

# Zygotic Loss of ZEN-4/MKLP1 Results in Disruption of Epidermal Morphogenesis in the *C. elegans* Embryo

Jeff Hardin,<sup>1,2\*</sup> Ryan King,<sup>2</sup> Christina Thomas-Virinig,<sup>3†</sup> and William B. Raich<sup>2</sup>

ZEN-4/MKLP1 is required maternally for cytokinesis in *Caenorhabditis elegans*, but was originally identified in a screen for zygotic lethal, enclosure abnormal (Zen) mutants. We report that *zen-4(w35)* homozygotes exhibit stochastic failures in cytokinesis in multiple lineages. Remarkably, multinucleate epidermal cells show directional migration, even when there are as few as half the normal number of cells. Temperature shift experiments and analysis of *zen-4::gfp* expression confirm that the epidermal requirement for *zen-4* function precedes morphogenesis. Driving expression of wild-type *zen-4* by means of an epithelial-specific transgene can rescue many epidermal morphogenetic defects in *zen-4* mutants. Early expression of *unc-119* in epidermal precursors made this promoter unsuitable as a neuronal-specific driver in this context. Our results indicate that zygotic *zen-4* function is required for correct division of epidermal precursors and, hence, indirectly for normal morphogenesis and that the epidermal morphogenetic program is surprisingly robust even in the absence of *zen-4* function. *Developmental Dynamics* 237:830–836, 2008. © 2008 Wiley-Liss, Inc.

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## INTRODUCTION

MKLP1 kinesins are important for the organization of spindle midzones in mitotic cells in animal cells (D'Avino et al., 2005; Glotzer, 2005). *zen-4* encodes the single MKLP1 kinesin in *Caenorhabditis elegans*. ZEN-4 is required for cytokinesis (Powers et al., 1998; Raich et al., 1998), localizes to the spindle midzone, and is a key component of the centralspindlin complex (Severson et al., 2000; Mishima et al., 2002). In postmitotic cells, ZEN-4 plays a role in de novo epithelializa-

tion of cells in the embryonic pharynx (Portereiko et al., 2004). Despite its well-documented role during cytokinesis, the original *zen-4* allele (*w35*), a protein null (Raich et al., 1998), was identified in a screen designed to identify zygotic, enclosure-abnormal (Zen) mutants (Ferguson et al., 1996). Here, we report that homozygous *zen-4(w35)* mutants undergo epidermal morphogenesis, despite possessing as few as half the normal complement of epidermal cells; morphogenetic defects during ventral enclosure and embryonic

elongation appear to arise from late cytokinesis failure in epidermal lineages.

## *zen-4* Mutants Display Cytokinesis Defects in Multiple Tissues but Carry Out Morphogenesis

Two major epidermal cell migrations occur during embryogenesis in *C. elegans* (Chisholm and Hardin, 2005). First, two rows of 10 cells at the dorsal midline intercalate to form a single row of cells (Williams-Masson et al.,

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<sup>1</sup>Department of Zoology, University of Wisconsin, Madison, Wisconsin

<sup>2</sup>Program in Cellular and Molecular Biology, University of Wisconsin, Madison, Wisconsin

<sup>3</sup>Department of Biomolecular Chemistry, University of Wisconsin, Madison, Wisconsin

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<sup>†</sup>Dr. Thomas-Virinig's present address is Department of Pathology and Laboratory Medicine, University of Wisconsin, Madison, WI 53706.

\*Correspondence to: Jeff Hardin, Department of Zoology, 1117 W. Johnson Street, Madison, WI 53706.

E-mail: [jddhardin@wisc.edu](mailto:jddhardin@wisc.edu)

