Nodal signaling regulates endodermal cell motility and actin dynamics via Rac1 and Prex1

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Embryo morphogenesis is driven by dynamic cell behaviors, including migration, that are coordinated with fate specification and differentiation, but how such coordination is achieved remains poorly understood. During zebrafish gastrulation, endodermal cells sequentially exhibit first random, nonpersistent migration followed by oriented, persistent migration and finally collective migration. Using a novel transgenic line that labels the endodermal actin cytoskeleton, we found that these stage-dependent changes in migratory behavior correlated with changes in actin dynamics. The dynamic actin and random motility exhibited during early gastrulation were dependent on both Nodal and Rac1 signaling. We further identified the Rac-specific guanine nucleotide exchange factor Prex1 as a Nodal target and showed that it mediated Nodal-dependent random motility. Reducing Rac1 activity in endodermal cells caused them to bypass the random migration phase and aberrantly contribute to mesodermal tissues. Together, our results reveal a novel role for Nodal signaling in regulating actin dynamics and migration behavior, which are crucial for endodermal morphogenesis and cell fate decisions.

Introduction

During the development of vertebrate organs, cells exhibit distinct morphologies and behaviors, such as cell migration, adhesion, and proliferation, that are indicative of their particular cell type and differentiation state. Although much work has been done to identify and characterize the signals that induce specific cell fates, how these developmental signals are translated into characteristic cellular behaviors is poorly understood. Cell migration is important for numerous processes, including embryonic development, immune function, and wound healing, as well as the progression of diseases such as metastatic cancer. The mode of cell migration can be persistent, in which cells migrate in the same general direction over time, or nonpersistent, in which cells frequently change direction (Pankov et al., 2005; Petrie et al., 2009). Not only do different cell types exhibit different modes of migration, but the same cell may also change the way it migrates at different developmental stages (Bak and Fraser, 2003; Pézeron et al., 2008). These observations suggest that the type of migratory behavior is a marker of differentiation, but its significance is poorly understood.

Endodermal cells in the early zebrafish embryo exhibit multiple modes of migration and thus constitute an ideal model for investigating how different migratory behaviors are regulated. Just before gastrulation, high levels of Nodal signaling at the blastoderm margin induce endoderm specification (Stainier, 2002; Zorn and Wells, 2009). As gastrulation begins, endodermal cells undergo ingestion and migrate between the yolk and epiblast. Initially, cells migrate in a random walk pattern, resulting in the dispersal of endodermal cells across the yolk surface in a discontinuous salt-and-pepper pattern (Pézeron et al., 2008). By 90% epiboly, endodermal cells begin a second phase of migration characterized by convergent movements toward the embryonic axis. Finally, these individual migratory cells must adhere together to ultimately form the epithelial lining of the gastrointestinal tract. These progressive changes in migration behavior are likely subject to tight regulation. However, although much work has been done to understand how developmental signaling molecules induce differential gene expression during endoderm differentiation and patterning (Stainier, 2002; Zorn and Wells, 2009), the downstream cellular responses, including migration, remain to be explored.

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Abbreviations used in this paper: DN, dominant negative; GEF, guanine nucleotide exchange factor; MO, morpholino; PBD, p21-binding domain; ROI, region of interest.

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Cell migration involves the complex rearrangement of the actin cytoskeleton, which is coordinated by numerous actin regulatory proteins (Rottner and Stradal, 2011). The Rho family of small GTPases, including RhoA, Rac1, and Cdc42, play several well-characterized roles in regulating actin dynamics during cell migration. For example, Cdc42 and Rac1 promote actin polymerization to drive membrane protrusion at the leading edge (Kozma et al., 1995; Wu et al., 2009), whereas RhoA induces actomyosin contraction, which provides the force necessary for cell translocation (Chrzanowska-Wodnicka and Burridge, 1996). The majority of studies investigating the molecular mechanisms underlying these actin dynamics have primarily used cells cultured on 2D or 3D substrates. However, it is known that cell migration can differ markedly in vivo (Yamada and Cukierman, 2007), but, until recently, it has been difficult to study subcellular actin dynamics within living organisms. In this study, we used a novel transgenic zebrafish line in which F-actin is fluorescently labeled specifically in endodermal cells. Using this line, we were able to track actin dynamics and cell motility at high resolution within the developing zebrafish embryo. We found that Nodal signaling can affect actin stability and retrograde flow in endodermal cells, which correlated with Nodal-dependent changes in cell migration. We further show that the effects of Nodal signaling on actin dynamics and cell migration are mediated by Rac1 and that Nodal signaling induces expression of the Rac activator Prex1. We found that similar to Nodal and Rac1, Prex1 is also required for the dynamic motility of endodermal cells and that it acts downstream of Nodal to drive random migration. Finally, we show that perturbing Rac1 activity in endodermal cells results in their aberrant contribution to mesodermal tissues, thereby revealing the importance of regulated cell motility to cell fate decisions.

