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The Inositol 1,4,5-Trisphosphate Receptor Regulates Epidermal Cell Migration in *Caenorhabditis elegans*

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Summary

Polarized migration and spreading of epithelial sheets is important during many processes in vivo, including embryogenesis and wound healing. However, the signaling pathways that regulate epithelial migrations are poorly understood. To identify molecular components that regulate the spreading of epithelial sheets, we performed a screen for mutations that perturb epidermal cell migration during embryogenesis in *Caenorhabditis elegans*. We identified one mutant (*jc5*) as a weak mutation in *itr-1*, which encodes the single inositol 1,4,5-trisphosphate receptor (ITR) in *C. elegans*. During the migration of the embryonic epidermis, *jc5* embryos display defects including misdirected migration or premature cessation of migration. Cells that halt their migration have disorganized F-actin and display reduced filopodial protrusive activity at their leading edge. Furthermore, some filopodia formed by epidermal cells in *itr-1(jc5)* embryos exhibit abnormally long lifetimes. Pharmacological studies with the inositol 1,4,5-trisphosphate antagonist xestospongin C phenocopy these defects, confirming that ITR function is important for proper epidermal migration. Our results provide the first molecular evidence that movements of embryonic epithelial cell sheets can be controlled by ITRs and suggest that such regulation may be a widespread mechanism for coordinating epithelial cell movements during embryogenesis.

Results and Discussion

Identification of Mutants Defective in Epidermal Cell Migration

The epidermis is born on the dorsal side of the *C. elegans* embryo after gastrulation. Two major epidermal cell migrations occur during embryogenesis: (1) dorsal intercalation, during which two rows of epidermal cells wedge between one another to produce a single row across the dorsal midline, and (2) ventral enclosure, during which the free edges of the epidermis migrate ventrally around the embryo. Ventral enclosure is initiated by two anterior pairs of cells, known as leading cells, which meet at the ventral midline in a bilaterally symmetric fashion. Then, posterior to the leading cells, cells known as pocket cells follow. Ventral epidermal cells extend filopodia as they migrate; this protrusive activity is required for the completion of ventral enclosure [1, 2]. The concurrent processes of dorsal intercalation and ventral enclosure almost completely encase the embryo in epidermis [3]. Disruption of ventral enclosure results in lethality caused by ruptures or severe defects during elongation of the embryo [1].

To identify genes involved in regulating epidermal migration in *C. elegans*, we screened for mutants that had the proper number of epidermal cells yet failed to complete ventral enclosure properly (see the Supplemental Data available with this article online). One mutant identified in this screen, *jc5*, displays cold-sensitive, maternal-effect defects in epidermal morphogenesis. The progeny of *jc5* homozygotes exhibit 95.4% (n = 1699) embryonic lethality at 15°C, but only 15.2% (n = 1894) at 20°C.

Epidermal Cells Fail to Migrate in *jc5* Mutant Embryos

We first examined *jc5* embryos for defects in cell lineage and muscle or gut differentiation with tissue-specific antibodies (see Supplemental Data). In typical embryos, we could detect no abnormalities in gut, muscle, or epidermal cell number or differentiation, suggesting that the *jc5* mutation results in defects specific to the epidermis. Progeny of *jc5* mutant mothers show a variety of phenotypes, including defects in epidermal morphogenesis, which we assessed in vivo by confocal microscopy with the apical junctional marker AJM-1::GFP [4]. At 15°C, approximately 11.4% arrest during enclosure. These display either complete retraction of the epidermis dorsally (1.4%) or partial enclosure (10%; n = 70); that is, some, but not all, epidermal cells meet ventrally, resulting in a characteristic ventral rupture. Moreover, approximately 16% (n = 89) of embryos have cells that are misdirected, failing to meet their proper contralateral neighbor. Multiphoton microscopy reveals that anterior ruptures occur in embryos in which leading cells halt prematurely (Figure 1).