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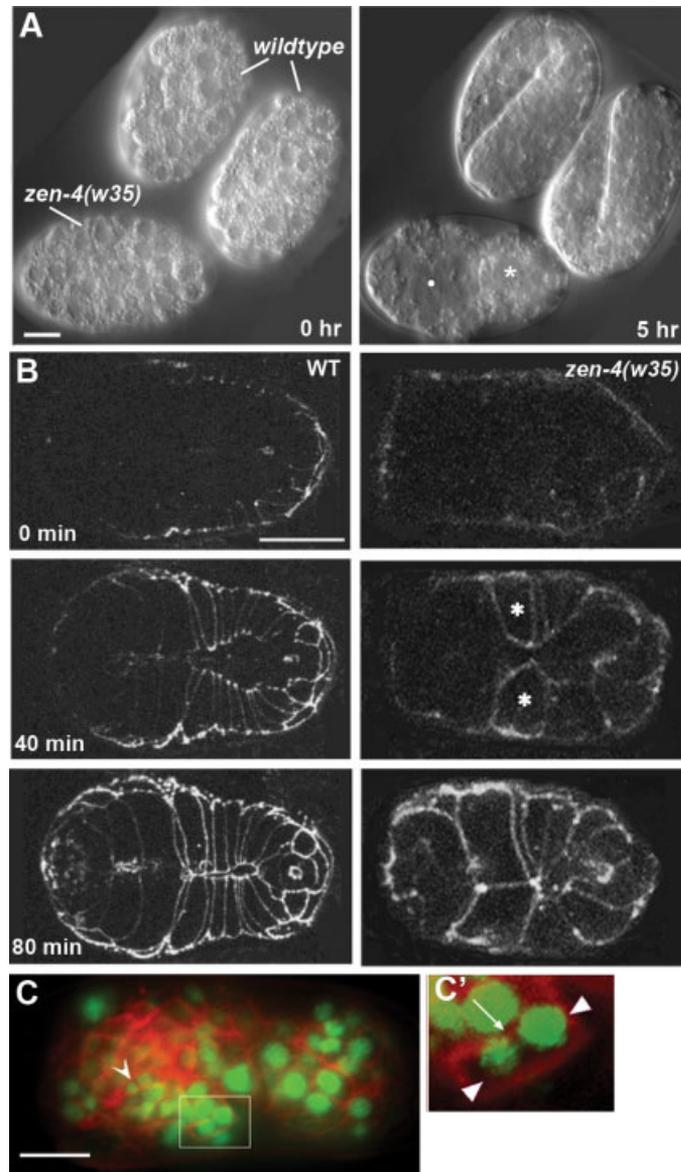
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1998). Shortly after dorsal intercalation is underway, ventral enclosure begins, as two pairs of epidermal cells (leading cells) migrate ventrally to meet at the ventral midline, followed by more posterior ventral cells (pocket cells; Williams-Masson et al., 1997). Ventral epidermal cells migrate over underlying neuroblasts. Prevention of epidermal cell migration (Williams-Masson et al., 1997; Sawa et al., 2003) or disruption of neuroblast organization (George et al., 1998; Chin-Sang et al., 2002) perturbs enclosure.

*zen-4(w35)* zygotic mutants display severe morphogenetic defects, including somewhat abnormal enclosure with subsequent body shape defects or rupture (Fig. 1A; Supplementary Movie S1, which can be viewed at <http://www.interscience.wiley.com/jpages/1058-8388/suppmat>), indicating a requirement for *zen-4* function before or during ventral enclosure. Based on *AJM-1::GFP* expression in *zen-4(w35)* homozygotes, it is apparent that *zen-4(w35)* mutants possess far fewer, but larger, epidermal cells compared with wild-type (Fig. 1B). Remarkably, embryos possessing too few epidermal cells can complete both dorsal intercalation (Supplementary Movies S2, S3) and ventral enclosure (Fig. 1B; Supplementary Movies S4, S5). In some cases, as few as 6–10 cells engage in dorsal intercalation, as compared with the normal 20 cells. Similarly, ventral enclosure can occur with as few as 8 cells, compared with the normal 20. Defects in epidermal cell number were observed in 100% of *w35* homozygotes observed ( $n > 25$  embryos).

Cytokinesis defects in ventral neuroblast precursors could contribute to defects in *zen-4(w35)* mutants, so we labeled homozygous *zen-4(w35)* embryos at the onset of ventral enclosure with the vital plasma membrane dye FM 4-64 and the vital DNA stain Hoechst 33342, and examined them before epidermal cells migrated to the ventral surface of the embryo. In every mutant embryo observed ( $n = 6$ ), several multinucleate ventral neuroblasts could be detected (Fig. 1C–C').

