C. elegans Enabled Exhibits Novel Interactions with N-WASP, Abl, and Cell-Cell Junctions

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Summary

Ena/VASP proteins are associated with cell-cell junctions in cultured mammalian cells [1] and Drosophila epithelia [2, 3], but they have only been extensively studied at the leading edges of migratory fibroblasts, where they modulate the protrusion of the leading edge [4]. They act by regulating actin-filament geometry, antagonizing the effects of actin-capping protein [5]. Embryos lacking the C. elegans Ena/VASP, UNC-34, display subtle defects in the leading edges of migrating epidermal cells but undergo normal epidermal morphogenesis. In contrast, embryos lacking both UNC-34 and the C. elegans N-WASP homolog have severe defects in epidermal morphogenesis, suggesting that they have parallel roles in coordinating cell behavior. GFP-tagged UNC-34 localizes to the leading edges of migrating epidermal cells, becoming redistributed to new junctions that form during epidermal-sheet sealing. Consistent with this, UNC-34 contributes to the formation of cadherin-based junctions. The junctional localization of UNC-34 is independent of proteins involved in Ena/VASP localization in other experimental systems; instead, junctional distribution depends upon the junctional protein AJM-1. We also show that Abelson tyrosine kinase, a major regulator of Enabled in Drosophila, is not required for UNC-34/Ena function in epithelia. Instead, our data suggest that Abelson kinase acts in parallel to UNC-34/Ena, antagonizing its function.

Results and Discussion

UNC-34 and WSP-1 Are Required for Dynamic Protrusive Activity

Previous work showed that UNC-34/Enabled is not required for epidermal morphogenesis and that unc-34 is genetically redundant with the gene encoding the sole C. elegans N-WASP homolog, wsp-1. Embryos lacking both proteins show defects in the ventral enclosure of the embryo [6]. We confirmed these results (Figures 1A–1B; Movies S1 and S2 in the Supplemental Data available online) and examined genetic interactions between WVE-1/WAVE, WIP-1/WIP, and UNC-34 (Supplemental Results and Discussion). We further investigated the wsp-1/unc-34 interaction by using dynamic analysis of protrusive activity. In wild-type embryos, the two pairs of ventral leading epidermal cells show extensive, relatively broad protrusions (Figure 1C). This protrusive region, which we refer to as the leading cell protrusive zone (LCPZ), has a dynamic perimeter and persists throughout migration (Movie S3). The pocket cells, in contrast, display much narrower projections (Figure 1C) whose lifetime is less than the 50 s interval we typically used for filming.

Protrusions in unc-34(gm104); wsp-1(RNAi) embryos appear to be quite different from those in the wild-type (Figures 1D and 1D; Movie S4). Although the overall actin morphology is comparable to that of the wild-type (Figure S3), the LCPZ in these embryos is less dynamic, shorter, and blunted, and quantitative analysis shows that this difference is statistically significant (Wilcoxon rank sum test; z = 0.05; Supplemental Results and Discussion). Pocket cell migration is less disrupted, but the failure to complete enclosure causes the arrest of pocket cells before they reach the ventral surface of the embryo, making protrusions difficult to score. Although unc-34 embryos enclose successfully and have normal LCPZ morphology (Figure 1E), pocket cell protrusions are somewhat shorter and are more infrequent than they are in the wild-type (z = 0.05; Supplemental Data). This phenotype is not scorable in unc-34; wsp-1 embryos. We did not detect any such defects in the pocket cells of wsp-1(RNAi) embryos.