Results

Tg(sox17:GFP-UTRN) expression labels F-actin in endodermal cells

To investigate the molecular mechanisms underlying endoderm migration in vivo, we generated a transgenic line in which the endoderm-specific sox17 promoter drives expression of a fluorescent actin probe consisting of the F-actin–binding domain of Utrophin (Burkel et al., 2007) fused to GFP (Tg(sox17:GFP-UTRN)). Tg(sox17:GFP-UTRN) expression readily labels actin-rich structures in vivo, including lamellipodia, filopodia, retraction fibers, dorsal ruffles, actin bundles, and cleavage furrows of dividing cells (Fig. 1 and Videos 1–4). Cells often contained multiple sites of GFP-UTRN fluorescence, suggesting that actin polymerization is not restricted to a single leading edge. To examine actin dynamics during active migration, we imaged Tg(sox17:GFP-UTRN) gastrulae by time-lapse spinning-disk confocal microscopy (Videos 1 and 2). We observed that GFP-UTRN fluorescence rapidly accumulated in protrusive areas of cells, presumably a result of actin polymerization, and rapidly disappeared at sites of membrane retraction. Within the larger protrusions, we sometimes observed fluorescent particles streaming back toward the cell center, indicative of retrograde flow (arrow in Video 1). Thus, using this transgenic line, we can track actin rearrangements with high resolution in living embryos and gain further insights into the in vivo regulation of cytoskeletal dynamics.

Endodermal cells exhibit progressive changes in migratory behavior and actin dynamics during gastrulation

A previous study has shown that endodermal cells undergo random migration during early gastrulation but switch to convergence movements in late gastrulation (Pézeron et al., 2008). We first confirmed that cells labeled by Tg(sox17:GFP-UTRN) expression exhibit similar migration behaviors. We quantified both the directional persistence of migration (defined as the ratio of net over total distance traveled) as well as the mean instantaneous velocity over 1-h intervals. During early stages (shield to 75% epiboly), cells migrated relatively randomly, although with a slight bias toward the dorsal side of the embryo (Fig. 2 [A and B] and Video 3). However, during late stages (90% epiboly to tailbud), endodermal cells moved with strong persistence in the dorsal direction, which was accompanied by a significant increase in migration velocity (Fig. 2, D and E). This switch from random to oriented migration was accompanied by a change in cell shape (Fig. 2 [F–H] and Video 3). In early stages, cells were mostly round with a few small lamellipodial protrusions (Fig. 2 F), but, by late stages, cells took on a flattened appearance with much broader lamellipodia (Fig. 2 G). By tail bud stage, the converging endodermal cells began to adhere to each other to form the endodermal sheet (Video 4).

By tracking GFP-UTRN fluorescence, we investigated the actin cytoskeletal rearrangements that occur during these changes in cell motility (Fig. 3 and Videos 1 and 2). First, we determined the dynamics of the actin cytoskeleton at early (70% epiboly) and late (90% epiboly) stages by measuring the persistence of GFP-UTRN fluorescence, focusing on the large fluorescent patches that often marked lamellipodia-like protrusions (Fig. 3, A and B). We found that these lamellipodia were relatively transient at 70% epiboly but were significantly more long lived at 90% epiboly (Fig. 3 C). This result suggests that the endodermal lamellipodia are more dynamic during early stages, which likely contributes to the ability of the cells to rapidly change migration direction. We also recorded the spatial orientation of lamellipodia within the cell with respect to the embryonic
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are less clear, although one study suggests that Nodal signaling may promote random migration of mesendodermal cells (Pézeron et al., 2008). Nodal is a member of the TGF-β superfamily of signaling proteins that is required for the specification of endoderm and mesoderm (Feldman et al., 1998; Stainier, 2002). Classically, the role of Nodal signaling during endoderm development has been to induce the expression of endoderm-specific transcription factor genes (Alexander and Stainier, 1999; Reiter et al., 2001; Poulain and Lepage, 2002; Stainier, 2002; Zorn and Wells, 2009). To determine whether Nodal signaling regulates the migration of endodermal cells in addition to its role in endodermal fate specification, we treated Tg(sox17:GFP-UTRN) embryos with the Nodal receptor/Alk4/5/7 inhibitor SB-505124 (Fig. 4; Hagos and Dougan, 2007). To focus on events subsequent to endoderm specification, inhibitor treatment started at 5 h after fertilization, which does not appear to interfere with the onset of endodermal marker gene expression (Fig. S1, A–D).