We were unable to find cells without cytokinesis failure that failed to migrate in the epidermis of *w35* homozygotes. However, it is possible that there are such cells, although they presumably occur very infrequently.



**Fig. 1.** *zen-4(w35)* embryos display epidermal and neuronal lineage defects. **A:** Frames from a 4d movie of wild-type and *zen-4(w35)* embryos, acquired as described previously (Raich et al., 1998). Time in hours relative to the start of the movie is shown. **B:** Frames from multiphoton movies of wild-type (left column) and *zen-4(w35)* embryos (right column) expressing *AJM-1::GFP*. Elapsed time is shown. The *zen-4(w35)* embryo undergoes proper polarized epidermal cell migration toward the ventral midline yet does not have the proper number of cells. Note the single pair of leading cells, as opposed to two pairs in wild-type, migrates to the ventral midline in the *zen-4(w35)* embryo (asterisks). **C–C':** Homozygous *zen-4(w35)* embryos contain multinucleate neuroblasts. In this focal plane, two binucleate cells are visible on the embryo's ventral surface (one in the box, the other denoted by a barbed arrowhead). The boxed region is shown at  $\times 3$  magnification in the inset. The arrow in the inset denotes the plasma membrane; arrowheads denote nuclei. Scale bars = 10  $\mu\text{m}$ .

To attempt to detect morphogenetically defective yet cytokinetically competent cells, we also examined a weaker allele, *px47*, carrying the *ajm-1::gfp* transgene. As previously reported, many *zen-4(px47)* homozygotes successfully develop into hatched larvae, but 5–6% are enclousure defective (Portereiko et al., 2004).

Of 47 embryos that we filmed using multiphoton microscopy, 2 were enclosure abnormal. In the one embryo where cell borders could be imaged clearly, the defect coincided with a large, presumably cytokinesis-defective ventral epidermal cell (data not

shown). Thus unlike the case for the pharyngeal primordium (Portereiko et al., 2004), epidermal defects in *px47* embryos probably result from stochastic lineage defects in the epidermis, as they do in *w35* homozygotes.

In summary, a hallmark of morphogenetic failure in *zen-4* mutant embryos is defective cytokinesis in epidermal and neuronal precursors. A dearth of molecular markers specific to dorsal or ventral epidermal cells precluded us from making more refined assessments of cell fate alterations in *zen-4* mutants, but based on their behaviors, they retain qualities commensurate with the basic epidermal fates appropriate to their position. We also conclude that, despite widespread late defects in cytokinesis of epidermal precursors, remarkably, some defective cells can nevertheless become correctly polarized and develop the intracellular machinery necessary to initiate and complete epidermal cell migrations.

### Requirement for *zen-4* Function Precedes Epidermal Morphogenesis

To determine whether *zen-4* is expressed during ventral enclosure, we used a *zen-4::gfp* translational fusion, driven by the endogenous *zen-4* promoter, to rescue *zen-4(or153)* temperature-sensitive mutants to viability. *or153* homozygous progeny of *or153* mothers show complete loss of function for *zen-4* within 1–2 min of being moved to the restrictive temperature and are indistinguishable from *zen-4(w35)* nulls (Severson et al., 2000; our unpublished observations). Thus, this construct is fully functional, and the locations in which it is expressed are sufficient for complete rescue. Moreover, this construct recapitulates the pattern of protein expression assessed by means of immunostaining in the early embryo as reported previously (Powers et al., 1998; Raich et al., 1998; Jantsch-Plunger et al., 2000). During mid-embryogenesis, ZEN-4::GFP is expressed in all dividing cells, consistent with its role in cell division (Fig. 2A,E,I). However, at the onset of dorsal intercalation, when epidermal precursors have ceased dividing, ZEN-4::GFP becomes undetectable in the epidermis (Fig. 2C,G,K). Neuroblasts, which underlie the epidermis,