We attempted to test for epidermal-specific requirements for wsp-1 and unc-34 in several ways. First, we used an epidermal-specific RNA interference (RNAi) strain [7] to knock down wsp-1 and unc-34 function, but we observed no lethality, perhaps because the promoter used in this strain is active too late in development to confer robust RNAi sensitivity. Attempts to drive expression of unc-34 in unc-34(gm104) mutants with a pan-epithelial promoter were likewise problematic because of selectively weak expression in the epidermis, although these results do suggest that at later stages, unc-34 acts specifically in epithelia (see below). Finally, we used a strain designed to reduce wsp-1 function in the epidermis, and this strain has been reported to result in some morphogenesis defects and lethality [8]. We could not replicate these results, nor have we ever observed similar effects when reducing wsp-1 function alone in any other context, including the putative null deletion, wsp-1(gm324). However, we did observe sporadic lethality in F3 embryos derived from unc-34 homozygous mothers carrying the wsp-1 knockout constructs, with phenotypes qualitatively similar to unc-34(gm104);wsp-1(RNAi) embryos (data not shown). This suggests that wsp-1 is specifically required in the epidermis. The synergistic genetic interaction between unc-34 and wsp-1 is consistent with several models at the molecular level (Figure S5).
UNC-34 and WSP-1 Modulate Epidermal-Sheet Sealing

Actin-based protrusions are critical to cell migration, but they also contribute to epithelial-sheet sealing [1, 9]. Some unc-34(gm104);wsp-1(RNAi) embryos display weaker migration phenotypes, in which all cells eventually reach the midline (Figures 1F–1G). These embryos often display gaps between junctions at the midline (Figures 1F and 1G) and ultimately arrest because of rupture. Such phenotypes are consistent with the disruption of protrusions involved in junction formation. Moreover, in ventral cells that fail to meet at the midline, junctional molecules are recruited to the leading edge in the absence of contact with a contralateral partner (Figure 1G). This cell-contact-independent "hemi-junction" formation appears to be in response to a general apical junction development program involving both the DLG-1/AJM-1 complex (Figure 1G) and the cadherin complex (data not shown), and it occurs in other unrelated enclosure mutants and occasionally in wild-type cells (M.S. and J.H., unpublished data).

UNC-34-GFP Localizes to the Leading Edge of Migrating Cells and to Apical Junctions

A polyclonal antibody against UNC-34 shows broad cortical localization, as well as enrichment near apical junctions in epithelial cells (Figures 2A and 2A'). To better determine the subcellular localization of UNC-34 in living embryos, we generated a strain expressing full-length UNC-34 protein, tagged with green fluorescent protein (GFP) at its C terminus, under the control of the HMR-1A promoter (PHMR-1A). This promoter is active in all major epithelia during embryonic and postembryonic development, as well as in a defined set of cells.

GFP expression is first detected throughout the cytoplasm of most cells in early embryos. Once the epidermis is formed, UNC-34::GFP becomes enriched at cell-cell junctions. As leading cells approach the ventral midline, UNC-34 accumulates at the leading edge (Figures 2B and 2C, Movie S5). UNC-34 is also present at the leading edge of migrating pocket cells, but at a lower level. As morphogenesis proceeds, the junctional localization becomes more pronounced, and it persists throughout subsequent development (Figures 2C–2E). It is tempting to speculate that the cessation of cell migration is functionally linked to the relocation of UNC-34 to nascent junctions and that the relocation of UNC-34 is part of a hierarchy of changes in actin dynamics that favor the formation of cell junctions rather than continued protrusion. UNC-34::GFP also surrounds apoptotic cells as they are engulfed by epidermal cells (Figure S6).
AJM-1 Participates in UNC-34 Recruitment to the Apical Junction

In primary keratinocytes, cadherin-catenin function is required for the recruitment of Mena and VASP to cell junctions [1]. We therefore examined UNC-34::GFP distribution in offspring of hmr-1 germline mosaics, which lack both maternal and zygotic HMR-1/E-cadherin. These embryos display junctional localization of UNC-34::GFP that is essentially indistinguishable from wild-type embryos (Figure 2K). We obtained similar results with hmp-1/α-catenin and hmp-2/β-catenin null mutants (data not shown). Thus, the recruitment of UNC-34::GFP

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Figure 2. UNC-34 localizes to the leading edge of cell protrusions and to apical junctions