We found that treatment with 50 µM SB-505124 significantly slowed migration velocity and increased migration persistence at early stages (70% epiboly) compared with DMSO-treated control (Fig. 4 [A–D] and Video 5). Nodal receptor inhibition also induced changes in actin dynamics. In particular, we found that SB-505124 treatment significantly increased lamellipodia lifetime and slowed the rate of retrograde flow (Fig. 4, E–J). However, we did not detect any directional bias in lamellipodia formation (unpublished data), suggesting that although Nodal inhibition can promote migration persistence, it likely does not provide guidance information.

Nodal signaling promotes Rac1 activity in endodermal cells

Our results suggest that Nodal signaling can regulate actin dynamics, but there are no known cytoskeletal regulators in the Nodal signaling pathway. To identify a link between Nodal and the actin cytoskeleton, we focused on the Rho family GTPase Rac1 as a candidate. Rac1 has well-characterized roles in many aspects of cell migration, including promoting actin polymerization and lamellipodia formation (Ridley et al., 1992). The characteristics of endodermal cells during early gastrulation—in particular, weak directionality and short-lived, nonoriented protrusions—are strikingly similar to cells expressing constitutively active forms of Rac1 (Pankov et al., 2005; Woo and Gomez, 2006). Moreover, expression levels of Rac1 were shown to be sufficient to modulate the migration persistence of fibroblasts in vitro, with high levels promoting random migration and low levels facilitating persistent migration (Pankov et al., 2005).

First, we determined whether Rac1 was required for early random migration by overexpressing dominant-negative (DN) Rac1 in Tg(sox17:GFP-UTRN) embryos. Injection of large amounts of DN Rac1 mRNA (10 pg) resulted in cessation of all cell movements (unpublished data). However, a low dose of DN Rac1 mRNA (2 pg) only moderately inhibited endodermal migration speed but significantly increased migration persistence at 70% epiboly, similar to what was observed with Nodal receptor inhibition (Fig. 4, K–N). This low dose of DN Rac1 expression did not appear to affect expression of the endodermal

Figure 2. Endodermal cells exhibit changes in migratory behavior and cell shape as embryos progress through gastrulation. (A–C) Representative migration tracks over a 1-h period of endodermal cells at shield (6 h after fertilization; A), 75% epiboly (8 h after fertilization; B), and 90% epiboly (9 h after fertilization; C). Dorsal is to the right. Bars, 25 µm. (D and E) Quantification of migration persistence (D) and instantaneous velocity (E) shows that migration persistence and speed increase as gastrulation proceeds. Shield, n = 49 cells; 75% epiboly, n = 74 cells; 90% epiboly, n = 95 cells. [F and G] Representative images of endodermal cells from Tg(sox17:GFP-UTRN) embryos at 70% epiboly [F] and 90% epiboly [G]. [H] Quantification of circularity shows that cells are significantly more rounded at 70% epiboly, a.u., arbitrary units. 70% epiboly, n = 63 cells; 90% epiboly, n = 66 cells. All error bars represent SEM. *P < 0.05.

axes (dorsal, ventral, animal, or vegetal; Fig. 3 D). At 70% epiboly, lamellipodia oriented at similar frequencies toward the dorsal, ventral, or vegetal directions but were less likely to occur toward the animal pole. However, at 90% epiboly, lamellipodia formation was significantly more biased in the dorsal direction (P = 0.00163 by χ2 test). Thus, the preferential initiation and persistence of dorsally oriented actin polymerization likely underlie the dorsal-directed movement of endodermal cells at late stages.

A study of migratory cells in vitro has shown that the rate of retrograde flow decreases as protrusion persistence increases (Lim et al., 2010). Therefore, we used kymography (Batchelder et al., 2011) to determine whether retrograde flow within protrusions varied from early to late stages (Fig. 3, E–I). We found that the rate of retrograde flow within endodermal cells was significantly faster during early compared with late stages (Fig. 3 I), correlating with the shift from random to oriented migration.