complete their terminal divisions slightly later than epidermal precursors (Sulston et al., 1983). ZEN-4::GFP is expressed for a correspondingly longer period, and is visible in spindle mid-bodies as ventral enclosure begins (Fig. 2D,H,L) and before it completes (Fig. 2M,Q,U). By the end of enclosure, little or no ZEN-4::GFP is detectable (Fig. 2N,R,V). As elongation of the embryo begins, virtually no ZEN-4::GFP can be detected, although occasional bright spots can be observed (Fig. 2O,S,W). By the 1.5- to 2-fold stage of elongation, no ZEN-4::GFP can be detected (Fig. 2P,T,X). Similar results were obtained using an anti-ZEN-4 antibody (Fig. 2Y–Z'). The lack of expression of the rescuing GFP construct or endogenous ZEN-4 in epidermal precursors during morphogenesis suggests that either the requirement for *zen-4* function in epidermal precursors precedes morphogenesis, or that epidermal morphogenetic defects result from indirect effects of loss of *zen-4* function in other cells, such as ventral neuroblasts.

To further investigate when and where *zen-4* is required for epidermal morphogenesis, we performed temperature shift experiments using *zen-4(or153)*. Animals shifted to the restrictive temperature of 25°C before the terminal divisions of epidermal precursors display severe morphogenetic defects ( $n = 14$ ; Fig. 3A,A'), whereas those shifted at the onset of dorsal intercalation survive to hatching ( $n = 4$ ; Fig. 2B,B'), indicating that ZEN-4 is not required during dorsal intercalation or ventral enclosure, but before the terminal divisions that generate the epidermis.

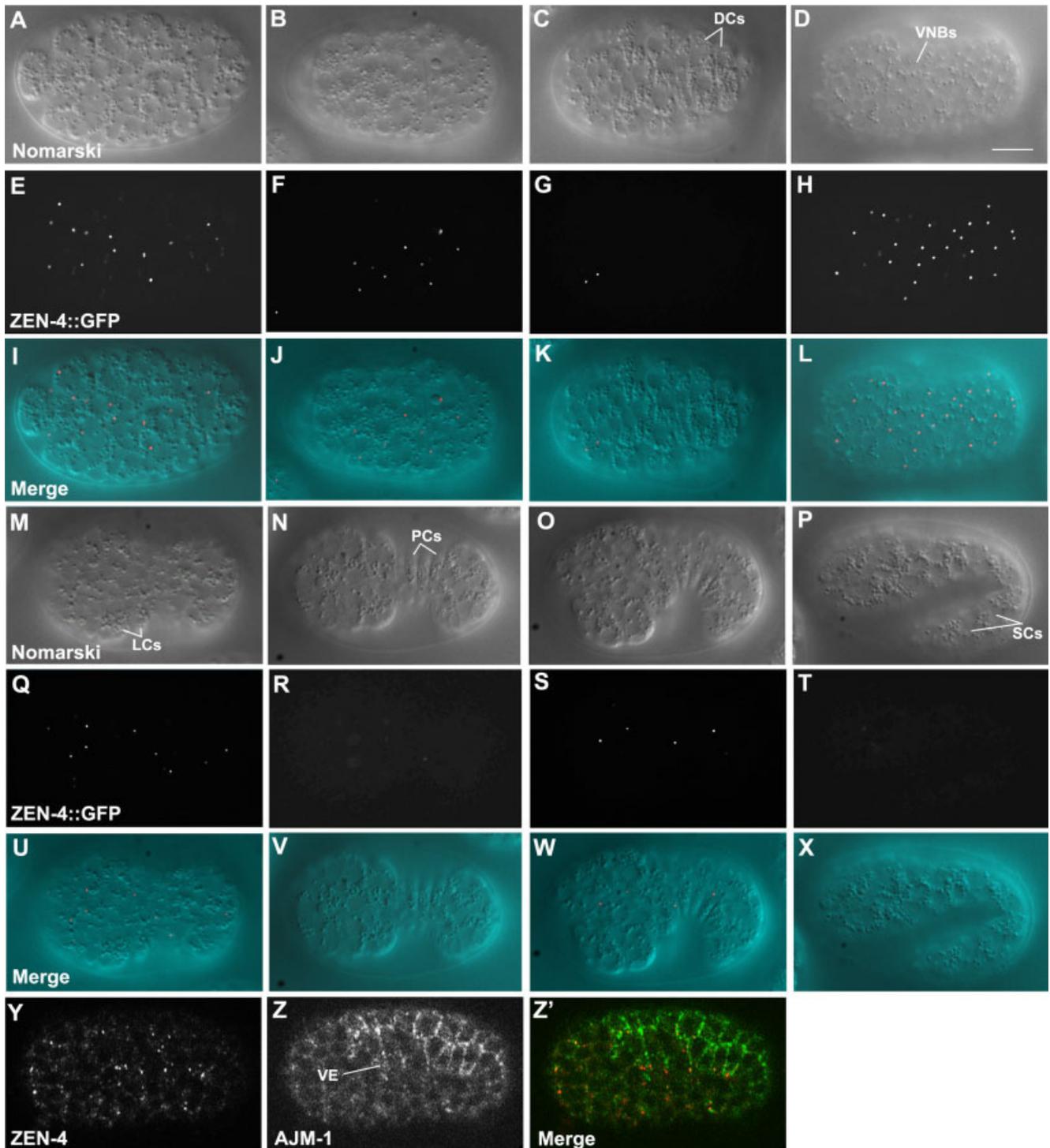
### Zygotic *zen-4* Function in Epithelial Cells Is Sufficient for Successful Enclosure and Intercalation