(A and A’) Confocal projections of dorsolateral (A) and ventrolateral (A’) surfaces (anterior is to the left) of the same embryo stained at mid-enclosure with anti-UNC-34 show broad expression throughout the embryo with enrichment at apical epidermal junctions. (B–E) Time-lapse sequence of projected confocal Z series, visualizing UNC-34::GFP in the ventral leading cell region (anterior is to the left) during enclosure. Besides apical junction enrichment, UNC-34::GFP is also present at the leading edge (arrowheads) of leading cell protrusions (C). Junctions enriched in UNC-34::GFP become apparent along the anterior and posterior borders of migrating cells (C, arrow). Protrusions in contralateral partners meet at the ventral midline (D, arrow), where apical junctions enriched with UNC-34::GFP eventually form (E, arrow). (F–H) Confocal images of a wild-type 3-fold stage embryo expressing UNC-34::GFP (F) costained for AJM-1 (G). A merged image is shown in (H). (I–K) Representative wide-field images of wild-type (I), ajm-1(ok160) (J), and hmr-1(zu389) maternal and zygotic loss (K) embryos expressing UNC-34::GFP. UNC-34::GFP at apical junctions is mislocalized into puncta in ajm-1 embryos (J, arrowheads) but is largely normal in hmr-1 embryos.

(L–O) Immunostaining of UNC-34 in embryos carrying a truncated ajm-1::gfp transgene lacking a putative consensus binding site for Ena/VASP proteins (ajm-1(102-868)::gfp). (L) and (M) show a wild-type embryo expressing AJM-1(102-868)::GFP. UNC-34 localizes to junctions (M, arrow). UNC-34 is also prominently expressed in neurons of the nerve ring (asterisk). (N) and (O) show an ajm-1(ok160) embryo rescued by ajm-1(102-868):gfp. Although the truncated AJM-1 localizes to junctions (N), it is insufficient to localize UNC-34 there (O), despite robust expression in neurons (asterisk).

Scale bars represent 10 μm.
to the apical junction is independent from the cadherin-catenin complex. Vinculin and zyxin have also been implicated as Ena/VASP recruitment factors [1, 10]. However, embryos lacking the only C. elegans zyxin family member show normal junctional localization of UNC-34::GFP (data not shown), and vinculin is not expressed in C. elegans epithelial cells [11].

Ena/VASP proteins are recruited to subcellular sites through their EVH1 domains, which bind specifically to the F/LPPPP motif [12]. We therefore examined known junctional proteins for such motifs. AJM-1, a coiled-coil protein with some similarity to the vertebrate protein tricohyalin [13], contains the sequence DLPPPP, strongly matching the consensus for EVH1-binding peptides [14] and also colocalizes with UNC-34::GFP (Figures 2F–2H), making it a good candidate. We examined the localization of UNC-34::GFP in embryos homozygous for an ajm-1 null allele, ok160. Although UNC-34::GFP still localizes to junctions in ajm-1(ok160) homozygotes, the intensity of the junctional signal is greatly reduced relative to the wild-type (Figure 2J), and its distribution along junctions is nonuniform, with the majority of UNC-34::GFP being concentrated at tri-cellular junctions.

We next tested whether the region of AJM-1 that includes the DLPPPP motif is necessary to maintain UNC-34 distribution by using ajm-1(ok160) mutants rescued with a transgene that does not contain this region but is sufficient for rescue of essential AJM-1 functions [13]. In wild-type embryos carrying the transgene, UNC-34 localizes to junctions (Figures 2L and 2M). In rescued ajm-1 null mutants, however, little or no UNC-34 is detectable at junctions via immunostaining, even though nonepithelial cell types express UNC-34 normally (Figures 2N and 2O). Thus, although it is not the only factor influencing the localization of UNC-34, AJM-1 is a major determinant of its recruitment to epithelial cell junctions. Ena/VASP proteins can directly bind F-actin [15], so UNC-34 might also be recruited to junctions through its association with actin filaments, accounting for its AJM-1-independent junctional localization.

