Abstract

Cell intercalation is a highly directed cell rearrangement that is essential for animal morphogenesis. As such, intercalation requires orchestration of cell polarity across the plane of the tissue. CDC-42 is a Rho family GTPase with key functions in cell polarity, yet its role during epithelial intercalation has not been established because its roles early in embryogenesis have historically made it difficult to study. To circumvent these early requirements, in this paper we use tissue-specific and conditional loss-of-function approaches to identify a role for CDC-42 during intercalation of the Caenorhabditis elegans dorsal embryonic epidermis. CDC-42 activity is enriched in the medial tips of intercalating cells, which extend as cells migrate past one another. Moreover, CDC-42 is involved in both the efficient formation and orientation of cell tips during cell rearrangement. Using conditional loss-of-function we also show that the PAR complex functions in tip formation and orientation. Additionally, we find that the sole C. elegans Eph receptor, VAB-1, functions during this process in an Ephrin-independent manner. Using epistasis analysis, we find that vab-1 lies in the same genetic pathway as cdc-42 and is responsible for polarizing CDC-42 activity to the medial tip. Together, these data establish a previously uncharacterized role for polarized CDC-42, in conjunction with PAR-6, PAR-3 and an Eph receptor, during epithelial intercalation.

Author Summary

As embryos develop, tissues must change shape to establish an animal’s form. One key form-shaping movement, cell intercalation, often occurs when a tissue elongates in a preferred direction. How cells in epithelial sheets can intercalate while maintaining tissue integrity is not well understood. Here we use the dorsal epidermis in embryos of the
nematode worm, *C. elegans*, to study cell intercalation. As cells begin to intercalate, they form highly polarized tips that lead their migration. While some mechanisms that polarize intercalating cells have been established in other systems, our work identifies a new role for CDC-42—a highly conserved, highly regulated protein that controls the actin cytoskeleton. We previously established that a related protein, Rac, is involved in tip extension during dorsal intercalation. CDC-42 also contributes to this process in addition to helping orient the extending tip. CDC-42 appears to work in conjunction with two other known cell polarity proteins, PAR-3 and PAR-6, and the cell surface receptor, VAB-1. Our work identifies a novel pathway involving proteins conserved from worms to humans that regulates a ubiquitous process during animal development.

**Introduction**

Understanding how the cellular behaviors that underlie embryonic morphogenesis are regulated is a longstanding and fundamental goal of developmental biology. One such cell behavior that can reshape tissues during embryogenesis is mediolateral intercalation, whereby cells within the same plane interdigitate along a preferred axis (reviewed in [1]). Mediolateral intercalation is a widespread process in animal development; it occurs in tissues derived from all three germ layers during gastrulation and organogenesis. There are multiple mechanisms by which cells can intercalate, including highly directed and coordinated protrusive activity and highly polarized apical junction rearrangement (reviewed in [1]). These mechanisms are unified by a fundamental theme: movement must occur in a highly polarized fashion.

One well studied regulator of cell polarity is the Rho family GTPase Cdc42 (reviewed in [2]). As a GTPase, Cdc42 cycles between active, GTP-bound and inactive, GDP-bound states. Cdc42-GTP has multiple molecular functions that allow it to influence cell polarity. Active Cdc42 can modify the actin cytoskeleton to produce long, thin filopodia [3], a process mediated through its interaction with the actin nucleation promoting factor Wiskott-Alrich syndrome protein (WASP) [4]. Furthermore, through interactions with the partitioning defective (PAR) complex [5–7], Cdc42 can regulate the microtubule cytoskeleton to reorient centrosomes in order to polarize cell division and regulate apical junctions in epithelia [8, 9]. Given these diverse and conserved molecular processes, it is reasonable to think that Cdc42 has roles in polarizing epithelia during intercalation.

While Cdc42 has documented roles in other intercalation events, it has not yet been implicated during embryonic epithelial morphogenesis. For example, Cdc42 helps orient intercalation in frog mesenchymal cells during convergent extension through the planar cell polarity pathway [10], in the ascidian notochord during convergent extension as dorsoventral polarity is established [11], in the fly mesoderm during gastrulation through fibroblast growth factor signaling [12], and during transendothelial migration as cancer cells metastasize [13]. While Cdc42 has a documented role in orienting cell divisions in an intercalating epithelium, the frog neuroepithelium [14], no role has been documented for Cdc42 in the rearrangement of postmitotic intercalating cells.

To study Cdc42 in an intercalating epithelium, we employed the *Caenorhabditis elegans* dorsal epidermis. During dorsal intercalation, two rows of ten epidermal cells interdigitate into a single row of twenty [15]. Dorsal intercalation is accompanied by the appearance of highly protrusive medial tips; in contrast, lateral (i.e., rear) cell borders are prosurvurally inactive [16]. Previous work made CDC-42 a good candidate for regulating polarity during dorsal intercalation. CDC-42 has roles in collective cell migration of ventral epidermal cells in the *C. elegans*...
embryo [17], suggesting that it is active in the epidermis. Moreover, we recently discovered that wsp-1/WASP, a key Cdc42 downstream effector, and wve-1/WAVE function redundantly to promote medial protrusive activity during dorsal intercalation [16].

In this study, we use a combination of two genetic approaches—epidermal-specific dominant-negative mutant CDC-42 and late loss of CDC-42 function—along with in vivo CDC-42 biosensor data to establish a role for CDC-42 in forming and fine-tuning the orientation of the medial tips of dorsal epidermal cells as they extend during intercalation. Additionally, we leverage results from large-scale genetic synthetic lethal screens [18] to implicate VAB-1/EphR receptor (EphR) as an upstream activator of CDC-42 in this context. Together, these results demonstrate the importance of CDC-42 as a key regulator of oriented cell migration and identify a genetic pathway involving highly conserved molecular components centered on CDC-42 that operates during epithelial cell intercalation.