In addition to ventral enclosure defects, other epidermal defects occur in *jc5* embryos. Of arrested embryos, 19% (n = 70) enclose properly but develop severe body shape defects as they attempt to elongate. Occasionally *jc5* homozygotes have defects in dorsal intercalation (see Figure 2J). The remaining arrested embryos possess fewer than the normal complement of epidermal precursors, suggesting defects in cytokinesis or cell fate specification.

jc5 Is an Allele of the Inositol 1,4,5-Trisphosphate Receptor, *itr-1*

We mapped the *jc5* mutation to the right arm of linkage group (LG) IV and cloned the gene with standard transformation rescue techniques. We found that *jc5* is an

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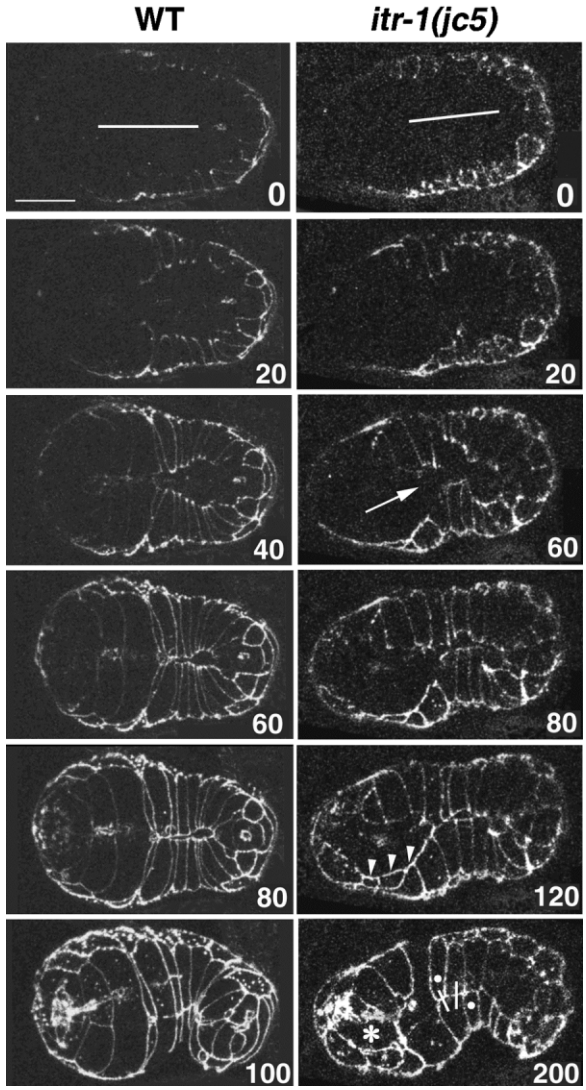

Figure 1. *itr-1(jc5)* Embryos Exhibit Epidermal Migration Defects

Elapsed time in minutes is shown in each panel. Left: a wild-type AJM-1::GFP-expressing embryo undergoing enclosure. Approximately 100 min after ventral epidermal cells begin migrating, they meet and seal at the ventral midline (marked in the first time point by a thick white line). Right: a representative *jc5* embryo expressing AJM-1::GFP. In the *jc5* embryo, leading cells halt prematurely, leaving the head of the embryo exposed (asterisk). In addition, some cells migrate at different rates toward the ventral midline (arrow), and many pocket cells do not align properly with their contralateral partner cell. In the example shown, the anterior-most P cell (P1) has not met with its contralateral partner (bullet). In addition, 2 cells form junctions with the same cell (vertical lines). The leading edges of some cells exhibit AJM-1::GFP localization, indicating that the cells have halted migration (arrowheads). The scale bar represents 10 μm.

allele of *itr-1*, the single inositol 1,4,5-trisphosphate (IP_3) receptor gene in *C. elegans* [5, 6]. Like other *itr-1* alleles, *jc5* larvae and adults show defects in defecation (see Supplemental Data); however, unlike other alleles, *jc5* preferentially affects epidermal morphogenesis (see Supplemental Data).

itr-1 has three alternative transcriptional starts, each followed by an exon that encodes three alternative N-terminal variants. It also has four alternative splice sites [7, 8]. We sequenced the entire 20 kb genomic region of the *itr-1* locus in *jc5* mutants; this included genomic sequence ~2.6 kb upstream of the 5′-most consensus start site and ~2.5 kb of the 3′ untranslated region. *jc5* mutants possess a single base pair deletion between exons 3 and 4. This deletion falls 39 bases beyond the end of exon 3, which also includes the third putative translational start site for *itr-1*(see Supplemental Data for a map of the *itr-1* genomic region). Given its position, this deletion could affect splicing of nascent transcripts produced from the upstream transcriptional start sites or the efficiency of translation from the upstream ATG. On the basis of sequencing of RT-PCR products with primers flanking the lesion, we could not detect mutant transcripts. It is possible that such transcripts are rapidly degraded and hence difficult to detect; alternatively, the *jc5* deletion affects a cryptic intronic enhancer. To assess the level of *itr-1* protein in *jc5* mutants, we performed Western blot analysis with an antibody known to recognize all ITR-1 isoforms [7]. Densitometry indicates that the amount of ITR-1 protein present in *jc5* mutants is 31.0 ± 7.5% (n = 3) of the amount found in the wild-type (Figure 2M). We observe a class of *jc5* embryos arrested prior to morphogenesis; this class is consistent with a quantitative reduction in ITR-1 protein levels. A previous study showed that embryonic lethality results when a dominant-negative IP_3 receptor is expressed in early *C. elegans* embryos [9].