**UNC-34 Contributes to Cadherin-Mediated Epidermal-Sheet Sealing**

In order to further investigate the role of UNC-34 at epithelial junctions, we took advantage of a hypomorphic mutation affecting the C. elegans α-catenin, HMP-1, which sensitizes cells to perturbations in cadherin function [16]. We constructed double-mutant combinations between hmp-1(fe4) and unc-34(gm104). hmp-1(fe4) mutants show variable defects in epidermal morphogenesis (Figures 3A and 3D): A small minority of animals arrest with defects in ventral enclosure, but the majority are viable and fertile. In contrast to the respective single mutant phenotypes (Figures 3A, 3B, and 3D), the unc-34 hmp-1(fe4) double-mutant combination exhibits completely penetrant maternal-effect lethality (Figures 3C and 3D). Significant numbers of arrested embryos display defects in ventral enclosure indicative of embryos lacking cadherin function. Embryos that successfully enclose display severe elongation defects (Hmp).

To investigate the basis of this lethality, we filmed unc-34(gm104) hmp-1(fe4) embryos. Of 15 embryos that subsequently failed to undergo ventral enclosure, four showed complete retraction of the leading edge of the ventral epidermises dorsally and 11 showed partial ventral enclosure but extruded their internal organs once elongation began (Figure 3C). In all cases, the leading cells migrated to the ventral midline; the defect is therefore likely due to the failure to establish stable junctions, rather than a prior failure in leading cell migration. This defect is characteristic of animals completely lacking cadherin-catenin function [9]. The unc-34::gfp transgene complemented the embryonic lethality of unc-34 hmp-1(fe4) double mutants (Figure 3D).

We next examined the localization of HMR-1 in gm104 fe4 double-mutant embryos. The distribution of HMR-1 is abnormal in hmp-1(fe4) embryos, showing occasional punctate accumulations and discontinuities [16]. However, there was no detectable difference in the distribution of HMR-1 in gm104 fe4 double mutants compared to either single mutant (Figure S7). Thus, the enhanced morphogenetic defects caused by the loss of unc-34 function are not attributable to loss of HMR-1/cadherin localization.

To determine whether unc-34/hmp-1(fe4) synergy occurs specifically in epithelial cells, we used the dlg-1 promoter to drive unc-34::gfp expression in epithelial cells (see Supplemental Experimental Procedures). This construct is weakly and incompletely expressed in the epidermis (J.P., unpublished data), but we observed partial rescue of the synthetic lethality in the brood of an unc-34(gm104) hmp-1(fe4) homozygote expressing unc-34::gfp. This result suggests that the loss of unc-34 function in epithelia is responsible for the observed synthetic lethality.

Recent work has led to the proposal that α-catenin acts to regulate actin dynamics at nascent adherens junctions, favoring the formation of unbranched, bundled actin filaments, and suppressing branched actin networks (reviewed in [17]). This would explain the requirement for UNC-34 in animals with impaired HMP-1 function; in this case, UNC-34 might partially compensate for reduced α-catenin activity. Given the association of Ena/VASP proteins with adherens junctions in other organisms, it is likely that there is a conserved relationship between α-catenin and Ena/VASP proteins.
the loss of abl-1 function partially alleviates the requirement for unc-34 function in hmp-1(fe4) embryos. Because unc-34(gm104) does not produce any detectable UNC-34 protein, abl-1(ok171) appears to act as a bypass suppressor, a genetic interaction that implies that unlike in Drosophila, Abelson kinase acts in parallel to UNC-34/Ena and that it antagonizes UNC-34 function.

In vitro experiments indicate that Ena/VASP proteins antagonize capping protein [5]; abl-1 loss of function could lead to a reduction of actin-capping activity and thereby partially relieve the requirement for UNC-34. In order to address this possibility, we attempted to suppress the synthetic lethality of unc-34(gm104) hmp-1(fe4) by reducing the expression of the two C. elegans capping proteins with RNAi. However, even a mild reduction in the expression of these proteins resulted in arrest during early embryonic development, precluding assessment of genetic suppression (data not shown).

In summary, our studies have uncovered novel interactions between UNC-34/Ena and components of both the leading edge and cell-cell junctions in the C. elegans epidermis. Future work aimed at identifying molecular components that recruit and modulate UNC-34/Ena function in these subcellular compartments should yield further insights into how this important actin regulator functions during morphogenesis.

Supplemental Data
Supplemental Results and Discussion, Experimental Procedures, seven figures, and five movies are available at http://www.current-biology.com/cgi/content/full/17/20/1791/DC1/.