**Results**

**CDC-42 is required for the intercalation of dorsal epidermal cells**

Cdc42 is a Rho family GTPase that polarizes cells during migration [19]. Previously, examination of the role of CDC-42 in cellular migrations in intact *C. elegans* embryos has been hampered by early requirements during anterior-posterior axis formation [6, 20]. To determine if CDC-42 function is required for the highly directed cell migrations that occur during dorsal intercalation (about 3 hours after the first division of the zygote), we sought to bypass these early requirements. To do so, we generated an epidermal-specific, inducible dominant-negative CDC-42(T17N) [21] (hereafter *cdc-42(DN)*) using the Nonsense Mediated Decay (NMD)-mediated system that we described previously for CED-10/Rac [16]. Briefly, transgenes expressing cDNA under control of the epidermal-specific *lin-26* promoter and an NMD-sensitive 3’UTR are generated in the temperature-sensitive *smg-1(cc546 ts)* (NMD defective) background; at the permissive temperature of 15°C, transcripts from these transgenes are degraded by the NMD RNA surveillance system. Conversely, at the restrictive temperature of 25°C the NMD system is inactivated, transcripts from the transgenes are stabilized, and protein is produced. In addition, we utilize a second, previously established transgene-mediated system to conditionally perturb CDC-42 and PAR-3/PAR-6 function. This system relies on the degradation of functional ZF1-tagged transgenes [22–24] in mutant backgrounds, which rescue the mutant phenotype in the early embryo, thus circumventing requirements [6, 20], but lead to loss of function during gastrulation and later in embryogenesis.

Upon incubation at 25°C, *cdc-42(DN)* embryos displayed two classes of intercalation defects, which were seen at significantly higher frequencies than in wild-type or in *gfp* expressing controls (*p*≤0.01, Fisher’s Exact Test) (Fig 1). First, adjacent dorsal cells often migrated together across the dorsal array, instead of interdigitating with contralateral neighbors (40%, *n* = 16), a phenotype we call “ipsilateral comigration”. This defect may be due to a mispolarization of the extending tip during intercalation. Second, medial edges of dorsal cells occasionally appeared blunt, rather than extending a pointed tip (7%, *n* = 16), as if opposing cells mutually block migration of its contralateral partner. We have described this phenotype previously [16]; here we refer to it as ‘medial delay’. These defects may result from a weak requirement for CDC-42 activity in early tip formation. Both types of defects sometimes occurred within the same pair of cells (i.e., cells tips are blunt, then migrate in the wrong direction); Fig 1B accordingly shows the pooled frequency of these two defects in *cdc-42* loss of function embryos.
Fig 1. *cdc-42* loss of function phenotypes during dorsal intercalation. A) DIC micrographs of *P*<sup>lin-26::gfp; smg-1(cc546)</sup> (top) and two different *P*<sup>lin-26::cdc-42(DN); smg-1(cc546)</sup> embryos. Left dorsal cells pseudocolored green, right cells pseudocolored blue. Arrowheads indicate direction of migration; yellow dotted lines outline blunt medial edges. Dorsal intercalation time measurements in Figs 1 and 2 were performed as in [16]: 0 min. was defined as 60 min. after terminal epidermal (Cpaa.a/p) divisions. Scale bar = 10 μm. Embryos incubated at 25˚C for 24 hours prior to mounting. B) Penetration of combined ipsilateral comigration and medial delay phenotypes in wild-type (WT), *P*<sup>lin-26::gfp; smg-1(cc546)</sup>, and *P*<sup>lin-26::cdc-42(DN); smg-1(cc546)</sup> embryos. All embryos imaged at 25˚C. Total defects in *cdc-42(DN)* and ZF1::cdc-42 embryos (see Fig 2) were significantly different from controls (p<0.01; **) but not each other (p = 0.749) (Fisher’s Exact Test). Gray numbers indicate number of embryos analyzed per genotype.

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To verify that these defects were due to reduction of CDC-42 function, we utilized a second approach to circumvent early requirements for CDC-42. Transgenic CDC-42 tagged with the ZF1 degron is stable during initial zygotic divisions but sensitive to degradation by the E3 ubiquitin ligase ZIF-1 during gastrulation in somatic cells, leading to late maternal loss of function [22, 23]. The ZF1::CDC-42-expressing transgene can be combined with the putative null allele, cdc-42(gk388) [25, 26] to additionally mediate strong zygotic loss of CDC-42 function. The resulting ZF1::cdc-42; cdc-42(gk388) embryos (hereafter ZF1::cdc-42) [23, 27] circumvent early requirements for CDC-42 while depleting CDC-42 in the later embryo. Both ipsilateral comigration and medial delay occurred in ZF1::cdc-42 embryos (20% and 24%, respectively, n = 35; Fig 2), and the penetrance of these defects was not significantly different from cdc-42(DN) embryos (p = 0.749, Fisher’s Exact Test). Together, these

![Image of cell morphology](https://example.com/image.png)

**Fig 2. Cell morphology in cdc-42 loss of function embryos.** A) Confocal images of an epidermal cytoplasmic reporter (P*plb-1::GFP*) in wild-type (WT) (A’) and ZF1::cdc-42; cdc-42(gk388) (A”) embryos, in which there is late maternal loss of CDC-42, 45 min. after terminal division. Yellow box outlines area enlarged in bottom image. Left cells pseudocolored cyan. Black dotted line connects tip (cyan star) and cell centroid (yellow star), which is quantified in B. Scale bars: top = 10 μm; bottom = 5 μm. B) Tip angle in wild-type and ZF1::cdc-42; cdc-42(gk388) cells. The distribution of tip angles is significantly less uniform in ZF1::cdc-42; cdc-42(gk388) than WT (Mardia-Watson-Wheeler, p = 0.002, **). Red text indicates number of cells. C) Box plot of aspect ratio in wild-type (n = 32) and ZF1::cdc-42 (n = 27) cells. Age-matched ZF1::cdc-42; cdc-42(gk388) cells were significantly less elongated than wild-type (Student’s T-test, p = 0.024, **).
data suggest that CDC-42 has roles in both tip extension and orientation during dorsal intercalation in *C. elegans*.