Nodal signaling promotes random migration and actin dynamics during early stages

A study has reported that the dorsally oriented migration of endodermal cells during late gastrulation depends on the chemokine Cxc12b and its receptor Cxcr4a (Mizoguchi et al., 2008). In contrast, the mechanisms controlling early random migration

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motility was assessed starting at 70% epiboly. Importantly, transplanted sox32-overexpressing cells display biphasic migration behaviors similar to those of endogenous endodermal cells, switching from random to persistent migration between early/mid and late gastrulation (Fig. S2, A–D). These cells also undergo the corresponding changes in cell shape (Fig. S2, E and F). However, when transplanted cells coexpressed DN Rac1, we found that directional persistence significantly increased during

Figure 3.  **Actin dynamics within endodermal cells change from early to late gastrulation.**  (A and B) Actin dynamics were analyzed by tracking lamellipodia through accumulations in GFP-UTRN fluorescence. Representative lamellipodia are highlighted in red in B from the cells in A. Bars, 25 µm. (C) Lamellipodial lifetime increases during late gastrulation. Early (70% epiboly), n = 523 lamellipodia from 45 cells; late (90% epiboly), n = 665 lamellipodia from 77 cells. (D) Orientation of lamellipodia formation with respect to the embryonic axes; V, ventral; A, animal; Vg, vegetal; D, dorsal. Lamellipodia formation is biased toward the dorsal direction during late gastrulation (P = 0.00163 by χ² test). Early (70% epiboly), n = 45 cells; late (90% epiboly), n = 77 cells from two independent experiments.  (E–I) Analysis of retrograde flow. Kymographs in F and H were generated along the red lines shown in E and G, respectively. Time is plotted horizontally, and the direction of membrane protrusion is oriented toward the top of the images. Red lines in F′ and H′ highlight retrograde-moving actin structures, which form streaks in the kymographs. The slope of these streaks was used to calculate the rate of retrograde flow (J), which decreases in late gastrulation. Early (70% epiboly), n = 12 cells; late (90% epiboly), n = 15 cells. Bars: (E–H) 10 µm; (F′ and H′) 5 µm. All error bars represent SEM. *, P < 0.05.

marker genes sox17 and sox32 (Fig. S1, E–H), suggesting that the effects on endodermal motility were not a result of mis-specification. To determine whether Rac1 was required cell autonomously within endodermal cells to promote dynamic migration, we performed cell transplantation experiments. Donor endodermal cells were generated by overexpression of sox32 either alone or combined with DN Rac1. Cells were transplanted into wild-type host embryos at 4–5 h after fertilization, and cell

Figure 4.  **Cell migration and actin dynamics during early gastrulation depend on Nodal and Rac1 signaling.**  (A and B) Representative migration tracks over a 1-h period from embryos treated with DMSO carrier (A) and 50 µM Nodal receptor inhibitor SB-505124 (SB; B). Dorsal is to the right. Bars, 25 µm.  (C and D) Quantification of migration persistence and instantaneous velocity shows that Nodal inhibition leads to significantly increased migration persistence and reduced migration velocity. DMSO, n = 74 cells; SB-505124, n = 48 cells.  (E) Nodal inhibition increases lamellipodial lifetime. DMSO, n = 191 lamellipodia from 28 cells; SB-505124, n = 324 lamellipodia from 46 cells.  (F–J) Nodal inhibition slows retrograde flow. Kymographs in G and I were generated along the red lines shown in F and H, respectively. Time is plotted horizontally, and the direction of membrane protrusion is oriented toward the top of the images. Bars: (F and H) 10 µm; (G and I) 5 µm. The rate of the retrograde flow is quantified in J. DMSO, n = 9 cells; SB-505124, n = 5 cells.  (K and L) Representative migration tracks over a 1-h period from control embryos (K) and embryos expressing DN Rac1 (L). Bars, 25 µm.  (M and N) Quantification of migration persistence and instantaneous velocity from control (Ctrl) and embryos expressing DN Rac1. Loss of Rac1 activity significantly increases migration persistence and moderately reduces migration velocity. Control, n = 76 cells; DN Rac1, n = 98 cells. All error bars represent SEM. *, P < 0.05.
The Rac-guanine nucleotide exchange factor (GEF) prex1 is a Nodal target gene and is required for random migration