To determine in which tissues *zen-4* function is essential, we attempted to use neuronal-specific (*unc-119*; Madero and Pilgrim, 1995) and epithelial-specific (*ajm-1*; Koppen et al., 2001) promoter constructs to drive expression of ZEN-4 in a *zen-4* null background.  $P_{unc-119}::zen-4$  constructs could rescue *zen-4(w35)* homozygotes to viability. Unfortunately, in our hands,

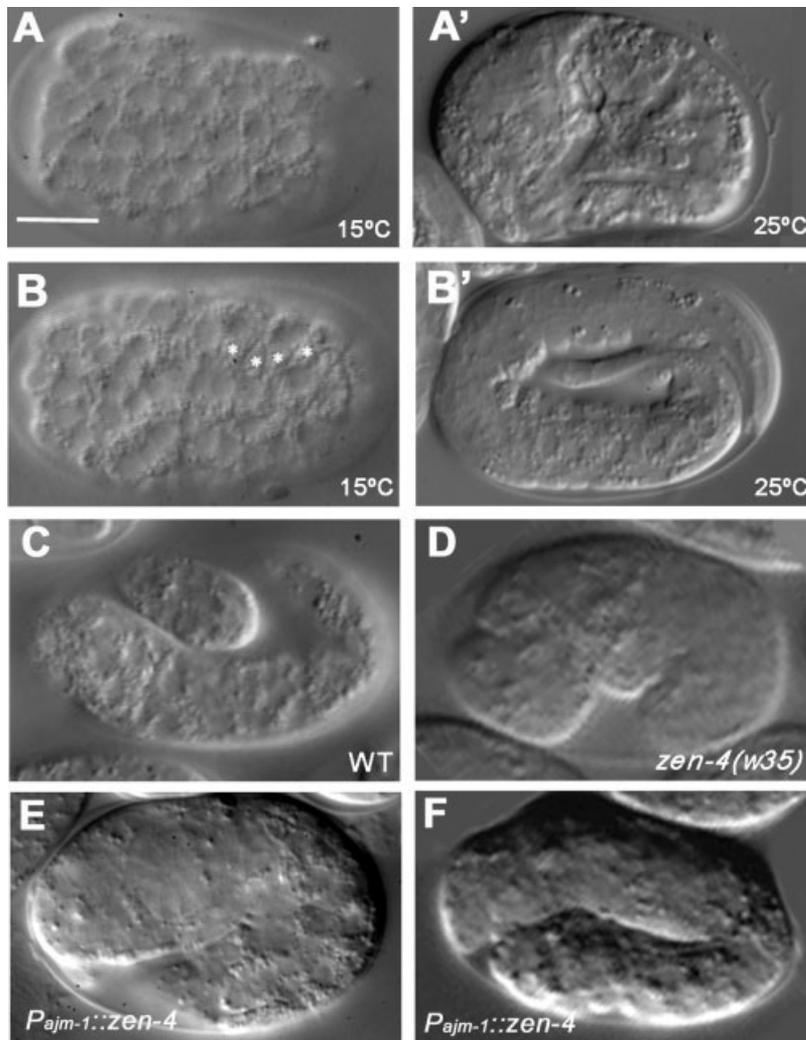
*unc-119::gfp* expression, although exclusively neuronal in later embryos, is widespread in the early embryo, and prominent in epidermal precursors (Supplementary Movie S6), making the *unc-119* promoter unsuitable for tissue-specific rescue of early zygotic loss of *zen-4* function in the epidermis. However, we found the *ajm-1* promoter to be exclusively expressed in epithelial cells or their precursors at all times, and its time of activation to be sufficiently early to drive *zen-4* in epithelia immediately before the terminal divisions that produce epidermal cells. In *zen-4(w35)* heterozygotes expressing  $P_{ajm-1}::zen-4$ , no decrease in homozygous dead embryos from the expected 25% occurred ( $26.0 \pm 3.0\%$  lethality, mean  $\pm$  SEM, progeny from  $n = 9$  worms) but elongation defects were partially rescued, so that embryos could progress to the twofold stage (Fig. 3C–F). Of 12  $P_{ajm-1}::zen-4$ ; *zen-4(w35)* embryos examined using 4d microscopy, 4 progressed to the twofold stage. Moreover, three of these rescued embryos did not possess the prominent large epidermal cells observed in *w35* homozygotes, based on analysis of Nomarski 4d movies. One of the partially rescued embryos was apparently a mosaic, because there were regions of the epidermis that contained large cells and in which elongation was defective, but other areas in which no large cells could be observed, and elongation appeared more fully normal. In contrast, no *zen-4(w35)* homozygotes examined elongated to twofold ( $n = 19$ ), and all possessed abnormally large epidermal cells ( $n = 15$  embryos). These results suggest that rescuing *zen-4* function in epidermal cells and their precursors is sufficient to rescue many of the defects observed in *zen-4(w35)* embryos, but suggests that there may be additional requirements for *zen-4* function in other tissues, or in epithelial precursors before the activation of the *ajm-1* promoter, for completion of morphogenesis.

