsible for the misalignment or disorganization of actin within the dorsal hypodermis. Terminal die-1 mutant embryos display thicker bundles of actin filaments as is observed in elongating wild-type embryos, but these filaments are discontinuous and nonuniform in appearance (Fig. 5D). These data suggest that DIE-1, or the process of intercalation, may be required for actin filaments to form a continuous, parallel array spanning the dorsal syncytium. In contrast, we could detect no significant differences in the organization of microtubules in die-1 embryos (Fig. 5E,F).

**die-1 encodes a C2H2 zinc finger protein**

die-1 was mapped and molecularly identified using standard methods (Fig. 6A,B; see Materials and Methods). Comparison of genomic and cDNA sequences revealed that the die-1 locus consists of six exons (Fig. 6C). The largest cDNA is believed to be full-length, based on identification of a polyA tract at the end of the 3’ UTR, the presence of a Genefinder predicted start codon and the presence of stop codons in all three frames of the sequence upstream of the predicted start codon. In addition, the only transcript detected on a Northern blot is 2.9 kb, the size predicted if the cDNA is assumed to be full-length (Fig. 6D). The transcript does not appear to be trans-spliced. To confirm that the predicted protein is encoded by this locus, we amplified and sequenced the region from w34 homozygous embryos. The w34 sequence contains a C to T transition in exon 2 that results in a premature amber stop codon following amino acid 89 (Fig. 6C).

die-1 encodes a predicted 645 amino acid protein containing four C2H2 zinc fingers (Fig. 7A). C2H2 zinc finger proteins are putative transcriptional regulators in which DNA binding is mediated by the zinc finger domain. Two hallmarks of C2H2 transcriptional regulators can also be identified (Ptashne 1988): serine/threonine rich regions in the amino terminal half of the protein,
which are approximately 50% serine or threonine (underlined in Fig. 7A), and two short glutamine rich regions (amino acids 58-90, and 229-251) upstream of the zinc fingers, in which 7 out of 23 residues are glutamine. All four fingers contain the conserved cysteine and histidine residues associated with C2H2 zinc finger proteins. Three out of four fingers have the conserved leucine residue at position 16, while only one of the four fingers has the typically conserved phenylalanine residue at position 10 (Fig. 7B). Typically C2H2 zinc finger proteins have a well conserved seven amino acid linker region between the individual fingers (Klug and Rhodes 1987; Schuh et al. 1986). The DIE-1 protein has much longer linker regions. Other examples of long linker regions are known, although their significance is uncertain (Wilson et al. 1994, and unpublished sequences submitted to Genbank). The zinc finger domain is located in the carboxy terminal half of the protein and there is no significant homology to other proteins outside the zinc finger domain.

The premature stop codon in \textit{w34} truncates the protein prior to the zinc finger domain of DIE-1 and is therefore predicted to destroy its ability to bind DNA. Two other lines of evidence suggest that \textit{w34} is a null allele: (1) the similarity in phenotype of \textit{die-1/mnDf71} embryos to that of \textit{w34} homozygous embryos (data not shown) and (2) the phenotypic similarity between \textit{w34} homozygotes and \textit{die-1 (RNAi)} embryos (data not shown).

\textbf{DIE-1 is expressed in dorsal hypodermal cells prior to and during intercalation and in additional cells that undergo display morphogenetic movements-defects in \textit{die-1} mutants}

We employed two methods to determine when and where DIE-1 protein is expressed during embryonic development. First, anti-DIE-1 polyclonal antibodies were generated. Second, a translational fusion between \textit{die-1} and \textit{gfp} was generated in which \textit{gfp} was inserted in frame between exons one and two of the \textit{die-1} coding sequence in the smallest rescuing vector. \textit{die-1::gfp} is capable of rescuing \textit{die-1} embryos to viability, indicating that the fusion protein is still functional and that it is expressed in the cells which require its function. The pattern of \textit{die-1} expression was very similar in both cases.

DIE-1 is expressed in tissues consistent with the phenotypes associated with \textit{die-1} embryos. Maternally expressed DIE-1 can be detected in all blastomeres of the early embryo (Fig. 8A data not shown). We infer that such expression represents translation of maternal mRNA based on two pieces of evidence. First, extrachromosomal arrays are not typically well-expressed in the \textit{C. elegans} germline (Kelly and Fire, 1998); that \textit{die-1::gfp} is not expressed until the ~ 50 cell stage suggests that zygotic expression begins at this time. Second, immunostaining of cross-progeny from
matings of wild-type males with die-1 germline mosaic hermaphrodites indicates that the first detectable expression of DIE-1 occurs at the same stage. In either case, expression is typically seen in a small number of cells at the posterior of the embryo (data not shown). Moreover, viable embryos result in either case; this result indicates that early expression of DIE-1 is not required for rescue of all die-1 phenotypes. DIE-1 expression remains prevalent in the posterior half of the embryo in muscle and hypodermal precursors immediately prior to the onset of jam-1::gfp expression (Fig. 8B\textsuperscript{A}). When jam-1::gfp expression first becomes visible, shortly after the hypodermal cells undergo their terminal divisions, DIE-1 can be detected very strongly in the dorsal hypodermis (Fig. 8C\textsuperscript{B}). As intercalation proceeds, DIE-1 expression becomes weaker in these cells. Interestingly, the "pointer" cells, which are the last dorsal hypodermal cells to intercalate, maintain elevated levels of DIE-1 longer than other dorsal hypodermal cells. DIE-1 is undetectable in C-derived dorsal cells by the time intercalation is complete (Fig. 8D\textsuperscript{C}).