Small GTPases such as Rac1 are activated by GEFs, which promote the dissociation of GDP, allowing GTP to bind. TGF-β1 has been shown to induce the expression of the Rho-GEF NET1, leading to increased RhoA activity and actin stress fiber formation (Shen et al., 2001). Therefore, we hypothesized that Nodal might similarly regulate expression of a Rac-GEF to control Rac1 activity. To identify endodermally enriched Nodal target genes, we performed microarray analysis using Tg(sox17:GFP) embryos treated with SB-505124 to inhibit Nodal signaling or overexpressing a constitutively active form of the acvr1b Nodal receptor (taram-a*). Of the genes identified, three were Rac-specific GEFs: arhgef25b, prex1, and tiam1. We verified these candidates by quantitative real-time PCR and found that only prex1 expression was consistently Nodal responsive (Figs. 6 A and S3 B). When embryos were treated with SB-505124, prex1 expression was down-regulated 2.8 ± 0.45 fold compared with DMSO-treated control. Correspondingly, when Nodal signaling was activated by expression of the constitutively active receptor taram-a*, prex1 expression increased 2.85 ± 0.5 fold compared with that in embryos expressing a control RNA.

Prex1 was initially identified in neutrophils as a protein required for phosphatidylinositol (3,4,5)-trisphosphate (PIP3)–induced Rac activation (Welch et al., 2002). It consists of a RhoGEF domain, a pleckstrin homology domain, two DEP (dishevelled, Egl-10, and pleckstrin) domains, two PDZ domains, and a C-terminal region with significant similarity to inositol polyphosphate-4-phosphatase but that is apparently catalytically inactive. Prex1 is synergistically activated by PIP3 and Gβγ (Welch et al., 2002; Barber et al., 2007; Zhao et al., 2007) and is important for neutrophil function (Welch et al., 2005), neurite formation (Waters et al., 2008), and motility of breast cancer cells (Sosa et al., 2010). By in situ hybridization, we found that at 70% epiboly, when endodermal cells are undergoing random migration, prex1 appears to be most highly expressed within the endoderm (Fig. 6 B).

We determined whether Prex1 functions as a Rac-GEF in zebrafish endodermal cells by examining the effects of morpholino (MO)-mediated knockdown of Prex1 on Rac1 activity (Fig. 6, C–E). Using the same aforementioned PBD fluorescence assay, we found that Prex1 knockdown resulted in a significant decrease in Rac1 activity (Fig. 6 E). We also examined the
prex1 is a target of Nodal signaling, promotes Rac1 activity, and regulates endodermal cell motility. (A) Expression of prex1 was measured by real-time quantitative PCR. Inhibition of Nodal signaling by SB-505124 treatment (SB) down-regulated prex1 expression (normalized to DMSO-treated controls), and overactivation of the Nodal pathway by expression of taram-A* (TA*) increased prex1 expression (normalized to control embryos expressing mCherry). The data shown are mean fold changes from six independent experiments. (B) Section through an embryo at 70% epiboly processed for prex1 in situ hybridization. prex1 appears to be enriched within the endodermal layer (arrows). Bar, 25 µm. (C and D) Representative ratiometric images of control (Ctrl; C) and Prex1 MO–injected (D) cells expressing RFP-PBD and colabeled with fluorescent dextran. Images are pseudocolored based on ratio value. Warmer colors indicate enrichment of PBD relative to dextran. Bars, 10 µm. (E) Quantification of the mean ratio of PBD to dextran indicates that Prex1 knockdown reduces Rac1 activity. Control, n = 124 cells; MO, n = 70 cells. (F and G) Representative migration tracks over a 1-h period from control (E) and Prex1 MO–injected (F) embryos. Dorsal is to the right. Bars, 25 µm. (H and I) Quantification of migration persistence (H) and instantaneous velocity (I) from control and Prex1 MO–injected embryos. Prex1 knockdown significantly increased migration persistence and moderately reduced migration velocity. Control, n = 80 cells; MO, n = 33 cells. (J and K) Overexpressing Prex1 can rescue random migration (J) and partially rescue migration velocity (K) in embryos treated with the Nodal inhibitor SB-505124. DMSO, n = 44 cells; SB-505124, n = 34 cells; SB-505124 + Prex, n = 52 cells. All error bars represent SEM. *, P < 0.05.