**CDC-42 is required for medial tip orientation and extension in intercalating cells**

We next sought to determine whether atypical cell morphology preceded the cell migration defects visible by DIC microscopy in cells with compromised CDC-42. To do so, we analyzed wild-type and *ZF1::cdc-42* cells expressing an epidermal cytoplasmic reporter (*P* *lbp-1::gfp*) [28] at a time prior to migration but when cells normally begin to take on a mediolaterally polarized morphology (45 min. after the terminal division; for comparison, the “0 min.” time point on all DIC images is 60 min. after the terminal epidermal divisions). Our analysis uncovered two abnormalities. First, we found that the angular orientation of medial tips was significantly less uniform in *ZF1::cdc-42* than in age-matched wild-type controls (Mardia-Watson-Wheeler, *p* = 0.002; Fig 3A and 3B). In *ZF1::cdc-42* cells, the tip was often oriented more anteriorly or posteriorly than in wild-type, suggesting that CDC-42 is involved in fine-tuning medial tip orientation. This initial misorientation also provides a potential explanation for ipsilateral comigration: if sufficiently misoriented, once cell migration ensues, then comigration could occur.

In addition to medial tip orientation defects, our analysis revealed a second abnormality. The length/width ratio (aspect ratio) of intercalating cells was significantly lower in *ZF1::cdc-42* than in age-matched wild-type controls (Student’s T-test, *p* = 0.02; Fig 3C). *ZF1::cdc-42* cells were often less elongated, suggesting that CDC-42 is required for cells to properly extend their medial tips during dorsal intercalation. Such extension defects may, in severe cases, result in the medial delay phenotype observed by DIC microscopy.

Since we had shown previously that CED-10/Rac is important for tip extension during dorsal intercalation [16], we next examined whether CED-10 and CDC-42 act together in this process. When we crossed the *ced-10(n3246)* missense mutation into the *P* *lin-26::cdc-42(DN)::smgS; *smg-1(cc546ts)* background, the resulting heat shocked embryos had fully penetrant intercalation defects (S1 Fig). The penetrance of total defects was significantly higher (Student’s T-test, *p* < 0.01) than similarly heat-shocked *smg-1(cc546ts), ced-10(n3246); smg-1(cc546ts)* or *P* *lin-26::cdc-42(DN)::smgS; *smg-1(cc546ts)* controls. Significantly, *P* *lin-26::cdc-42(DN)::smgS; *ced-10(n3246); smg-1(cc546ts)* loss-of-function embryos had a significantly greater penetrance of medial delay than controls (ANOVA, *p* < 0.02), while the penetrance of ipsilateral comigration remained the same, suggesting that CED-10/Rac and CDC-42 normally cooperate specifically to extend medial tips. Taken together, these data support a minor role for CDC-42 in tip extension during dorsal intercalation.

**par-6 and par-3 are also required for dorsal intercalation**

Cdc42 forms a complex with Par6 and Par3 to influence cell polarity [7], so we next compared the phenotypes of *cdc-42* and *par* loss of function during dorsal intercalation. We again utilized existing ZF1 transgenes to abrogate *par-3* and *par-6* function [8, 9, 24]. Late maternal loss of function in *par-3::ZF1::gfp; par-3(it71)* embryos led to defects closely resembling those in *ZF1::cdc-42* and *cdc-42(DN)* embryos, including ipsilateral comigration and medial delay (37% and 5%, respectively, n = 19) (Fig 3). Late maternal *par-6* loss of function—through *par-6(tm1425)* allele—resulted in similar phenotypes (33% ipsilateral comigration and 17% medial delay, n = 18) (Fig 3). Together, these results suggest that CDC-42 and the PAR complex both act to orient and extend medial tips during dorsal intercalation.
Fig 3. PAR complex loss of function phenotypes during dorsal intercalation. A) DIC images of wild-type (WT) and representative par-6::ZF1; par-6(tm1425), par-3::ZF1; par-3(it71), and ZF1::cdc-42; cdc-42(gk388) embryos. Left dorsal cells pseudocolored green, right cells pseudocolored blue. 0 min. was defined as 60 min. after terminal epidermal (Cpaa.a/p) divisions. Light arrows indicate direction of migration, while yellow dotted lines outline blunt medial edges. Scale bar = 10 μm. Embryos maintained at 20°C. B) Distribution of intercalation phenotypes in WT, ZF1::cdc-42; cdc-42(gk388), par-3::ZF1; par-3(it71), and par-6::ZF1; par-6(tm1425), embryos. Total defects in par-3::ZF1; par-3(it71), par-6::ZF1; par-6(tm1425), and ZF1::cdc-42; cdc-42(gk388) embryos were significantly different from wild-type controls (p<0.01, **) but not each other (p>0.7) (Fisher’s Exact Test). Gray numbers indicate number of embryos analyzed per genotype.

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CDC-42 activity is enriched at the medial tip

If CDC-42 is involved in medial tip formation, it might be expected that its activity would be enriched at sites of tip formation. As a Rho family GTPase, CDC-42 cycles between active (GTP-bound) and inactive (GDP-bound) states. We determined the localization of active CDC-42 using a validated CDC-42 biosensor (wsp-1(G-protein binding domain)::gfp [29]), under the control of its endogenous promoter (hereafter wsp-1(GBD)::gfp). WSP-1(GBD)::GFP localization was significantly enriched at the medial edge relative to a uniformly distributed membrane marker, PH::mCherry [30] (Student's T-test, p = 0.03, Fig 4C). WSP-1(GBD)::GFP localization was polarized from the time of tip formation (about 30–60 min. post-terminal division) through tip extension (about 90–120 min. post-terminal division; Fig 4A). The timing of this medial enrichment of active CDC-42 coincides with the timing of tip orientation and extension, and with the defects in intercalation we observed in cdc-42 loss of function backgrounds described above (see Fig 2).