In addition to expression in the dorsal hypodermal cells, DIE-1 is strongly expressed in ventral hypodermal cells during the process of enclosure (Fig. 8D); expression ceases after enclosure has completed, and prior to the process of elongation (see Fig. 8G). The absence of expression during elongation suggests that DIE-1 may not play a direct role in elongation and that the elongation defect is a secondary result of other morphogenetic abnormalities. DIE-1 is also expressed strongly in pharyngeal cells and cells at the anterior of the embryo prior to and during the process of pharyngeal attachment, and in the gut primordium (Fig. 8E, F). DIE-1 is also expressed in muscle cells, as confirmed by double-staining die-1::gfp expressing embryos with anti-GFP and muscle-specific antibodies. Nuclei containing GFP indicative of DIE-1 expression were consistently observed within the rows of muscle (Fig. 8F\textsuperscript{G}). After enclosure is complete, DIE-1 expression completely disappears in all tissues until late in embryogenesis (Fig. 8G\textsuperscript{H}), at which time ~15 cells between the anterior and posterior bulbs of the pharynx are observed to express die-1::gfp (data not shown). Based on immunostaining, DIE-1 is also present in numerous cells along the ventral midline in L2-L4 larvae (data not shown).

Mosaic analysis suggests that DIE-1 acts within the C lineage to promote intercalation of posterior dorsal hypodermis

To determine whether DIE-1 acts in the dorsal hypodermis in a cell-autonomous or cell-non-autonomous fashion, we employed two methods. First, since the most prevalent defects in die-1 mutants involve the posterior dorsal hypodermis and posterior muscle, we asked whether intercalation movements
might be dependent on the presence of underlying muscle cells in the posterior. In order to assess whether the presence of underlying posterior body muscle is required for intercalation, we ablated Cap and Cpp in wild-type animals. These ablations eliminate 32 of the 81 body muscle cells present at the end of embryogenesis and approximately half of the dorsal muscle cells which are present in the two quadrants that underlie the dorsal hypodermis. Three embryos were ablated and intercalation was observed to proceed normally in each case (Fig. 9A-C). These results suggest that intercalation occurs independently of the underlying muscle tissue.

Second, we used a strain which expresses a rescuing \textit{die-1::gfp} as well as \textit{jam-1::gfp} as an extrachromosomal array (\textit{jcEx23}) for mosaic analysis. Mosaics can be identified that lack the array in specific epithelial lineages by identifying embryos in which \textit{jam-1::gfp} expression is absent. \textit{jam-1::gfp} is expressed in some progeny of four of the six founder cells in \textit{C. elegans} (AB, C, E and MS) and allows us to identify early losses of the array within these lineages. Since the defects in \textit{die-1} mutant embryos are more pronounced in the posterior, we focused on identifying rare losses in the C lineage. The C blastomere generates posterior dorsal hypodermal cells and muscle cells; since our ablation results suggested that C-derived dorsal muscle is dispensable for dorsal intercalation, we expected mosaic losses in C-derived hypodermis to provide information about requirements for DIE-1 within posterior dorsal hypodermis. We were able to identify two embryos in which expression of the array was lost from the C lineage, and one in which the array was lost from its precursor, P2. In all of these embryos intercalation defects were apparent in the posterior dorsal cells but not in the AB-derived anterior dorsal cells (Fig. 9D). The phenotype of these embryos is consistent with a requirement for DIE-1 function within individual cells that intercalate.

In contrast, DIE-1 does not appear to be required in ventral hypodermal cells. Eleven mosaics were identified that represent losses in lineages derived from ABp, which generates ventral hypodermal cells among other cell types. All of these embryos appeared to elongate normally (Fig. 9E), suggesting that DIE-1 function within the ABp lineage is not critical for morphogenesis of the embryo. As an additional byproduct of this analysis, other mosaic losses in epithelial cells were identified. In two embryos complex mosaic losses were sustained in cells that generate the gut (one in EMS, another in E), as well as cells in AB lineages; both exhibited misshapen guts with an abnormally wide lumen and abnormal organization (data not shown). Finally, in three cases we observed losses in subsets of the AB lineage that include the rectum. These embryos displayed detached or severed intestinal rudiments similar to those observed in \textit{die-1} mutants or offspring of \textit{die-1} germline mosaic mothers (data not shown), suggesting that rectal attachment requires DIE-1 in some AB progeny.
DISCUSSION

die-1 encodes a regulator of dorsal intercalation in posterior dorsal hypodermal cells

Dorsal intercalation is a simple example of directed epithelial cell rearrangement because it involves the rearrangement of a small number of cells in a predictable alternating pattern that is essentially invariant from embryo to embryo (Williams-Masson et al., 1998). Although there is not dramatic extension of the tissue along the anterior-posterior axis, dorsal intercalation in *C. elegans* is reminiscent of the movements of convergent extension in vertebrates because the intercalating cells converge toward the dorsal midline. Subsequent cell shape changes within the hypodermis elongate the tissue after intercalation is complete (Priess and Hirsh, 1986; reviewed in Simske and Hardin, 2000).

die-1 is the first cloned gene that has been shown to be directly required for cell rearrangements within the dorsal hypodermis. Our data indicate that *die-1* encodes a C2H2 zinc finger protein containing four zinc fingers. Zinc finger proteins typically act as transcriptional regulators; given its nuclear localization, we believe DIE-1 regulates the expression of other genes that are necessary for intercalation and other aspects of morphogenesis.