ITR-1 Is Expressed at High Levels in the Epidermis

IP_3 receptors (ITRs) localize to both the endoplasmic reticulum (ER) and the plasma membrane [10, 11]. To determine the level of expression and subcellular distribution of ITR-1 in the epidermis, we performed immunostaining. ITR-1 is expressed at significant levels in the embryonic epidermis during the process of ventral enclosure. The reticular pattern of staining within these cells suggests that ITR-1 is localized to the ER [12] and close to the plasma membrane (Figure 2B). Consistent with Western blot analysis, ITR-1 is also present in *jc5* embryos derived from mothers grown at 15°C, although the overall membrane staining is reduced and more signal is present in the cytoplasm. The presence of ITR-1 at or near the plasma membrane may provide a mechanism for the close apposition of upstream receptors and *itr-1* and/or the localized release of calcium near the leading edge of migrating cells. The limited diffusion of Ca^{2+} within the cytoplasm may thus restrict its effects to targets near the leading edge. Alternatively, the close apposition of ITRs to the plasma membrane has been proposed to play a role in store-operated calcium (SOC) entry in models that depend on conformational coupling of ITRs and SOC channels [13, 14]. Thus, the localization we observe may also act to allow or induce a local sustained calcium response.

ITR Function Is Required during Ventral Enclosure

The previous results strongly suggest that ITR-1 is specifically required in epidermal cells, but they do not establish the precise time during which ITR function, and

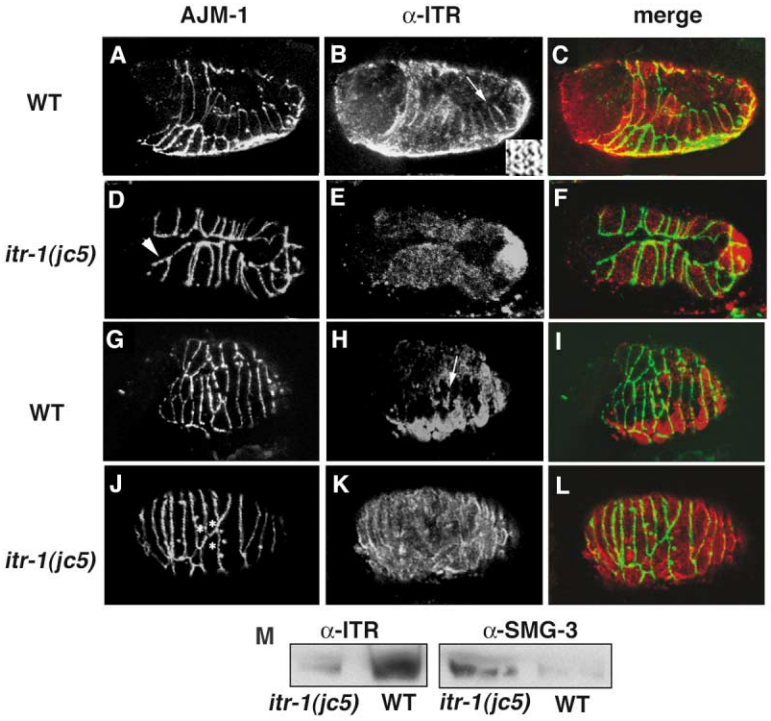

Figure 2. ITR-1 Expression during Ventral Enclosure

Images show representative wild-type and *itr-1(jc5)* embryos expressing AJM-1::GFP; the embryos were stained for ITR-1 (antibody 263, a gift from K. Mikoshiba).

(A, D, G, and J) AJM-1::GFP; (B, E, H, and K) anti-ITR immunostaining; (C, F, I, and L) merge.