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Note Added in Proof

While this work was under review, Gates et al. published a report describing the requirement for and dynamic localization of Ena during morphogenesis in the Drosophila embryo [20]. They found that Ena is dispensable for dorsal closure and for general cell-cell adhesion but that Ena modulates protrusive activity at the leading edge of the epithelium, where it localizes to filopodia. This work is consistent with our analysis of lamellipodial localization of UNC-34 during ventral enclosure and with our analysis of roles for UNC-34 during cadherin-mediated events in C. elegans.
Supplemental Data

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Supplemental Results and Discussion

UNC-34 Aids CeSCAR/WAVE in Initiating Cell Migration

Several barbed-end modulators act via the Arp2/3 complex, which nucleates new actin filaments via the formation of side branches from existing filaments [S1]. Reducing the function of various members of the Arp2/3 complex results in embryonic lethality in C. elegans well before the initiation of morphogenesis [S2]. However, in order to perform its role as an actin nucleator, Arp2/3 must be bound and activated by one of several families of molecules, allowing for the localized activation of Arp2/3 in specific places and at specific times during development.

The SCAR/WAVE family of molecules is one such family of Arp2/3 activators [S3]. SCAR/WAVE forms part of a polypeptide complex that responds to activated Racs, or other membrane-associated signals, which in turn activates Arp2/3, thereby promoting actin-filament nucleation [S4–S7]. The C. elegans genome contains one predicted member of this family, wve-1 [S8]. wve-1(RNAi) results in embryonic lethality (75.6%, n = 1018), with affected embryos displaying severe defects in embryogenesis (Figure S1B). wve-1(RNAi) does not affect the formation of the epidermis, but cells fail to perform the migrations necessary for proper dorsal intercalation and enclosure. Embryos ultimately arrest without completing morphogenesis with a terminal phenotype identical to Gex (gut on the exterior) mutants [S9].

Performing wve-1 RNAi in unc-34(gm104) animals yielded both moderately increased lethality (90.2% n = 112) and slightly more severe phenotypes. Epidermal cells appeared rounded and did not initiate cell migrations (Figure S1C). In C. elegans, the loss of function of only one Rac, ced-10, yields extensive embryonic defects [S10]. ced-10(n3417), a maternal-effect allele, causes lethality (89.8%, n = 430) and phenotypes strikingly similar to those in unc-34(gm104); wve-1(RNAi) embryos (Figure S1D). These phenotypes suggest that WVE-1, perhaps augmented by UNC-34, mediates the bulk of Rac signaling to the actin cytoskeleton during morphogenesis.

Ena/VASP and WASP/WIP Act in Parallel during Ventral Enclosure

The WASP family of molecules constitutes another important group of Arp2/3 activators [S11]. This activation can occur by WASP binding to active Cdc42 and/or PIP2 at the membrane, and it often leads to the formation of

![Figure S1. Initiation of Epidermal Migration Requires WVE-1 and UNC-34](image)

Nomarski time-lapse images. Ventral is up, and anterior is to the left.
(A) Wild-type enclosure. Ventral cells migrate around the lateral surfaces (arrowheads) of the embryo to meet at the ventral midline (arrow).
(B) wve-1(RNAi) embryos in which epidermal cells fail to migrate and never enclose (asterisk).
(C) unc-34(gm104); wve-1(RNAi) embryos also fail during morphogenesis, with epidermal cells becoming slightly rounded and never migrating to enclose the animal (asterisk).
(D) ced-10(n3417) embryos phenocopy unc-34(gm104); wve-1(RNAi) embryos, including enclosure defects (asterisk).
The scale bar represents 10 μm.
filopodia [S12, S13]. In our hands, the removal of the function of the sole C. elegans N-WASP, wsp-1, via RNAi resulted in embryos that were largely phenotypically normal, with a low level of lethality (15.6%, n = 596); dead embryos displayed phenotypes consistent with disruptions in cell division (data not shown). However, as previously reported [S8], the reduction of WSP-1 in an unc-34 null background yielded 100% lethality (n = 125); affected embryos displayed substantially disrupted morphogenesis (see Figure 1).