Although identification of transcriptional targets for DIE-1 awaits further experiments, several possibilities exist. Given the preponderance of defects in the posterior of *die-1* mutants, one possibility is that *die-1* acts in concert with posterior patterning genes. Several such genes are required for embryogenesis, including vab-7 (Ahringer, 1996), pal-1 (Edgar et al., 2001), and nob-1 (Van Auken et al., 2000). However, there is currently no evidence that dorsal intercalation is defective in mutants for any of these genes. Thus far we have not detected obvious differences in DIE-1 expression in posterior patterning mutants or misexpression of posterior patterning gene reporters in *die-1* mutants (our unpublished observations). Based on the lack of overt patterning defects in *die-1* (w34) homozygotes, we favor the hypothesis that DIE-1 regulates structural genes whose products are required for morphogenesis. If this is the case, then why are anterior dorsal hypodermal cells less affected in *die-1* mutants? One possibility is that a second regulatory process operates in parallel with DIE-1 within the anterior, AB-derived dorsal cells. If so, then a lineage-restricted difference in regulatory pathways between AB- and C-derived hypodermal cells may account for the greater sensitivity of posterior dorsal cells to loss of DIE-1 function.

Two examples of transcription factors that affect deep cell rearrangement in other organisms have been described in some detail. First, the BTB domain transcription factor BRIC-A-BRAC is
required for intercalation of terminal filament precursors in Drosophila (Godt and Laski 1995); currently, there are no identified targets of BRIC-A-BRAC regulatory activity. Second, a precedent for a spatially restricted transcription factor regulating structural genes required for deep cell rearrangement in the zebrafish embryo is the T-box transcription factor encoded by spadetail. SPADETAIL is necessary for cells from the ventrolateral region of the zebrafish embryo to converge towards the dorsal midline (Griffin et al. 1998). One suggested target of SPADETAIL is the protocadherin papc. Convergent extension movements are disrupted when a dominant negative form of PAPC is overexpressed in zebrafish embryos (Yamamoto et al. 1998). Although there are several protocadherin family members in C. elegans (Pettitt et al., 1997; Tepass, 1999; Hutter et al., 2000), there is currently no evidence for a protocadherin-mediated mechanism of cell rearrangement in the hypodermis of C. elegans analogous to that in zebrafish deep cells (J. Pettit, personal communication). Mutations in one protocadherin family member, cdh-3, do not result in any intercalation defects in the dorsal hypodermis, although it is possible that cell rearrangement is affected in the tail hypodermis (Pettit et al., 1996).

**Defects in die-1 embryos show that the steps of intercalation are genetically separable into early and late phases**

The first detectable defect in die-1 mutants is the failure of dorsal hypodermal cells to complete intercalation. Although DIE-1 is expressed maternally there is no evidence for a maternal requirement for the gene. When maternal die-1 function is removed, either in germline mosaics (our unpublished observations) or via RNA interference, the phenotypes of the resulting embryos are identical to those of die-1 mutants. In addition, germline mosaic animals mated with wild-type males produce viable progeny, indicating that zygotic expression is sufficient to rescue die-1 phenotypes. In addition to defects in dorsal intercalation, die-1 mutant embryos also display abnormalities in the processes of ventral enclosure, seam cell organization, pharynx attachment, muscle organization, and gut formation. All of the observed defects appear to be primarily morphogenetic in nature, since antibody staining and Nomarski analysis of various tissues provides no evidence that any cell types are mis-specified in die-1 mutants. The presence of multiple morphogenetic defects in die-1 mutants, and the appearance of DIE-1 in the affected tissues prior to their morphogenetic movement, suggests that DIE-1 may act to regulate multiple morphogenetic processes within the embryo. However, since most of these defects are not observed until after the intercalation defect is apparent, it is also possible that some or all of the additional defects are
secondary defects that arise because the process of intercalation has been perturbed. The expression pattern of DIE-1 is consistent with either possibility, and further experiments will be required to determine which defects reflect direct requirements for DIE-1 in specific tissues. Based on our multiphoton microscopic analysis, dorsal hypodermal cells appear normal in several respects. First, they are correctly polarized along the right-left axis, since they can extend protrusions in a polarized fashion that initially appears indistinguishable from wild-type siblings. Second, basolateral protrusions are initially extended in a normal fashion by die-1 mutant cells, so the machinery required for this process must function normally, at least initially. Third, the apical junctional domains of mutant cells become wedge-shaped, indicating that initially the cells are capable of modulating their junctional connections with one another. However, subsequent translocation of the cell body, which is crucial for successful completion of cell rearrangement, is defective in die-1 mutants. This result implies that the process of epithelial cell rearrangement can be subdivided into several steps genetically in C. elegans, and that die-1 regulates late events in the intercalation process (Fig. 10A).