### Zygotic Loss of *zen-4* Leads to Morphogenetic Defects in the *C. elegans* Epidermis

Based on the timing of its requirement, partial rescue of defects using an epithelial-specific promoter, and



**Fig. 2.** Loss of *zen-4::gfp* expression correlates with onset of morphogenesis. Embryos expressing *zen-4::gfp* were imaged, and color merges of the Nomarski and fluorescence images were produced as described in the Experimental Procedures section. Anterior is to the left in all images. **A,E,I:** Dorsal view, early intercalation. A few spindle mid-body remnants (small dots in E, I) are visible. **B,F,J:** Ventral view, onset of enclosure. No epidermal cells are visible at this time on the ventral surface. A small number of ventral neuroblast precursors with mid-body remnant expression are visible. **C,G,K:** Dorsal view, late dorsal intercalation. Dorsal epidermal cells (DCs) are completing intercalation. Virtually no cells have ZEN-4::GFP expression. **D,H,L:** Ventral view, early enclosure. Some ventral neuroblast precursors (VNBs) show mid-body expression of ZEN-4. **M,Q,U:** ventral view, late enclosure. Few ventral neuroblasts, and no epidermal cells express ZEN-4::GFP. **N,R,V:** Ventral view, enclosure complete. No ZEN-4::GFP is visible. **O,S,W:** Lateral view, comma stage. Four cells express ZEN-4::GFP. **P,T,X:** Lateral view, twofold stage of elongation. No ZEN-4::GFP is visible. **Y-Z':** Immunostaining for ZEN-4 and AJM-1, a junctional marker (lateral view). Mid-body signal is visible in neuroblasts (Z', red). The leading edge of the ventral epidermis is visible (VE, green in Z'). No epidermal ZEN-4 is detectable. Scale bar = 10  $\mu$ m.