We next addressed the functional relationship between CDC-42 and the PAR proteins during dorsal intercalation. Construction of strains for standard epistasis testing proved technically challenging due to maternal requirements for CDC-42, PAR-3, and PAR-6, and complexities in genotyping, and we were unable to recover transgenic lines following injection of DNA encoding the CDC-42 activity biosensor into par-3::ZF1::gfp; par-3(it71) worms. However, we were able to obtain one line of par-6::ZF1::gfp; par-6(tm1425) worms expressing wsp-1(GBD)::gfp. Based on the previous literature, there were two possible predicted outcomes from this experiment. Since PAR-6 is typically considered a downstream effector of Cdc42 [2], loss of par-6 function might be predicted to have little effect on CDC-42 activity. However, a study in Drosophila neuroblasts [31] and results from the one-cell C. elegans zygote [29] (reviewed in [32]) suggested another possibility. These studies indicated positive feedback between CDC-42 and PAR-6. In this case, loss of par-6 activity might be expected to lead to loss of CDC-42 activity at the midline due to disruption of the feedback loop. As Fig 4B and 4C indicates, par-6::ZF1::gfp; par-6(tm1425) embryos show loss of accumulation of WSP-1(GBD)::GFP at the dorsal midline, suggesting that CDC-42 and PAR-6 may engage in positive feedback to polarize intercalating cells in the dorsal epidermis.

VAB-1/EphR is required for medial enrichment of CDC-42 activity

We next sought to identify additional components of the CDC-42 pathway that regulates dorsal intercalation. To do so, we mined the literature for genes that might be associated with activation of CDC-42. We identified one gene expressed in the dorsal epidermis during intercalation, vab-1/EphR [33], which had a genetic interaction with cdc-42 in a previous large-scale study [18]. Indeed, when we imaged GFP::WSP-1(GBD) in vab-1(dx31) null embryos during intercalation, we observed a significant decrease in the medial enrichment of active CDC-42 (Student’s T-test, p = 0.03; Fig 4B and 4C). These data suggest that VAB-1/EphR leads to activation of CDC-42 at the medial edge during dorsal intercalation. We next examined spatial localization of VAB-1 in dorsal epidermal cells using a rescuing, GFP-tagged vab-1 fosmid. VAB-1::GFP was enriched at medial edges in dorsal cells (S2 Fig) in a manner similar to GFP::WSP-1(GBD).

Loss of vab-1/EphR function phenocopies cdc-42 loss of function

If VAB-1/EphR is required for medial CDC-42 activity, loss of vab-1 function would be expected to lead to defects similar to those following loss of cdc-42 function. When we imaged vab-1(dx31) null mutants, we also observed the comigration and medial delay phenotypes (Fig 5; Fisher’s Exact Test to wild-type, p = 0.038) seen in ZF1::cdc-42 and cdc-42(DN) mutants.
**Fig 4.** Localization of a CDC-42 biosensor (GFP::WSP-1(GBD)) during dorsal intercalation A) in wild-type embryos. Micrographs pseudocolored according to fluorescence intensity using the “Fire” lookup table in ImageJ. As the medial edge becomes pointed, it accumulates active CDC-42 (white arrows), which is maintained during intercalation. Scale bar = 5 μm. B) Comparison of PH::mCherry in wild-type embryos and CDC-42 biosensor localization in par-6::ZF1, vab-1, and otherwise wild-type embryos during dorsal intercalation as in A). Scale bar = 10 μm. C) Quantification of the medial versus lateral enrichment of active CDC-42 in various genotypes. Medial enrichment was significantly greater for GFP::WSP-1(GBD) than PH::mCherry controls (Student’s T-Test, p = 0.01; **). Medial enrichment of GFP::WSP-1(GBD) was significantly decreased in both the par-6::ZF1, par-6::lm1425 and vab-1(dx31) backgrounds (Student’s T-Test, p≤0.01; **). Numbers indicate number of cells analyzed per genotype.

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Given the wide array of mapped genetic lesions available within the vab-1 locus [34], we were additionally able to ask whether certain domains were required for VAB-1 function. We found that animals homozygous for mutations in the kinase domain (e118 and e2), which prevent phosphorylation and activation of VAB-1 [35], also displayed dorsal intercalation defects (Fisher’s Exact Test, p ≤ 0.007). This suggests that, in contrast to some other VAB-1-dependent
processes, such as hyp6 cell fusion [35] and amphid axon guidance [36], dorsal intercalation requires VAB-1/EphR kinase activity.

We next examined the potential roles of Eph ligands in dorsal intercalation. First, we examined embryos homozygous for a vab-1 mutation (e699), which causes an amino acid substitution (T63I) within the extracellular domain that binds to canonical ephrin ligands [34, 35, 37, 38]. In contrast to putative kinase-dead mutants, vab-1(e699) homozygotes displayed intercalation defects at intermediate levels that were not significantly different from either wild-type or vab-1(dx31) null mutants (Fisher’s Exact Test, p = 0.135 and p = 0.796). Given the surprisingly mild defects in vab-1(e699) mutants, we next analyzed ephrin mutants directly. Surprisingly, neither triple ephrin mutants (vab-2(ju1), efn-2(ev658); efn-3(ev696)) [39] nor putative null mutants of the more divergent ephrin, efn-4(bx80) [40], displayed intercalation defects (S3 Fig). Taken together, these data suggest that while kinase activity is important for VAB-1 function during intercalation, interaction with traditional ephrin ligands is dispensable.