How cell translocation occurs during dorsal intercalation is currently unknown. One possibility is that DIE-1 indirectly regulates the reorganization of the cytoskeleton in rearranging cells. It is known that aspects of convergent extension in Xenopus require microtubules (Lane and Keller, 1997) and actin microfilaments (Tamai et al., 1999). Likewise, dorsal intercalation depends on both actin filaments and microtubules (Williams-Masson et al., 1998). We have not detected obvious differences in microtubule organization within the hypodermis of die-1 mutants (J.H., unpublished observations). However, nuclear migration, which likely depends on alignment of microtubules (Malone et al., 1999), appears incomplete in die-1 mutants, and so although it is possible that a subtle defect in microtubule organization exists in the mutants. Although we were unable to determine whether actin is disorganized during the process of intercalation; we have demonstrated defects in the actin cytoskeleton in slightly older die-1 mutants. Such defects could arise in several ways. One possibility is that DIE-1 indirectly regulates the reorganization of the actin cytoskeleton in rearranging cells. A second possibility is that cytoskeletal disorganization in die-1 mutants may be purely a secondary result of the failure of dorsal hypodermal cells to intercalate. In this case, nuclear migration may simply be physically blocked due to the narrowness of the medial tips of the cells that fail to intercalate. Although we were unable to determine whether actin is disorganized during the process of intercalation, our demonstration that die-1 mutant embryos show defective actin organization during elongation (see below) is Our results are
consistent with either possibility. Finally, although it is known that the classical cadherin-catenin complex is not required for dorsal intercalation to occur (Costa et al., 1998; Raich et al., 1999), little else is known about the events that occur on the surface of migrating dorsal cells as they rearrange. It is possible that cell-substrate or cell-cell interactions are affected in die-1 the mutants.

It is also possible that cell-substrate or cell-cell interactions are affected in die-1 the mutants. It is known that the classical cadherin-catenin complex is not required for dorsal intercalation to occur (Costa et al., 1998; Raich et al., 1999), but little else is known about the events that occur on the surface of migrating dorsal cells as they rearrange. There is no evidence for the presence of a basal-lamina underneath the dorsal hypodermis at the time of intercalation (our unpublished observations), so it is unclear what migratory substrate dorsal cells could use to intercalate other than themselves. The basolateral protrusions extended by intercalating dorsal cells lie immediately beneath the hypodermal cell junctions, near the apical surface (Williams-Masson et al., 1998; our results), suggesting that the cells exert traction by attaching to neighboring hypodermal cells instead of a substratum basal to the dorsal hypodermis. DIE-1 could regulate the cellular machinery required for this process.

**DIE-1 functions autonomously to regulate intercalation**

Since die-1 encodes a putative transcriptional regulator, it is likely that DIE-1 acts autonomously within the posterior dorsal hypodermis to regulate factors necessary for intercalation. Analysis of mosaic embryos in which die-1 function was lost in progeny of the C blastomere supports this suggestion; in such embryos intercalation fails among those cells lacking DIE-1. Laser ablation experiments also support the view that dorsal cells, once polarized, intercalate in a predominantly autonomous fashion of cells surrounding the dorsal hypodermis. In mosaic embryos that failed to express die-1::gfp within any cells of the C lineage but did express it within the AB lineage, all of the AB-derived dorsal hypodermal cells intercalated normally, while most of the C-derived cells failed to intercalate. The anterior-most pair of C-derived cells also intercalated; it is not entirely clear whether this indicates that expression of DIE-1 in AB-derived dorsal cells can rescue the ability of the nearest C dorsal cells to intercalate, or whether there is a less stringent requirement for DIE-1 in this group of C-derived hypodermal cells. In die-1 mutant embryos there was a tendency for cells in this region of the C-derived dorsal hypodermis to intercalate; this favors the second possibility. Laser ablation studies are also consistent with dorsal intercalation being a tissue-autonomous process. Dorsal cells can intercalate
in the absence of underlying muscle cells (our results), and our previous studies have shown that intercalation can occur in the absence of half of the lateral hypodermal cells or among anterior dorsal cells in the absence of posterior dorsal cells (Williams-Masson et al. 1998). We infer from these additional experiments that the observed DIE-1 expression within the posterior muscle and lateral hypodermal tissues is not required for dorsal intercalation, since the cells themselves are dispensable.

In addition to defects in dorsal intercalation, *die-1* mutant embryos also display abnormalities in the processes of ventral enclosure, pharynx attachment, muscle organization, and gut formation. It is possible that some of the additional defects arise secondarily because the process of dorsal intercalation has been perturbed. Our mosaic analysis identified one case in which such indirect defects appear to occur, the ventral hypodermis. We identified mosaic embryos that elongated completely normally yet displayed losses in the ABp lineage, which generates ventral hypodermal cells. This suggests that the ventral hypodermal defects associated with *die-1* mutants do not result from a requirement for DIE-1 within the ventral cells, but are indirect. Further experiments will be required to determine with certainty what defects in *die-1* mutants reflect direct requirements for DIE-1 in the tissues in which it is expressed.