(M) Western blot analysis of ITR (left) and SMG-3 (right) (a gift from P. Anderson) as a loading control.

(A)–(F) are ventral views; (G)–(L) are dorsal views. All embryos are shown with anterior to the left and posterior to the right.

(A–C) Wild-type embryo undergoing enclosure.

([B], inset) 2.5× magnification of a portion of two ventral epidermal cells from another embryo immunostained for ITR-1; the magnified region shows reticular staining.

(C) ITR-1 staining is evident at epidermal cell borders, as shown by overlapping signal (arrow).

(D–F) *itr-1(jc5)* embryo undergoing enclosure. Leading cells are starting to retract (arrowhead). There is no ITR-1 staining evident at epidermal cell membranes.

(G–I) Wild-type embryo undergoing dorsal intercalation.

(H) ITR-1 localizes near the plasma membrane (arrow) and in a reticular pattern.

(J–L) *itr-1(jc5)* embryo undergoing dorsal intercalation. Dorsal cells have not intercalated (asterisks).

(K) Where dorsal cells have not intercalated properly, ITR-1 staining appears more diffuse, and membrane staining is not visible. The scale bar represents 10 μm.

presumably calcium signaling, is required for epidermal migration. We perturbed calcium levels during the migration of epidermal cells by laser permeabilizing embryos in the presence of 100 μM BAPTA-AM, a known calcium chelator. Permeabilization was performed after all epidermal cells were born and just before the start of enclosure. In the presence of BAPTA-AM, epidermal migration was compromised. Phenotypes ranged from no migration of ventral epidermal cells (4/9) to the complete or partial retraction of the epidermis (n = 5/9) (Figure 3), indicating that calcium is required at the onset of enclosure. Control embryos permeabilized in medium alone enclosed normally (n = 13). Embryos were treated with 100 μM xestospongin C, a specific inhibitor of ITRs, to confirm that ITR-1 activity is also required at this time [15]. In the presence of xestospongin C, ventral epidermal cells either did not migrate (3/11) or retracted dorsally (n = 7/11) (Figure 3). In one embryo, migration commenced but internal cells were extruded before enclosure was completed. These results indicate that ITR function is required at the time of enclosure and not simply for a cellular event prior to epidermal morphogenesis.

To date, no one has succeeded in visualizing calcium dynamics in the *C. elegans* epidermis. Although we were also unable to detect measurable calcium transients in the epidermis with a variety of calcium-sensitive dyes and a cameleon construct (data not shown), we presume

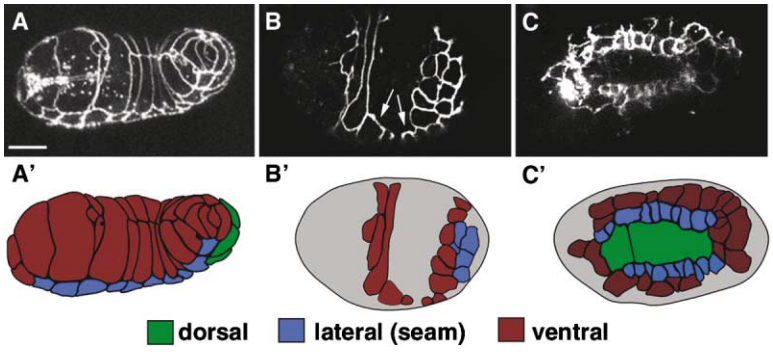

Figure 3. Pharmacological Disruption of Calcium and ITRs Causes Epidermal Migration Defects

All embryos are terminals expressing AJM-1::GFP, with anterior to the left and posterior to the right. (A)–(C) are representative embryos from pharmacological inhibition experiments.

(A) A representative control embryo, laser-permeabilized in the absence of drug, that has enclosed normally.

(B) A BAPTA-AM-treated embryo in which the pocket cells have halted their migration prematurely (arrows; ventral view).

(C) A xestospongin C-treated embryo in which the epidermis initiated migration but retracted dorsally before junctions formed at the ventral midline (dorsal view).

(A′–C′) Schematics of the embryos shown above. Red, ventral epidermal cells; blue, lateral (seam) cells; green, dorsal cells. The scale bar represents 10 μm.