vab-1/EphR genetically interacts with cdc-42 during dorsal intercalation

The phenocopy of cdc-42 loss of function in vab-1 mutants, medial enrichment of VAB-1, and the requirement of VAB-1 function for CDC-42 medial enrichment suggests that these two genes lie in the same pathway to promote oriented cell migration during dorsal intercalation. To examine this possibility, we crossed the weak allele, vab-1(e2), into the cdc-42(DN) background. We predicted that at a semi-permissive temperature (20°C)—when induction of transgenes using the NMD-sensitive system is weak (16)—the combination of weak vab-1 and cdc-42 loss of function would enhance intercalation defects to levels seen at the restrictive temperature (25°C) for cdc-42(DN) alone or in ZF1::cdc-42 embryos. Indeed, at 20°C, intercalation defects in cdc-42(DN) were significantly less frequent than at 25°C (Fisher’s Exact Test, p = 0.006, Fig 6). Furthermore, the penetrance of defects was significantly enhanced in the vab-1(e2); cdc-42(DN) background relative to either cdc-42(DN) or vab-1(e2); smg-1(cc546) grown at 20°C (Fisher’s Exact Test, p ≤ 0.004, Fig 6 and S4 Fig). Moreover, the frequency of enhanced intercalation defects was not significantly different from either cdc-42(DN) at 25°C or ZF1::cdc-42, suggesting that CDC-42 and VAB-1 function together during intercalation. Combined with the loss of medial enrichment of the CDC-42 biosensor in vab-1(dx31), these results suggest that vab-1 and cdc-42 function co-linearly in the same genetic pathway during intercalation to promote proper cell guidance and cell migration.

Discussion

Cell intercalation comprises a series of highly directed cell rearrangements. Given that Cdc42 is a highly conserved regulator of cell guidance, it is surprising that relatively little is known about the role of Cdc42 in this process. While Cdc42 has a known role in regulating adhesion of mesenchymal cells during convergent extension in Xenopus [10], there is little evidence for a role for Cdc42 during the intercalation of other cell types, particularly epithelia. Here, using conditional loss of function experiments and an in vivo biosensor, we describe a role for CDC-42 at the medial tips of intercalating cells in the dorsal epidermis of C. elegans. Dorsal intercalation is an epithelial intercalation event that occurs via cell migration and protrusive activity rather than predominantly by spatially-restricted apical junctional rearrangement [15, 16]. Our study suggests that CDC-42 functions with PAR-6, PAR-3, and VAB-1 during dorsal intercalation, particularly through formation and orientation of medial tips.

Loss of cdc-42 function—either using tissue-specific expression of a dominant-negative (NMD-sensitive system) or degradation of a functional, early maternally-rescuing transgene (ZF1)—can result in intercalating cells that fail to form a medial tip (“medial delay”) and/or...
that fail to interdigitate properly ("ipsilateral comigration"). Detailed analysis of a cytoplasmic reporter in ZF1::cdc-42 cells uncovered defects in the orientation of medial tips and the extension of dorsal cells. These results suggest that CDC-42 has two roles during dorsal intercalation: 1) to help form and 2) to orient the medial tips of intercalating cells (Fig 7). When cell tips do not form efficiently, the medial edges of cells appear blunt, leading to the medial delay phenotype. These blunt medial edges often resolve eventually, albeit sometimes through comigration of adjacent cells across the dorsal array. When cells do appear to form tips efficiently, they nevertheless often become misoriented, which also leads to comigration of cells that appear otherwise normal.

Since the roles of CDC-42 in tip formation and orientation are separable, we propose two separate roles for CDC-42 during intercalation. Previously, we uncovered a role for ced-10/Rac and mig-2/RhoG in directing tip formation through actin polymerization mediated by wve-1/WAVE and wsp-1/WASP, respectively [16]. We hypothesize that CDC-42 plays a minor role in this tip formation process through WASP, which is a well-documented downstream effector of active Cdc42. This hypothesis is consistent with the synergistic effects of weak loss of ced-10 function combined with perturbation of cdc-42 function, which specifically affects tip extension.

In addition to its minor role in supporting tip extension in intercalating cells, CDC-42 plays a role in orienting the tips of intercalating cells. We cannot rule out the possibility that subtle orientation defects occur in dorsal epidermal cells prior to the formation of protrusive tips, although as Fig 7 indicates, we favor roles for CDC-42 immediately prior to intercalation. What CDC-42 effector pathways lead to reliable alternation of contralateral cells during dorsal intercalation is an important area of future study. One potential mechanism by which
comigration could occur is through abnormal cell adhesion; perhaps adjacent ipsilateral cells adhere too strongly or contralateral cells do not adhere strongly enough in \( \text{cdc-42} \) loss of function embryos. This idea is supported by findings regarding the PAR complex in epithelial cells in \( \text{C. elegans} \): while junctional proteins acquire their normal apicobasal localization in \( \text{par-6} \); \( \text{ZF1} \) and \( \text{par-3}:\text{ZF1} \) embryos, they do not form continuous belt-like structures and cells in such embryos display subsequent defects in cell adhesion \([8, 9, 24]\). While the comigration phenotype has not been reported upon loss of components of the apical junction \([41–43]\), it is possible that inherent local asymmetries of adhesion are required for interdigitation, which would not be revealed by experiments involving simple loss of function. Indeed, the \( \text{Drosophila} \) E-cadherin homologue, Shotgun/DE-cadherin, is asymmetrically localized in the intercalating germ-band \([44]\), and local junctional disassembly is an important driver of tissue rearrangement \([45]\). Alternatively, the PAR complex may direct microtubule reorganization (reviewed in \([3, 46]\)) to either form or orient the medial tip. In the single reported drug study, microtubule depolymerization was reported to block intercalation \([15]\) consistent with this possibility. Additional experiments will be required to address these potential mechanisms.

In this work we also describe an upstream role for the sole \( \text{C. elegans} \) Eph receptor, \( \text{VAB-1} \), in \( \text{CDC-42} \) regulation during dorsal intercalation. \( \text{vab-1} \) mutants not only phenocopy \( \text{cdc-42} \) loss of function embryos but also display contiguous belt-like structures and cells in such embryos can still comigrate if tips are misoriented.