In contrast to the ventral hypodermis, our mosaic analysis is consistent with the view that DIE-1 is required cell autonomously in the gut. It is interesting to note that the cells within the *C. elegans* gut primordium undergo a type of intercalation (Leung et al., 1999), although the process is somewhat different from that in the dorsal hypodermis. Failure of gut progenitors to intercalate properly could lead to a subsequent morphogenetic defect, including the detachment phenotype we observed in *die-1* mutants and offspring of *die-1* germline mosaic mothers. Although we did not recover mosaic animals in which a clean loss of DIE-1 function occurred within E, the gut progenitor cell, we did recover mosaics in which complex losses occurred that included E. In each case gut abnormalities were observed, consistent with an autonomous role for DIE-1 in the gut. It is possible that DIE-1 regulates intercalation in both the gut and the dorsal hypodermis. It is intriguing to note that the other tissue in which defects are observed in *die-1* mutants, i.e., pharynx and body wall muscle, undergo coordinated changes in cell position along a preferred axis as well. Muscle cells organize into quadrants and elongate along the anterior-posterior axis (reviewed in Moerman, 1997). Similarly, pharyngeal precursors, which arise as a ball of cells attached to the forming midgut, elongate anteriorly to connect to the buccal cavity (ref??). In addition to its role in the dorsal hypodermis, DIE-1 may also be autonomously required in the gut. Although the precise
morphogenetic movements of these tissues have not been examined carefully, they may involve movements that resemble intercalation. If this is the case, then DIE-1 may be a regulator of several similar morphogenetic be a general regulator of intercalation processes in *C. elegans*.

**Elongation defects in die-1 embryos may result from defects in actin filament formation**

It has previously been shown that elongation of *C. elegans* embryos is dependent on actin, and that actin filaments organize into a circumferential, uniformly spaced array within the hypodermis during elongation (Priess and Hirsh 1986). In *die-1* mutant embryos in which dorsal cells had recently fused, short stretches of organized actin were observed around the periphery of the dorsal syncytium, while the central region of the dorsal syncytium did not display organized, circumferentially organized actin filaments. That actin can organize to some extent in mutant embryos suggests that DIE-1 may not directly regulate actin filament formation, but rather that the failure of cells to intercalate prevents actin from aligning across the entire dorsal hypodermis. In this model, once intercalation is complete in wild-type embryos, cells span the width of the dorsal hypodermis, allowing the forming actin filaments to stretch across the entire dorsal surface. In *die-1* mutants, individual actin filaments may be able to span the length of a cell, but if the cell fails to intercalate the individual filaments may not be able to span the entire dorsal hypodermis. In this case, the central area where the tips of cells were located might be expected to lack organized actin (Fig. 10). Once the dorsal cells fuse, actin filaments would be interrupted by gaps at the dorsal midline; the lack of organized actin in turn could lead to defects in subsequent circumferential constriction of the actin network associated with elongation.

The model we propose provides a role for intercalation in morphogenesis in *C. elegans*, and suggests that intercalation events may be generally required for the production of columns of cells with aligned arrays of cytoskeletal elements that bear mechanical loads anisotropically. In this sense, the dorsal hypodermis is similar to the dorsal involuting marginal zone of *Xenopus*. There, cells that have undergone convergent extension are measurably stiffer along the anterior-posterior axis than those that have not (Moore et al., 1995). Moore et al. (1995) proposed that tension-resisting elements (i.e., actin filaments) reinforce converging and extending cells mediolaterally (i.e. perpendicular to the axis of extension) as they rearrange, and that microtubules may bear compressive loads as the cells extend. We have provided direct evidence for defects in similar tension-resisting elements within the hypodermis of *C. elegans* when directed cell rearrangement is disrupted. Thus, such mechanical changes in rearranging cells may be a feature common to both
deep (non-epithelial) and epithelial cells. As this and other morphogenetic processes regulated by DIE-1 are unraveled, significant insights will be gained into the process of epithelial cell rearrangement. This in turn should deepen our understanding of this ubiquitous morphogenetic process.

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REFERENCES


**Table 1. Timing of cell fusion and enclosure**

<table>
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<tr>
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<th>Time to First Fusion&lt;sup&gt;b&lt;/sup&gt; (min ± SEM)&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Time to Last Fusion&lt;sup&gt;c&lt;/sup&gt; (min ± SEM)</th>
<th>Time to Enclosure&lt;sup&gt;d&lt;/sup&gt; (min ± SEM)</th>
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<tr>
<td>wild-type</td>
<td>121 min ± 4.2 (n=4)</td>
<td>167 min ± 16.9 (n=3)</td>
<td>74 min ± 2.3 (n=8)</td>
</tr>
<tr>
<td>die-1(w34)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>75 min ± 5.8 (n=3)</td>
<td>133 min ± 6.0 (n=3)</td>
<td>73 min ± 2.4 (n=3)</td>
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<sup>a</sup>Embryos from heterozygous w34 animals expressing *jam-1::gfp* were analyzed.
<sup>b</sup>Time to first fusion was defined as the first time point at which a cell appeared completely or partially fused in the dorsal hypodermis.
<sup>c</sup>Time to last fusion is the first time point at which all dorsal hypodermal cells appeared fused.
<sup>d</sup>Time to enclosure is defined as the first time point at which the leading cells of the ventral hypodermis have clearly met at the ventral midline.
<sup>e</sup>min = time from initial expression of *jam-1::gfp* to described phenomenon.
FIGURE LEGENDS