**Fig. 3.** Temporal and tissue-specific requirements for *zen-4*. A–B'. **A:** *zen-4(or153)* embryo shifted before final epidermal divisions occur. **A':** Terminal phenotype of the embryo in A, which has not completed morphogenesis. **B:** *zen-4(or153)* embryo just before being shifted to 25°C. Note that the process of dorsal intercalation has begun (asterisks), indicating that all epidermal cells have already been born. **B':** Subsequent development of the embryo in B. The embryo has elongated into a full-length worm and, therefore, has performed morphogenesis properly. C–F: Partial rescue of *zen-4* phenotypes using a full-length *zen-4* construct driven by the *ajm-1* promoter. **C:** Wild-type embryo at the threefold stage of embryogenesis. **D:** Representative *zen-4(w35)* embryo at a comparable developmental age; arrest occurs immediately after ventral enclosure. E, F: Representative *zen-4(w35); P<sub>ajm-1</sub>::zen-4* embryos. **E:** Embryo rescued for ventral enclosure, but arrested at 1.5-fold elongation. **F:** An embryo rescued to the twofold stage. Note that twofold embryos still have body shape defects that are not completely rescued. Scale bar = 10  $\mu$ m.

phenotypic defects, our results indicate that loss of zygotic *zen-4* function results in morphogenetic defects due to stochastic late cytokinesis defects in epidermal precursors. These stochastic failures result in a disorganized epidermis that cannot completely execute the normal epidermal morphogenetic program. Although there are multinucleate neuroblasts in *zen-4(w35)* embryos, we believe it is unlikely that these subtle defects are

the main cause of morphogenesis failure. The availability of a truly neuroblast-specific promoter that could drive wild-type ZEN-4 in a *zen-4(w35)* mutant background could definitely resolve this issue in the future.

Perhaps more remarkable than the defects in *zen-4(w35)* embryos is their surprisingly robust morphogenesis, despite lacking as many as half of their epidermal cells. Previous studies have suggested that cytokinesis de-

fects in neuronal lineages in *C. elegans* can result in cells that perform largely normal functions. In cases in which a terminal division would result in two different daughter cells, tetraploid neuronal cells appeared to adopt one or the other of the two normal daughter cells' fates (White et al., 1982). The lineages of lateral and ventral epidermal cells, as well as anterior dorsal cells, are complex; they are derived from the founder cell AB. Posterior dorsal cells have a less complicated lineage; they are derived from the C founder cell (Sulston et al., 1983). However, in almost all cases, examination of the lineage indicates that terminally differentiated sister cells give rise to the same basic type of epidermal cells (i.e., ventral, lateral, or dorsal) in both cases. Thus, one might expect that tetraploid cells resulting from a cytokinesis failure at the division before the terminal division might maintain a largely normal fate, as we observed.

### Zygotic Loss of *zen-4* as an Example of “Molecular Vitalism”

While the timing of cytokinesis defects inferred from comparison to wild-type embryos suggests that multinucleate cells in the epidermis retain their essential character as epidermal cells, it is more surprising that many aspects of dorsal intercalation and ventral enclosure still occur in *zen-4* mutants possessing such cells. This result suggests that polarization occurs normally in the abnormally large epidermal cells we observe in the mutants. Because the positions of these cells are abnormal, this finding suggests that at least some aspects of their polarization, changes in shape, and directed migration are determined by means of local signals.

These results are strongly reminiscent of classic experiments in the 1940s by Gerhard Fankhauser (1945). Fankhauser studied the effects of ploidy on newt embryos by examining the effects of suppression of early cleavages on subsequent morphogenesis. The resulting polyploid embryos had fewer cells that were significantly larger. Remarkably, the overall dimensions of specific tissues, and indeed the entire adult animal, were

normal. Perhaps most famously, Fankhauser examined the development of the pronephric duct and found that the average number of cells spanning the circumference of the duct in cross-section decreased with increasing ploidy, even though the overall dimensions of the duct remained the same. Most dramatically, in pentaploid embryos as few as one to three cells spanned the circumference of the duct, whereas in normal diploid cells, this was accomplished by three to five cells. Such compensatory mechanisms have been termed "molecular vitalism" (Kirschner et al., 2000), that is, they represent a robust network of molecular and cellular regulatory processes that can compensate for extensive perturbation. *zen-4* mutant embryos appear to exhibit such "vitalistic" regulation of morphogenesis. It remains to be seen what signals may be causing their cells to act in such a manner.