Fig 7. Model of \( \text{Cdc42}'s \) roles during dorsal intercalation. Posterior intercalating cells in wild-type are shown in the center, with left cells in green and right cells in purple. As the medial tip forms, it must also become oriented. We hypothesize that \( \text{Cdc42} \) has roles in both tip formation and orientation (this work, red). Tip formation is highly dependent on \( \text{Rac/RhoG} \) (large text, see \([16]\)). \( \text{cdc-42} \) and \( \text{vab-1} \) loss of function embryos display blunt medial edges, implicating \( \text{Cdc42} \) and \( \text{EphR} \) in tip formation as well. Ipsilateral comigration in \( \text{cdc-42} \) and \( \text{vab-1} \) loss of function embryos also implicates \( \text{Cdc42} \) and \( \text{EphR} \) in tip orientation. These defects are separable. Even when tip formation is partially defective, cells can still interdigitate; conversely, when tip formation is normal, cells can still comigrate if tips are misoriented.

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loss of function, but the localization of a biosensor for active CDC-42 to the medial tip is lost in \textit{vab-1} mutants. Nevertheless, the phenotype of \textit{vab-1} null mutants is milder than that resulting from loss of \textit{cdc-42} function. This result suggests that other signaling pathways may impinge on CDC-42 in addition to \textit{VAB-1}, and warrants further investigation in the future. It is unclear by what mechanism \textit{VAB-1} and CDC-42 interact. One straightforward explanation is that \textit{VAB-1}/EphR activates or localizes a guanine nucleotide exchange factor (GEF) that can activate CDC-42. There are several vertebrate GEFs with such reported specificities: ephxin, intersectin, and Vav2. \textit{ephx-1}/ephxin interacts with EphR to direct Cdc42 activation in neurons in \textit{C. elegans} [47, 48]. However, homozygous null \textit{ephx-1} mutants intercalate normally (S5 Fig). Intersectin is a Cdc42 GEF [49, 50] that also binds EphR and WASP [51, 52]. However, the GEF domain is not conserved in worm \textit{ITSN-1} [53]. Vav2 is a GEF that mediates endocytosis of Eph-ephrin complexes in neurons [54]. The sole Vav family member in \textit{C. elegans}, VAV-1, is required for rhythmic contractions in multiple cell types [55]. Additionally, VAV-1 functions in a pathway with VAB-1 during oocyte maturation [56] without changing VAB-1 localization [39]. A \textit{vav-1} transcriptional reporter [57] is present in the dorsal epidermis [16]. While \textit{vav-1} (RNAi) did not result in intercalation defects [16], it is possible that \textit{vav-1} is functioning redundantly with another GEF during dorsal intercalation.

Another open question regarding \textit{VAB-1}/EphR is which of its ligands functions during dorsal intercalation. Surprisingly, we found that \textit{vab-1(e699)}, which disrupts the ephrin binding domain, leads to intercalation defects at frequencies between wild-type and \textit{vab-1(dx31)} (Fig 5). However, neither canonical ephrin (\textit{vab-2 efn-2; efn-3}) triple mutants nor a divergent ephrin (\textit{efn-4}) mutant had significant intercalation defects (S3 Fig). There are two possible explanations for these results. First, all four ephrins could function redundantly during dorsal intercalation. Second, dorsal intercalation could be an ephrin-independent process and the \textit{e699} mutation disrupts a portion of the extracellular domain that also binds to other, non-canonical ligands. While the former possibility is difficult to test because EFN-4 has VAB-1 and EFN-1 independent roles that make \textit{vab-1; efn-4} and \textit{efn-1; efn-4} mutants synthetic lethal [40], we sought to investigate the latter possibility. Recently, a noncanonical VAB-1/EphR ligand, VPR-1/VAPB, was identified. VPR-1/VAPB is a secreted protein; mutations in \textit{vpr-1} have pleiotropic effects [58–60]. The progeny of \textit{vpr-1(tm1411)} null homozygotes are maternal effect lethal and display the comigration phenotype (S6 Fig); however, given the widespread disruption of epidermal cell positioning in such embryos we were not able to rule out earlier defects during cell specification and gastrulation as contributing factors. Future studies involving conditional loss of \textit{vpr-1} function will be needed to definitively determine whether \textit{vpr-1} acts in the same pathway as \textit{cdc-42} during dorsal intercalation.

While roles for Cdc42 are well established for migrating cells in culture, roles for Cdc42 during morphogenesis are difficult to establish due to earlier Cdc42-dependent processes, such as gastrulation and polarized cell division. Our results implicate polarized Cdc42 activity in orienting an intercalating epithelium during morphogenesis. Dorsal intercalation is only one of many examples of epithelial intercalation that involve basolateral protrusion [1, 61]. Given its ubiquitous role in establishing cell polarity and in regulating the actin cytoskeleton, it is likely that Cdc42 has widespread functions during these epithelial intercalation events.

\section*{Materials and Methods}

\subsection*{Nematode strains and genetics}

Worms were maintained on \textit{Escherichia coli} OP50, as previously described [62]. The wild-type strain used in this study was Bristol N2. Experiments were performed at 20°C unless otherwise specified. The following genetic lesions were utilized in this study. LGI: \textit{par-6(tm1425)}, \textit{smg-1}
(cc546ts), vpr-1(tm1411). LGII: cdc-42(gk388), ephx-1(ok494), vab-1(dx31), vab-1(e699), vab-1(e118), vab-1(e2). LGIII: par-3(it71), unc-119(ed3). LGIV: efn-2(ev658), efn-4(bx80), vab-2(ju1). LGX: efn-3(ev696). Additionally, the following transgenic arrays were made for or utilized in this study: jcEx200[P

lin-26::cdc-42(T17N/DN)::smg sensitive 3'UTR, sur-5::mCherry], ojEx99[P

cdc-42::gfp::wsp-1(G-protein binding domain); unc-119(+)] based on the cdc-42 biosensor described in [29], jcEx273[P
cdc-42::gfp::wsp-1(G-protein binding domain); P

lin-26::gfp::smg sensitive 3'UTR, rol-6(su1006)] [16], xnIs83[P
cdc-42::2xHA::ZF1::cdc-42; unc-119(+)] [22], zuIs20[P

par-3::par-3::ZF1::gfp; unc-119(+)] [24], zuIs43[P

pie-1::gfp::par-6::ZF1; unc-119(+)] [9].