Figure 1. Schematic diagram of hypodermal morphogenesis in *C. elegans*. Images are only intended to portray relative location, movements and sequence of morphogenetic events occurring within the hypodermis. For clarity, cell borders have been omitted in the lateral and ventral hypodermis. All diagrams represent dorsal views of unenclosed embryos with the exception of the bottom cartoon, which represents a lateral view of an enclosed and partially elongated embryo. Anterior is to the left in all images. The hypodermis arises as a patch of cells on the dorsal/posterior surface of the embryo; the cells organize into six rows. Intercalation involves the rearrangement of the two central (green) rows of cells into a single row. As intercalation nears completion, enclosure begins with the movement of the outer (red) rows of cells to the ventral midline, followed by anterior spreading of the hypodermal sheet to cover the anterior end of the embryo. This is followed by elongation of the embryo into a worm-like shape. Many of the dorsal and ventral hypodermal cells later fuse into syncytia.
Figure 1

Intercalation

Enclosure begins

Completion of enclosure

Elongation and Fusion

Hypodermal Cell Type
- Dorsal
- Lateral (seam)
- Ventral
**Figure 2.** Intercalation is defective in homozygous *die-1* embryos. Wild-type ((left column) and *die-1 (w34)* mutant (right column) embryos at various stages of morphogenesis. Alternating pairs of figures are Nomarski and multiphoton laser scanning microscopy views of different, but comparably staged embryos. Embryos in this and all figures are presented with anterior to the left. A, B. Wild-type and *w34* embryos shortly after all hypodermal cells have formed. Arrows indicate dorsal cells that have become wedge shaped (A,B). C. Wild-type embryo 35 minutes after the onset of JAM-1 expression. Intercalation has just begun. Dorsal posterior hypodermal cells marked with asterisks have wedged between one another. D. *w34* embryo 65 minutes after the onset of *jam-1::gfp* expression. Many of the anterior dorsal hypodermal cells have intercalated; however, most of the posterior dorsal cells are wedged and have not intercalated completely (asterisks). Arrows indicate cell boundaries that will fuse prematurely (see panel H). E. Same embryo as A, 60 min later. Intercalation is essentially complete. Arrows indicate dorsal cells that have become narrower along the anterior/posterior (A/P) axis, elongated along the left/right (L/R) axis and have finished intercalation. F. Same embryo as B, 120 min. later. Arrows indicate cells that fail to complete intercalation. Wedged tips have not moved to make contact with the opposing lateral hypodermal cells. Cells have extended along the L/R axis, but have not become significantly narrower in the A/P axis. G. Completely intercalated dorsal hypodermis in a wild-type embryo 100 min. after the onset of *jam-1::gfp* expression. H. Same embryo as in panel B, 80 minutes after the onset of *jam-1::gfp* expression. Arrows indicate cell boundaries that have started to fuse. I. Same embryo as in A 200 min later, lateral view. The embryos has elongated to twice its original length. J. Same embryos as B, 200 min. later. Elongation has failed. K. *jam-1::gfp* expression in an *elf-1 (oj55)* embryo comparable to I. Elongation is normal. L. Reconstruction of *jam-1::gfp* expression in a terminal *die-1(w34); elf-1 (oj55)* embryo; for clarity, the gut and intestinal expression has been omitted by "coring" the embryo as in Mohler and White (1998). Unintercalated cells in the posterior are clearly visible (arrows). Siblings had fully elongated. Scale bar = 10 µm.
Figure 2

WT vs. die-1(w34)

(A, C, E, G, I, K) WT

(B, D, F, H, J, L) die-1(w34)
Figure 3. Analysis of protrusive activity in dorsal hypodermal cells in w34 embryos. Wild-type (left, A-E) and die-1 (w34) (right, F-J) embryos carrying a lbp-1::gfp transcriptional reporter (Plenefisch et al., 2000) were analyzed using multiphoton microscopy. Times in minutes are indicated in the lower right corner of each frame; in each case, filming commenced within 5-10 minutes of completion of the terminal divisions of the C-derived dorsal hypodermal cells. To suppress cell fusion, both strains were made homozygous for the elf-1 (oj55) allele. Small arrows indicate basolateral protrusions extended by the dorsal hypodermal cells. In w34 embryos, protrusions are extended (G-I), but cell translocation does not occur (J). Scale bar = 10 µm.
Figure 3
**Figure 4.** Additional defects associated with *die-1* embryos. Wild-type embryos are shown in the left column; *die-1* (w34) on the right. A, B. Lateral views of wild-type and *die-1* embryos stained with MH27 antibody. A. In wild-type embryos, lateral cells form linear rows of ten cells per side. B. *w34* embryos typically have lateral cells pinched out of the row (arrow). C, D. Ventral views of N2 and *w34* embryos expressing *jam-1::gfp* as viewed with multiphoton microscopy. Both embryos are enclosed with hypodermis and are approximately the same age. Asterisks indicate the same cells in both embryos. C. Hypodermal cells in a wild-type embryo meet at the ventral midline in a pairwise manner. The arrow indicates a junction at the midline between a specific pair of cells. D. A *w34* embryo; cells have met at the midline out of register. Small arrowheads indicate cells that failed to reach the midline. The arrow indicates the missing junction between the same pair of cells highlighted in panel C, which have fused prematurely. E, F. Wild-type (E) and *die-1* (F) embryos expressing *jam-1::gfp* as viewed by multiphoton microscopy. E. An embryo displaying normal attachment of the pharynx to the buccal cavity (arrow) F. *die-1* embryo in which the pharynx has failed to attach to the buccal cavity. Arrow indicates absence of JAM-1 expressing cells between the pharynx and buccal cavity. G, H. Wild-type (G) and *w34* (H) embryos stained with the body wall muscle specific antibody NE8/4C6.3. G. Body muscle cells separate into distinct quadrants prior to elongation of the animal. The two dorsal quadrants can be seen in this image. H. *die-1* embryos also display four muscle quadrants but often exhibit crossover of tissue between rows (arrows). I, J. 3d reconstructions of wild-type (I) and embryos derived from a *die-1* germline mosaic mother (J) stained with MH27. The exterior focal planes have been removed and the focal planes containing the gut have been reconstructed. Arrows in J indicate sites at which the posterior intestine have detached from the anterior intestine (upper arrow) and the anal region (lower arrow). Scale bar = 10 µm.
Figure 4