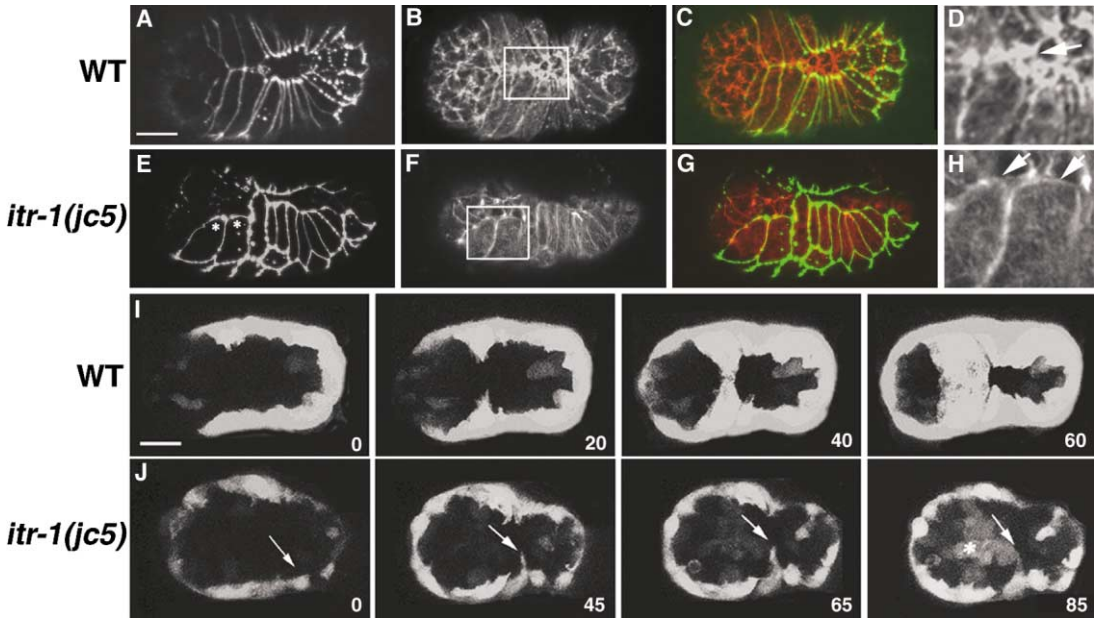

Figure 4. Epidermal Cells in *itr-1(jc5)* Embryos Both Display a Disrupted Actin Cytoskeleton and Have Fewer, More Persistent Filopodia at the Leading Edge

Embryos are shown with anterior to the left and posterior to the right.
(Top) (A and E) AJM-1::GFP. (E) is a three-dimensional projection of a confocal Z series due to the orientation of the embryo.
(B and F) Phalloidin staining.
(C and G) Merge.
(D and H) Enlargements of the boxed areas in (B) and (F), respectively.
(A–C) A wild-type embryo undergoing enclosure.
(D–F) A similarly staged *itr-1(jc5)* embryo undergoing enclosure.
(E) Leading cells have halted migration (asterisks) while pocket cells continue migration.
(F) Ventral epidermal cells have reduced actin accumulation at the leading edge ([H], arrows) when compared to those in the wild-type ([D], arrow). Note also that F-actin in a nonmigrating cell (F) appears disorganized compared to that in migrating cells (B), in which F-actin accumulates at the tip of the migrating cell.
(Bottom) A cytoplasmic GFP expressed under the control of the *dlg-1* promoter was used to visualize filopodia. Filopodial dynamics at the leading edge in wild-type and *itr-1(jc5)* embryos are shown. Elapsed time in minutes is shown in each panel. One filopodium (arrow) in the *itr-1(jc5)* embryo persists for over 80 min, in contrast to wild-type embryo filopodial protrusions, which never last for more than 10 min. The gut of the *itr-1(jc5)* embryo is eventually extruded (asterisk). The scale bar represents 10 μm.

that ITR-1 regulates release of calcium from internal stores during epidermal migration. ER-dependence of calcium transients is typically shown pharmacologically with thapsigargin, which disrupts the calcium ATPase (SERCA) responsible for reuptake into the ER and which is also known to perturb the motility of mesodermal cells in *Xenopus* embryos [16]. However, regulation of calcium homeostasis in the *C. elegans* epidermis is unlikely to depend crucially on this channel. The *C. elegans* SERCA homolog, *sca-1*, is not expressed in the epidermis, and *sca-1* deletion mutants and *sca-1(RNAi)* embryos show no enclosure defects [17]. Moreover, embryos treated with up to 10 μM thapsigargin at the time of enclosure develop normally (data not shown). Calcium levels must presumably be regulated via another, unidentified pathway in the *C. elegans* epidermis.