To make par-6(M/Z) mutants, gfp::par-6::ZF1; par-6(tm1425) embryos were obtained through crossing, as described previously [9]. par-6(M/Z) mutants die late in embryogenesis [9], so only embryos that died at this stage were analyzed for intercalation defects.

VAB-1 cloning

The vab-1 tagged fosmid was obtained through the C. elegans TransgeneOme project [63]. The vab-1 fosmid was injected into wild-type animals at 30ng/μL along with 50ng/μL odr-1::rfp coinjection marker to obtain transgenic strain IC1403 quEx531[vab-1::2xTY1::gfp::3xFLAG fosmid; odr-1::rfp]. The quEx531 array was crossed into vab-1(dx31) to create strain IC1487 vab-1(dx31); quEx531 and tested for functionality.

Microinjection

To incorporate the epidermally-enriched cytoplasmic reporter, P

lbp-1::gfp, into ZF1::cdc-42 worms, we microinjected pSL500[P

lbp-1::gfp::unc-54 3'UTR] [64], directly into the gonads of ZF1::cdc-42 worms, as described previously [65].

CROSSES

Genetic crosses were used to incorporate the CDC-42 biosensor (gfp::wsp-1(GBD)) into the vab-1(dx31) background. To analyze efn-4(bx80), an outcross was performed to remove the him-5(e1490) marker from the background. Six outcrosses were performed to remove background mutations from ephx-1(ok494) (allele generated in [66]). Additionally, crosses were performed to incorporate vab-1(e2) into the CDC-42 dominant-negative and constitutively active backgrounds.

NMD-dependent conditional expression system

The plasmid backbone (pCM1.3) for the epidermal-specific, NMD-sensitive expression system was described previously [16]. The wild-type cdc-42 cDNA was amplified with primers DJR491, 5' TTTTTTTTggccggcct ggcATGCAGACGATCAAGTG CGTCGTCG 3', and DJR512, 5' TTTTTTcccgggTTAGA GAATATTGCACTTCTTCTTC 3', digested with FseI and XmaI, and cloned into pCM1.3 digested with FseI/XmaI to make pCM7.4. Restriction sites in the primers are underlined. The dominant-negative cdc-42, cdc-42(T17N/DN) (pEWS26), was made with pCM7.4 (cdc-42(+)) as a template for PCR site-directed mutagenesis, using the following primers: Forward 5' ATTGTCTCCTGATC AGCTATACC 3' Reverse 5' TTTTACCGA CAGCTCCATCTC 3'. Underlined bases denote those mutated relative to wild type.

As described previously [16], gonads of wild-type animals were microinjected with these constructs at 40 ng/μL and a coinjection marker [65]. Resulting lines in the wild-type background were screened for defects using smg-1 feeding RNAi [67]. Representative lines were
crossed into the smg-1(cc546ts) background, which can be detected by PCR using Forward: 5’ CAGTCGTGAGCTTTGGATGCTGGC 3’ and Reverse: 5’ TCGGGGATACGCAGATTTCTT TCCCTCC 3’ followed by digestion specifically of wild-type product using MslI. At least three lines were analyzed per construct. The resulting lines were maintained at 15°C and heat shocked at 25°C for 24 hours to induce transgene expression prior to filming. Crosses were performed at 15°C. Filming was performed at 25°C. For vab-1(e2) enhancement and suppression experiments, a semi-permissive temperature of 20°C was used for heat shock and filming.

**DIC imaging and phenotypic scoring**

Four dimensional DIC movies were gathered on either a Nikon Optiphot-2 connected to a QImaging camera or Olympus BX50 connected to a Scion camera. ImageJ plugins (available at http://worms.zoology.wisc.edu/research/4d/4d.html) were used to compress and view movies.

Embryos scored "medial delay" appeared to have blunt medial edges for greater than five time points (corresponding to 15 minutes). Embryos were scored “ipsilateral comigration” if at least one pair of two adjacent cells intercalated together across the dorsal array. Some embryos had both defects. For this reason, comigration and medial delay phenotypes are pooled into “intercalation defects”. Though nuclear migration defects were observed in some embryos, they were not considered “intercalation defects” as nuclear migration failure does not prevent successful intercalation [68]. For strains with extrachromosomal arrays—cdc-42 (DN) and cdc-42(CA)—only embryos that inherited the arrays were analyzed, as determined by inheritance of a co-injected nuclear fluorescent protein (SUR-5::mCherry), assayed by epifluorescence. For statistical analysis of intercalation defects among genotypes, Fisher’s Exact Test—a form of chi-squared specialized for low n values that has been used previously to compare vab-1 alleles [36]—was used.

**Immunostaining**

Freeze-cracking was used to permeabilize embryos [69] for antibody staining. Staining was performed as described previously [70]. Embryos were incubated with primary antibodies in PBST+dry milk overnight at 4°C. Embryos were incubated with secondary antibodies in PBST + dry milk for two hours at room temperature. The following primary antibodies were used: 1:1000 rabbit-anti-GFP (Invitrogen), 1:200 mouse-anti-AJM-1 (MH27). The following secondary antibodies were used: 1:50 anti-mouse IgG Texas Red (Jackson ImmunoResearch) and 1:50 anti-rabbit FITC (Jackson ImmunoResearch). Images of stained embryos were acquired as described below.