Because these defects are not observed in either single mutant, these results show that during morphogenesis, unc-34 and wsp-1 act in a genetically redundant manner. In order to support the genetic specificity of this interaction, we examined the role of the C. elegans WIP (WASP-interacting protein) during enclosure. The precise molecular role of WIP is unclear (reviewed in [S14]). It is capable of binding WASP family members [S15] and often appears to be important for WASP function [S16, S17], but it also participates in actin dynamics independent from WASP. C. elegans has a single WIP homolog, wip-1 [S18]. In wild-type animals, wip-1(RNAi) results in low levels of embryonic lethality similar to wsp-1 (RNAi), and double RNAi of wsp-1 and wip-1 does not enhance this lethality. unc-34(gm104); wip-1(RNAi) and unc-34(gm104); wsp-1(RNAi) animals are phenotypically indistinguishable (Figures S2A and S2B; cf. Figures 1B and 1B), strongly suggesting that wip-1 and wsp-1 act together during enclosure and that, in this context, WIP acts as a positive regulator of WASP function. One caveat to these experiments is that the RNAi knockdown for wip-1 might have been incomplete. Future experiments involving wsp-1 and wip-1 null mutants would definitively confirm these results.

Overall Actin Morphology Is Grossly Normal in unc-34(gm104); wsp-1(RNAi) Embryos

The presumptive roles of UNC-34 and WSP-1 involve the modulation of the actin cytoskeleton. Actin is known to be generally required for ventral enclosure [S19], so we performed phalloidin staining to investigate the gross structure of actin in unc-34; wsp-1 embryos (Figure S3). Phalloidin-stained unc-34; wsp-1(RNAi) embryos resemble wild-type embryos. Although leading-edge protrusions are less readily apparent, actin is enriched at the leading free edges and cell-cell borders of ventral cells that fail to migrate, and the bundling of cellular actin into nascent circumferential cables appears to initiate normally (Figure S3B).

Models for the Genetic Interaction between unc-34 and wsp-1

Our results regarding the genetic interaction between unc-34/Ena and other components involved in leading-edge migration, collated with those of others, is shown in Figure S5A. The synergistic genetic interaction between unc-34 and wsp-1 is consistent with several models at the molecular level (Figure S5B). WSP-1 is known to activate the C. elegans Arp2/3 complex [S20] to promote actin-filament nucleation via side branching. UNC-34 presumably acts in a manner similar to other members of the Ena/VASP family because unc-34 mutants display phenotypes [S8] that appear to be similar to neuronal phenotypes associated with the loss of Ena/VASP family proteins in other systems (reviewed in [S21]). One model of filopodium formation involves actin branches in the dendritic network becoming privileged via the presence of filament tip complexes that include Ena/VASP proteins [S22], which allow pre-existing filaments to elongate and ultimately become bundled into filopodia. So that redundancy between UNC-34 and WSP-1 can be integrated into this model, in C. elegans, most of the dendritic network could be generated via WVE-1-mediated branching. In addition, a local microenvironment with elevated WSP-1 activity—possibly because of active Cdc42 or another signal from the membrane—combined with UNC-34/tip complex activity could lead to a more elaborate protrusion structure.
provide insight into the molecular nature of this genetic interaction.

Supplemental Experimental Procedures

Strains
The N2 Bristol strain was used as wild-type and was the basis for all strains. The following mutations were used: hmr-1(zu389) I [S24], unc-34(e315, e566, gm104) V [S8], hmp-1(zu278, fe4) V [S24, S25], abl-1(ok171) X [S26], and ajm-1(ok160) X [S27]. The mis10 chromosome rearrangement (an integrated transgenic array consisting of the myo-2::GFP, pes-10::gfp and F22B7.9::gfp transgenes), which suppresses recombination over a large region of LG V, was used for the balancing of unc-34,hmp-1(fe4) double-mutant chromosomes. hT2[bli-4(e937) let(q782) qIs48] [S28] was used for the balancing of hmr-1(zu389). SU93 contains an integrated array (jcIs1) on linkage IV that includes a truncated ajm-1::gfp encoding amino acids 102–866 of the predicted protein [S27]. SU159 carries the same transgene carried as an extrachromosomal array in an ajm-1(ok160) null mutant background [S27]. Strain SU166 includes jcIs1 and unc-34(gm104) V. SU220 has an extrachromosomal array (jcEx60) including dgl-1::t7::gfp [S29], SU226 is unc-34(gm104) V, and carries jcEx60. jcEx60 was subsequently integrated, and the strain was outcrossed to make strain SU276. Strain LE179 carrying ceo-10(n3417) / lin-1(e1279ts),dpy-13(e184) IV was a gift from Erik Lundquist.