Motility at the Leading Edge Is Compromised in Epidermal Cells of *itr-1(jc5)* Mutants

Surprisingly little is known about the regulation of motility by ITRs. One study indicates that ITRs play a role in neurite outgrowth [18]. Consistent with this, transient elevations of calcium can induce filopodia [19]. Because epidermal cells extend filopodia as they migrate toward the ventral midline [3], we next examined the cytoskeleton in epidermal cells of *jc5* mutants. Interestingly, in *itr-1(jc5)* mutants, actin is depleted at the tips of aberrantly migrating cells (Figures 4E–4H), whereas there is a high concentration of F-actin at the leading edge in cells that migrate normally (Figures 4A–4D). Moreover, the F-actin retained in cells that halt their migration appears to be disorganized compared to that in neighboring cells that continue to migrate. Cytoskeletal defects at the leading edge were confirmed by imaging HMP-1/α-catenin::GFP in *itr-1(jc5)* embryos (Supplemental Data). In contrast, microtubule organization is normal in *itr-1(jc5)* mutants (data not shown).

To visualize filopodial dynamics, we expressed a cytoplasmic green fluorescent protein (GFP) in epithelial cells (Figures 4I and 4J) [20]. Both the number of filopodia and their persistence were measured in wild-type and *jc5* mutant embryos. The number of filopodial protrusions is reduced in *itr-1(jc5)* embryos when compared to that in the wild-type (see Supplemental Data). In *itr-1(jc5)* embryos, 1.0 $\pm$ 0.1 new filopodium/embryo could be counted every five minute interval (n = 358 filopodia

in 10 embryos); in contrast, wild-type embryos averaged 2.5 ± 0.1 (n = 434 filopodia in 8 embryos). The filopodia in *itr-1(jc5)* embryos are also more persistent. In particular, the percentage of filopodia that persist for more than 15 min is significantly increased in *itr-1(jc5)* embryos, with many lasting far longer. Nearly 15.2% of total filopodia in *itr-1(jc5)* embryos last for more than 15 min, whereas only 1.0% last that long in the wild-type (see Supplemental Data). Increased filopodial persistence in *itr-1(jc5)* embryos may account for the inhibited migration of epidermal cells during ventral enclosure. Our observations thus support other studies [18, 19] suggesting that IP_3-mediated signaling has a role in the formation and maintenance of filopodia.

Both ITR-1 and calcium may exert their effects on the actin cytoskeleton by regulating actin binding proteins such as nonmuscle myosin [21, 22]. The nonmuscle myosin NMY-2 was recently shown to interact with ITR-1 [21]. Both NMY-1 and NMY-2 have roles in embryonic elongation [23], and elongation is defective in some *itr-1(jc5)* embryos. It is currently unknown whether ITR-1 regulates morphogenesis through NMY-1 and -2 or whether the NMYs play a role in epidermal migration. Another potential calcium effector is calmodulin. RNAi against the *C. elegans* calmodulin homolog, *cmd-1*, results in defects in ventral enclosure (data not shown) [24], suggesting that CMD-1 may play a role during epidermal migration. CMD-1 could regulate the actin cytoskeleton through proteins such as myosin [25], calcineurin [26], and IQGAP1 [27, 28]. To date, a role for calcineurin in *C. elegans* development has not been demonstrated. RNAi against the best IQGAP homolog in *C. elegans* does not result in obvious phenotypes (data not shown) [29].

The concerted movement of cells to form three-dimensional structures requires complex cell signaling networks to coordinate directional cell migrations. Calcium and IP_3 signaling have been implicated in the migration of neuronal growth cones [18, 27, 30] and the nonepithelial mesodermal cells of *Xenopus* [16]. Our investigation provides the first molecular evidence that ITRs also play a central role in regulating protrusive activity during epithelial morphogenesis. It will be interesting to investigate whether this is an evolutionarily conserved mechanism in higher eukaryotes. Future studies focusing on the identification of effectors downstream of ITRs could ultimately provide a direct link between ITRs, the actin cytoskeleton, and filopodial dynamics during epithelial migration.

Supplemental Data

Supplemental Experimental Procedures and Figures are available with this article online at www.current-biology.com/cgi/content/full/14/20/1882/DC1.

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