## EXPERIMENTAL PROCEDURES

### Temperature Shift Experiments

Temperature shift experiments used the temperature-sensitive allele *zen-4(or153ts)* (Severson et al., 2000). Homozygous *zen-4(or153ts)* embryos were mounted on a 5% agar pad as described previously (Raich et al., 1998), with the exception that the mount was prepared in a room cooled to 15°C. Mounts were then monitored at 15°C using Nomarski microscopy until the desired stage, at which time mounts were moved to a 25°C incubator. Mounts were monitored periodically for progress and pictures of terminal embryos taken.

### Labeling of Cells With FM4-64

Homozygous *zen-4(w35)* embryos were labeled with 5 µg/ml Hoechst 33342 (Molecular Probes, Eugene, OR) and 10 µg/ml FM 4-64 (Molecular Probes), following laser permeabilization of the eggshell, as described in (Williams-Masson et al., 1998). Wide-field fluorescence images of embryos at the onset of ventral enclosure were obtained using a Nikon Optiphot mi-

croscope equipped with a Hamamatsu Orca cooled CCD camera.

### Immunostaining and Imaging of *gfp* Strains

Antibody staining using anti-ZEN-4 antibodies was performed as previously described (Raich et al., 1998). MH27 antibodies were a gift from R. Waterston and were used at a dilution of 1:100. All imaging was performed on a Bio-Rad MRC1024 microscope.

For analysis of morphogenesis in *zen-4(w35)* mutants, heterozygous *w35* hermaphrodites were cotransformed with pBR957 and pJS151 (*ajm-1::gfp*; Mohler et al., 1998) by standard protocols (Mello and Fire, 1995). The extrachromosomal array was integrated into the genome using gamma rays as described (Mello and Fire, 1995). For integration, the worms were exposed to ~3,000 rad. Three lines containing integrants were identified, and the line demonstrating the brightest fluorescence was outcrossed four times before observation by live fluorescence microscopy, which was performed using multiphoton microscopy as previously described (Raich et al., 1999). Strain SM831 [*zen-4(px47)*; *ajm-1::gfp*] (Portereiko et al., 2004), was a gift from S. Mango.

Imaging of strain MG170, a *zen-4(or153)* mutant strain carrying a rescuing *zen-4::gfp* transgene (Jantsch-Plunger et al., 2000), and DP132, which carries *edIs6* [pDPMMUGF12(*unc-119::gfp*), pRF4(*rol-6<sup>D</sup>*)] (courtesy D. Pilgrim, University of Calgary), was performed using spinning disc confocal microscopy using a Perkin-Elmer Ultraview LCI system mounted on a Nikon E600 upright microscope as described (Sheffield et al., 2007). Overlays of Nomarski and fluorescence images were performed using Adobe Photoshop.

### Production of Tissue-Specific *zen-4* Constructs

To address the tissue specificity of *zen-4* function, a construct was made using the *ajm-1* promoter to drive wild-type *zen-4* expression. To construct pBR980 [*P<sub>ajm-1</sub>::zen-4*], 5 kb upstream of the *ajm-1* transcriptional initiation site was amplified from

C25A11 (Koppen et al., 2001) using the following primers: MH27 5' *Bam*HI, GTTGGATCCCGATTGACCGTTCGATAAGACG; MH27 3' *Xho*I: TCCCTCGAGTCGTCGGTAGTACTC-GTCC.

The polymerase chain reaction (PCR) product was cloned into PCR-Script according to the manufacturer's instructions (Stratagene, La Jolla, CA), generating pBR979. The *zen-4* coding region and 3' untranslated region were amplified from plasmid pBR938 using the following primers: ZEN-4 5' *Xho*I, TCCCTCGAGATGTCGTCGCGTAAACGAGGAATAACTCC; ZEN4 3' *Kpn*I, GCGGTACCCATCATGTCTCGAACAAAGCTCAG.

The PCR product was cloned into PCR-Script according to the manufacturer's instructions, (Stratagene), and subsequently cloned into the *Xho*I/*Kpn*I sites in pBS SKII (Stratagene), generating plasmid pBR978. To generate pBR980, the *ajm-1* promoter from pBR979 was then ligated into *Bam*HI and *Xho*I sites in pBR978. pBR980 was then injected into young adult worms to make *jcEx28* transgenic animals using standard methods (Mello and Fire, 1995). Terminal phenotypes of *zen-4(w35)* and *zen-4(w35);jcEx28* embryos were scored from 4d Nomarski movies.

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