WT  

A

B

C

D

E

F

G

H

I

J
Figure 5. Actin and microtubule organization in the hypodermis of die-1 embryos. Insets are 3X enlargements of the boxed area in each panel. Intense phalloidin staining running horizontally across the embryo indicates the underlying dorsal muscle in all images A-D (asterisks). A. A wild-type embryo shortly after dorsal cells have fused (embryo approximately 2-fold its original length); dorsal view. Fine actin filaments can be seen within the hypodermis in a mediolaterally oriented pattern (arrowheads). Arrows indicate the boundary between dorsal and lateral hypodermis that runs horizontally across the image. B. Dorsal view of a completely elongated wild-type embryo. Actin filaments are thicker and organized into distinct fibrils within the dorsal hypodermis and display uniform spacing. Arrows indicate the boundary between dorsal and lateral cells. C. Dorsal view of a die-1 embryo shortly after fusion has occurred. This embryo is equivalent in age to the embryo in panel A. Small regions of organized actin can be discerned (arrows); however, the central region of the dorsal hypodermis does not display organized actin filaments. D. Terminal die-1 embryos display regions of actin filament organization within the hypodermis; however, filaments often appear discontinuous (arrowheads). E. Microtubule organization in a wild-type embryo. Partially parallel arrays of microtubules are visible. F. Microtubule organization in a die-1(w34)mutant. Similar arrays are visible. Scale bar = 10 µm.
Figure 5
Figure 6. Mapping and cloning of die-1. A. Genetic map of the region surrounding die-1. Three factor mapping and complementation tests against deletion mutants place die-1 between egl-43 and daf-19. B. Cosmids spanning the die-1 region were injected, and a 10kb rescuing fragment was isolated (pPH31). C. Top: intron/exon structure of the only predicted ORF in the smallest rescuing fragment. The arrow indicates the location of a premature stop codon. Bottom: intron/exon structure of the die-1::gfp fusion construct generated by insertion of gfp coding sequence (represented by gray box) into the first intron of the predicted gene. D. Northern blot probed with die-1 specific probe. A single band was detected at approximately 2.9kb (arrow).
Figure 6

A

\[\text{mnDf71} \quad \text{mnDf14} \quad \text{mnDf83}\]

\[\text{LGII} \quad \text{unc-4} \quad \text{egl-43} \quad \text{die-1} \quad \text{daf-19}\]

B

\[\text{T28D10} \quad \text{ZK892} \quad \text{F52H3} \quad \text{K10A2} \quad \text{pPH31}\]

\[\text{rescue} \quad - \quad - \quad + \quad +\]

C

\[\text{1kb}\]

D

\[\text{7.46 kb} \quad \text{9.69 kb} \quad \text{4.4 kb} \quad \text{2.9 kb} \quad \text{2.37 kb} \quad \text{1.35 kb} \quad \text{0.24 kb}\]
Figure 7. *die-1* encodes a zinc finger protein. A. Predicted amino acid sequence of DIE-1. The protein contains four well-conserved C2H2 zinc fingers (underlined), as well as two serine/threonine rich regions (boxes). These regions were 50% S and T. An asterisk denotes the position of a premature stop codon which truncates the protein to 89 amino acids. B. Alignment of the four zinc fingers detected in *die-1* with the consensus sequence for C2H2 zinc fingers. Conserved residues are bold.
Figure 7

A

MANAVDMQQL LMSMDPSSAN QFNMMNKGGG CVMAPSGGSA SPTSSSGAPS 50
SSSSNAETDE KDMFMPSRLI QNLANQNLQM SMILQQQSNNQ ADSNIADAGH 100
NNNSGAEKNII ELLMSGNADL MKFATQFAQQ AKSVQDEPEE QRESSSPPPP 150
AVVSLSNLAS LDFVTPTTSTAT ECSAASSTSS VDSAESTIV VNGHSNSQNSNT 200
DDDDVSTQPS AKKQRTGDEQ PLTPSNQQNL MQSSLAMGIL SNPLHQNNQX 250
QMROESMMSF GLFPPQGLAGI PLMFQPLHQP QFAGMQDFEP LSALSTPNKG 300
SGVKRQYSSN NKNFCDCICNK EVCNKYPFRT HMLKMHGIVI DENKTVIANI 350
DTSIKEREGE LTFRCDTCHR MKTRNQLRQ HRQDGVSQVL LSDKPRNPNK 400
SSVPTTPNGA NNNSSPSNAS MSEEKCALCD KRVSPSMML KMAQDFGA 450
AANGDLNMGMM AIL/QQRNQST EKMKSSNLLE CTDMSYKTRD PKNLHMER 500
HIKMSAAGG NDEDDETVALQ LITEAALQMV VQQNQQYQDG DTAANLNTF 550
KNDGKKEIGE EKATGNHER NSHTSGSISP SGSIEPEGFQK NIGEKAFTPQ 600
SFLIKCNDDS GEFlTEFLAQ LVRSVIDGP RQLVFNLHPA PPTTS 645