So that germline mosaics of hmr-1(zu389) and hmp-1(zu278) could be obtained, individual adult hermaphrodites derived from transgenic lines carrying extrachromosomal arrays containing wild-type hmr-1 or hmp-1 genes, respectively, were picked to separate plates, and their progeny were examined for 100% embryonic arrest. Animals with this phenotype were placed on fresh plates without food for approximately 16 hr, and their broods were processed for immunostaining.

Generation of unc-34::gfp Transgenic Lines
The HMR-1A promoter (P_HMR-1A) was fused to the unc-34 gene via PCR fusion, and the resultant ampiclon was fused to the gfp gene and unc-34 3’ untranslated region (UTR) via a second polymerase chain reaction (PCR) fusion reaction. A list of the primer sequences used to generate the fusion ampiclons is available on request to J.P. The P_HMR-1A::unc-34::gfp fusion ampiclon was co-injected along with the rol-6(sv1006) marker gene into unc-34(gm104) V hmp-1(fe4)/mis10 hermaphrodites, and three independent lines carrying both transgenes as an extrachromosomal array were established. All three gave identical patterns of expression.

To express unc-34::gfp under the control of the dgl-1 promoter, the upstream region of dgl-1 was amplified from wild-type DNA and cloned into a modified version of pPD95.75 (provided by A. Fire, Stanford University), which can be used as a destination vector in the Gateway cloning system (Invitrogen). The primers used to amplify the dgl-1 upstream region were dgl1PL, 5’-CGAAGCTTCAAGTTTACCAAACAGTCGCT-3’, and dgl1PR, 5’-GGCATCGGCTCCCTTCCGCGT-3’. The unc-34 cDNA was then cloned into this vector via recombination. This construct produced a fusion of the GFP gene to the C terminus of UNC-34. The resultant vector, pPEKJP101 was injected into unc-34(gm104) hmp-1(fe4)/mis10 hermaphrodites.

RNAi
Double stranded RNA-mediated interference was performed as previously described via either injection [S30] or feeding [S31] methods. Ambion MegaScript T7, T3, and SP6 kits were used for the preparation of injection samples. Plasmid pBG1 (described in [S8]) is a partial wsp-1 cDNA cloned into Fire vector kit plasmid L4440 for feeding RNAi. R144.4 RNA was made with partial cDNA yk453c4, from the laboratory of Yui Kohara, as a template. wve-1 RNA was made with a genomic fragment amplified with primers 5’-AAGTTAAGCTGAAGCGCAGAGCCTGCG-3’ and 5’-GAACATCCTCGGAGAGATTATCAGACG-3’ as the template.

Microscopy
4D Nomarski microscopy was performed as previously described [S32]. In brief, gravid adults were bisected in M9 solution so that embryos could be collected. Embryos were transferred by mouth
pipette to a 5% agar pad on a glass slide, submerged in M9, and sealed with a glass coverslip for filming. Embryos were filmed on a Nikon Eclipse E600 or Optiphot-2 upright microscope equipped with DIC optics and Ludl shutter controllers. Dage-MTI analog video cameras collected the data onto Macintosh G3 computers with Scion AG-5 frame grabbers. NIH Image software with custom macros was used for the compression of 3D time-lapse data into 4D QuickTime movies. Two-photon excitation microscopy was performed as previously described [S33] with a custom setup for the collection of images into Bio-Rad software, which were then compressed into 3D projected time-lapse movies with NIH Image and custom macros. Spinning-disc confocal microscopy on live GFP specimens was performed with a Yokogawa CSU10 scanhead attached to a Nikon Eclipse E600 microscope. Data were collected with a Hamamatsu ORCA-ER CCD camera with Perkin Elmer Ultra-view software on a Pentium 4 PC. Fixed specimens were viewed either with spinning-disc confocal microscopy or laser-scanning confocal microscopy with a Bio-Rad MRC1024. All live specimens were filmed at 20° C.