**Confocal microscopy**

Spinning-disk, confocal images were acquired with a Z-slice spacing of 0.4 μm using Micromanager software [71, 72] and a Nikon Eclipse E600 microscope connected to a Yokogawa CSU10 spinning disk scanhead and a Hamamatsu ORCA-ER charge-coupled device (CCD) camera.

**Medial enrichment.** Medial enrichment measures were obtained by calculating the ratio of average intensity measurements at the medial and lateral edges of each cell in ImageJ. Specifically, for each cell, the lasso tool was used to manually select the medial cell edge, lateral cell edge, and a random area in the cytoplasm (non-nuclear) as background. The average intensity of the background was subtracted from the medial and lateral edge measurements prior to calculating the fold enrichment. Projections of 5 focal planes, spaced 0.4 μm apart, were used for this analysis. Only cells that had both a visible medial and lateral edge were analyzed. JMP was used for statistical analysis. For CDC-42 biosensor images, background subtraction and pseudocoloring were performed in ImageJ.
Cell Morphology. Tip angles were calculated using trigonometric functions as the angle of a line connecting the tip to the cell centroid. The cell centroid, nuclear center, and aspect ratios were measured in ImageJ from the manual outline of each cell/nucleus. PAST was used for angular statistics and graphs [73]. JMP software (SAS; Cary, North Carolina) was used for the remaining statistical analysis.

Supporting Information

S1 Fig. CDC-42 and CED-10/Rac function together during intercalation. A) Dorsal images of smg-1(cc546), ced-10(n3246); smg-1(cc546), dominant-negative cdc-42(T17N), and triple cdc-42(T17N/DN); ced-10(n3246); smg-1(cc546) mutants at 25°C. Arrows within cell nuclei point in the direction of migration (left arrows green, right arrows blue). Left-hand cells pseudocolored green, right-hand cells pseudocolored blue. Cells that do not migrate are marked with a white “X”. Scale bar is 10 μm. B) Penetrance of dorsal intercalation defects in ced-10, cdc-42, smg-1, double and triple loss-of-function. Perturbation of ced-10 function significantly increased cdc-42(DN) defects (based on total mount defect frequency, ANOVA, significantly different from all other groups p < 0.005).

S2 Fig. vab-1/Eph is expressed in the dorsal epidermis. A) Left: α-GFP immunostaining to detect VAB-1::GFP expressed from a rescuing GFP and FLAG-tagged vab-1 fosmid in posterior intercalating cells. Right: MH27/α-AJM-1 immunostaining in the same embryo. Green arrows denote medial edges. Scale bar = 2.5 μm. B) Quantification of medial/lateral fold enrichment of α-GFP staining relative to MH27/α-AJM-1 staining. Numbers denote number of cells analyzed. * denotes significant difference using Student’s T-test (p = 0.045).

S3 Fig. Dorsal intercalation in ephrin mutants. A) The penetrance of total intercalation defects in neither vab-2(ju1) efn-2(ev658); efn-3(ev696) nor efn-4(bx80) is significantly different from wild-type (p ≥ 0.50, Fisher’s Exact Test). Gray numbers indicate number of embryos analyzed per genotype. B) Intercalation time in neither vab-2(ju1) efn-2(ev658); efn-3(ev696) nor efn-4(bx80) is significantly different than wild-type (p ≥ 0.232, Student’s T-test).

S4 Fig. DIC images of dorsal intercalation in cdc-42(DN); smg-1(cc546ts), cdc-42(DN); vab-1(e2); smg-1(cc546ts), and vab-1(e2); smg-1(cc546ts) at 20°C. Left dorsal cells pseudocolored green, right cells pseudocolored blue. Yellow dotted lines outline the blunt medial edge. Scale bar = 10 μm.

S5 Fig. Dorsal intercalation phenotypes in ephx-1/ephexin embryos. A) The penetrance of intercalation defects in ephx-1(ok494) is not significantly different than wild-type (p = 1, Fisher’s Exact Test). Small gray numbers indicate number of embryos analyzed per genotype. B) Intercalation time in ephx-1(ok494) is not significantly different than wild-type (p = 0.133, Student’s T-test).

S6 Fig. Maternal effect phenotypes for vpr-1(tm1411). A) DIC images of a vpr-1(tm1411M/Z) embryo with intercalation defects. Left dorsal cells pseudocolored green, right cells pseudocolored blue. Light arrows indicate direction of migration. The “0 min.” time point is one hour after the terminal epidermal divisions. Scale bar = 10 μm. B) Many vpr-1(tm1411M/Z) die before intercalation. The portion of those remaining that display intercalation defects (~12%)...
is significantly different than wild-type (WT) (p = 0.01, Fisher's Exact Test).

(TIFF)

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**Author Contributions**

Conceptualization: EWS JH.

Formal analysis: EWS JH.

Funding acquisition: JH ICS DR.

Investigation: EWS BL HC WB.

Methodology: EWS DR KK.

Project administration: JH.

Resources: EWS ICS WB KK DR JH.

Supervision: JH.

Validation: EWS HC BL.

Visualization: EWS JH.

Writing – original draft: EWS JH.

Writing – review & editing: EWS BL ICS DR KK JH.

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Figure S2
Figure S3

(A) Intercalation Defects (%)

(B) Intercalation Time (min.)

Genotype

WT       vab-2(ju1)   efn-2(ev658); efn-3(ev696)   efn-4(bx80)

N.S.     N.S.         N.S.                        N.S.
Figure S4
Figure S4

Panel A: Intercalation Defects (%)
- WT: 51
- ephx-1(ok494): 40

Panel B: Intercalation Time (min.)
- WT: 100
- ephx-1(ok494): 80

Both panels show the effects of different genotypes on intercalation, with comparable results indicated by 'N.S.'