B

Consensus C--C----F------L--H-----H
Finger 1 CDICNKEVCNKYFLRTHMLKMH
Finger 2 CDTCRTMPKTRNQLRQHRQDVH
Finger 3 CALCDKRVSFSMML HMAQDH
Finger 4 CTDMSYKTRDPKNNLHMER H
Figure 8. DIE-1 expression. Confocal images of fixed wild-type embryos stained with anti-DIE-1 polyclonal antibodies (A, D, E, F) or of fixed homozygous die-1 embryos expressing die-1::gfp, jam-1::gfp from an extrachromosomal array that were subsequently immunostained with anti-GFP antibodies (B, C, G-I A-D, G-H) were obtained. A. 4-cell embryo. Expression in all four cells is nuclear. B. Embryo with ~100 cells. Note higher levels of DIE-1 in posterior nuclei. C. Middle focal plane of an embryo immediately prior to intercalation. Strong staining is observed in presumptive hypodermal precursors and muscle precursors. B. An embryo during the early phase of dorsal intercalation. Note the elevated levels of DIE-1 in dorsal hypodermal nuclei (arrows). C. An embryo near the completion of dorsal intercalation. DIE-1 expression has declined in posterior dorsal hypodermal cells (arrowheads), but is now higher in the "pointer cells", which are the last dorsal cells to intercalate (arrows). D. Expression of DIE-1 (green) and JAM-1 (green MH27 immunostaining, red) in dorsal cells during intercalation. Arrowhead indicates the position of the dorsal midline. Arrows indicate the two lateral rows of hypodermis. E. Expression of DIE-1 in lateral (arrowhead) and ventral (arrow) hypodermal cells (lateral view). Lateral view of an enclosing embryos double labeled for DIE-1 (red) and JAM-1 (green). E. Expression of DIE-1 in the pharyngeal (ph) and intestinal (int) primordia. DIE-1 is present in the nuclei of all gut cells; the lumen is identifiable by MH27 immunostaining (arrow). F. Pharyngeal expression of DIE-1. An enclosed embryo expressing DIE-1 (red green) and JAM-1 (green red). Middle focal plane, lateral view. Arrow indicates jam-1::gfp expression associated with the epithelium lining the pharyngeal lumen. G. An unenclosed embryo double stained with anti-GFP antibody (green) and muscle-specific antibody (red). DIE-1-GFP is detected in nuclei of muscle cells (arrows). H. An enclosed embryo double stained with anti-GFP antibody (red) and a muscle-specific antibody (green). Epithelial cell junctions are observed in both channels (detecting JAM-1-GFP), but no nuclear GFP expression is observed, indicating die-1::gfp expression has ceased. Scale bar =10 µm.
Figure 8
Figure 9. Tissue-specific requirements for DIE-1. A-C. Time course of development of a single wild-type embryo in which the muscle precursor cells, C\text{ap} and C\text{pp}, were ablated (dorsal view). Despite the absence of \(\sim 1/3\) of the body muscle (primarily posterior and dorsally positioned cells), intercalation occurs normally. Intercalating dorsal cells in A and B are indicated by arrows. A. Start of intercalation. B. Intercalation complete. C. Interior focal plane; arrowheads indicate undifferentiated ablated material. Times indicate amount of time elapsed from the beginning of intercalation. D. DIE-1 is required within progeny of C for their intercalation. Embryos from a homozygous \textit{die-l} strain rescued by an extrachromosomal array expressing \textit{die-1::gfp}, \textit{jam-1::gfp} were analyzed. Embryos were stained with anti-GFP antibody (green) and anti-MH27 (red). Mosaic embryos were identified as embryos that showed normal MH27 staining in the red channel, but expressed \textit{jam-1::gfp} only in a subset of the epithelial cells in the green channel. No \textit{jam-1::gfp} expression is observed in the posterior dorsal hypodermis, which is derived from the C lineage and includes several unintercalated cells (arrows). AB derived cells have intercalated normally. E. Mosaic loss in ABp. This embryo elongated normally. Arrows indicate ventral hypodermal cells that are not expressing the array, based on the absence of signal in the green channel. Arrowhead indicates a lateral hypodermal cell that does not contain the array. Thorough analysis of the embryo showed that none of the ABp-derived hypodermal cells that could be analyzed contained the array, while all ABa-derived and non-AB cells that could be analyzed expressed the array. Scale bar = 10 \(\mu\text{m}\).
Figure 9
Figure 10. Model for DIE-1 function during intercalation and elongation. During elongation, loss of DIE-1 function results in incomplete intercalation, leading to failure of actin filaments to span the entire dorsal hypodermal array. After fusion, the filament network is discontinuous at the dorsal midline, leading to elongation failure.
Figure 10

WT

die-1

fusion

fusion