Quantitative Protrusion Analysis
Embryos expressing dlg-1::GFP [S29] were filmed with spinning-disc confocal microscopy. Twenty focal planes were captured from the ventral-most plane moving 9.5 μm into the specimen in 0.5 μm intervals. Such Z stacks were taken every 50 s throughout morphogenesis. Stacks were projected into a single image and compressed into AVI movies. These movies were then viewed on a Macintosh G4 computer with ImageJ software. Movies were expanded to 200% zoom so that analysis could be facilitated. The perimeter of the LCPZ was measured at specified time points; this measurement was divided by the width of the cell bodies so that slight differences in viewing angle and differences in cell width in the mutants could be internally controlled for (Figure S4A). These data were collected over five consecutive time points (at 50 s intervals) per embryo: the time point at which the leading cell protrusions first touch their contralateral partners and the four preceding time points for the wild-type and the last five time points of productive migration toward the ventral midline for mutant embryos. For leading cell analysis, the perimeter of the protrusions on one side was traced...
in ImageJ with a Wacom Graphire3 input tablet, and the path length of the perimeter was measured. The width of the bodies of the leading cells was then measured with a segmented straight line tool, and the perimeter was divided by the width to yield an internally normalized metric. This process was repeated on the other side and again at each of the other time points in the analyzed sample. The same process was repeated for the pocket cells. Data were compared between genotypes for leading cells or pocket cells with a Wilcoxon rank sum test with \( \alpha = 0.05 \). A nonparametric test was chosen largely because of concerns that the population values might not be normally distributed and probably did not have equal variances; blunted mutant protrusions probably have lower variance values than do the highly dynamic wild-type protrusions. The resulting Z statistic value for leading cells was \( Z = 2.7085 \), and a similar test on pocket cells between wild-type and \( \text{unc-34(gm104)} \) yielded \( Z = 2.723 \), which are both well below the cutoff value for a two-tailed test with an \( \alpha = 0.05 \).

Antibody Staining
UNC-34 staining was performed with a modified Finney-Ruvkun [S34] protocol with 1:100 diluted polyclonal anti-UNC-34 and overnight room temperature incubations. Phalloidin staining was performed in a manner similar to previous descriptions [S19] but with fixations and incubations performed on a poly-L-lysine coated slide. Texas Red phalloidin (Molecular Probes) was applied with an overnight 4°C incubation.

Supplemental References

Figure S6. UNC-34::GFP Localizes Around Cell Corpses as They Are Engulfed
Ventral view of a postventral enclosure embryo at successive 2 min time points. Each frame is a Z projection of ten focal planes, encompassing 2 \( \mu \)m. Arrows indicate newly formed rings of UNC-34::GFP around cell corpses. Numbers indicate the time in minutes. The scale bar represents 10 \( \mu \)m.

Figure S7. Junctional Localization of HMR-1/E-Cadherin Is Not Affected by the Loss of \( \text{unc-34} \) Function
Immunofluorescent detection of HMR-1 at epithelial junctions during embryonic elongation in \( \text{unc-34(gm104)} \) (A), \( \text{hmp-1(fe4)} \) (B), and \( \text{unc-34(gm104) hmp-1(fe4)} \) (C and D) embryos. (B) and (C) show a Hmp phenotype, and (D) shows a Hmr phenotype. The arrow indicates the same lateral epidermal cell (V1) in each panel to allow for comparisons between embryos (two V1 cells are visible in [D] because this is a dorsal view of the completely retracted epidermis). Anterior is to the left in all panels. The scale bar represents 10 \( \mu \)m.