Prex1 can rescue random migration (J) and partially rescue migration velocity. Control, n = 80 cells; MO, n = 33 cells. Overexpression of Prex1 rescued the effects on directional persistence (H) and instantaneous velocity (I). Overexpression of Prex1 rescued the effects on directionality and partially rescued the effects on migration velocity, suggesting that Prex1 at least partially mediates signaling downstream of Nodal to control endodermal cell motility.

All together, these results suggest that prex1 is an endodernally expressed Nodal target gene that activates Rac1 and mediates the Nodal-dependent dynamic motility of endodermal cells.

Random migration is required to maintain endodermal identity
It is not clear how an initial phase of random migration contributes to subsequent steps of endodermal morphogenesis. To address this question, we expressed low levels of DN Rac1 to bypass the random migration phase and promote precocious persistent migration and then assessed the effects on later stages of endoderm development (Fig. 7). Control endodermal donor cells labeled by Tg(sox17:GFP-UTRN) expression were transplanted together with DN Rac1–expressing cells labeled by Tg(sox17:dsRed) expression into unlabeled wild-type hosts after gastrulation (4–5 h after fertilization). The distribution of GFP- and dsRed-labeled cells was then assessed at 22–24 h after fertilization. We found that the majority of both control and Rac1-deficient cells were located within the gut tube and pharyngeal endoderm (Fig. 7, A–D). However, a significant proportion of cells expressing DN Rac1 was found within mesodermal tissues such as the somites and notochord (arrows in Fig. 7 [A, C, E, and F]). The percentage of cells residing in such nonendodermal positions was significantly higher among DN Rac1–expressing donor-derived tissue than control (Fig. 7 G). Intriguingly, these cells were still Tg(sox17:GFP) positive but exhibited the characteristic cell shapes and expressed molecular markers of the
tissues in which they resided (Fig. 7, C–F). To better understand how Rac1-deficient cells became mislocalized to the mesoderm, we performed time-lapse imaging soon after transplantation (Fig. 7 [H–K] and Video 7). We observed that at 75% epiboly, control cells were spread out along the dorsal–ventral and animal–vegetal axes. In contrast, DN Rac1–expressing cells appeared dispersed along the animal–vegetal axis only (Fig. 7 I). As a result, during the switch to dorsally oriented migration beginning at 90% epiboly, the Rac1-deficient cells reached the dorsal end of the embryo first, whereas control cells were still relatively spread out dorsally (Fig. 7 J). Subsequently, we observed some of the dorsal-most Rac1-deficient cells extruding away from their neighbors and taking on an elongated cell shape reminiscent of notochord cells (boxed region in Fig. 7 K). These experiments suggest that the migration behavior of endodermal cells during gastrulation is important for maintaining endoderm identity.

**Discussion**

In this study, we have shown that during gastrulation stages, endodermal cells undergo developmentally regulated changes in migration behavior, which are driven by corresponding changes in actin cytoskeletal dynamics. We have also shown that the increased actin dynamics and random motility of cells during early gastrulation stages depend on Nodal signaling and Rac1 activity. Furthermore, we showed that Nodal signaling induces the expression of the Rac-specific GEF prex1 and that Prex1 functions downstream of Nodal signaling to promote random migration at early gastrulation stages. Together, these observations indicate that the early random migration of endodermal cells is driven by Nodal-induced Rac1 activation.

Interestingly, our data also suggest that the transition to directed migration during late gastrulation may not be simply a result of down-regulation of Nodal and/or Rac1 signaling. First, we observed that Rac1 activity increases rather than decreases during late gastrulation (Fig. S4 I). This increase in Rac1 activity may correlate with the onset of Cxcl12–Cxcr4 chemokine signaling (Mizoguchi et al., 2008), which has been reported to signal through Rac1 (Xu et al., 2012). Second, when we examined endodermal cell migration during late gastrulation in Nodal- or Rac1-inhibited embryos, we found that although cell migration was not severely affected, directional persistence was slightly increased (Fig. S4, C and G). This result suggests that Nodal-dependent signals may still be operating to promote random motility, but, at late stages, they are now superseded by directional cues provided by putative chemotactants such as Cxcl12. Therefore, we propose a model in which Nodal, via Prex1, induces global Rac1 activation, which results in directionally random cell migration during early gastrulation stages. Then, as endodermal cells become responsive to directional cues during late gastrulation, these cues may lead to strongly polarized Rac1 activation that overwheels the Nodal-dependent global Rac1 activation, leading to highly persistent, dorsal-directed migration. Thus, we speculate that by promoting global Rac1 activation, the function of Nodal/Prex1 during early gastrulation stages is to generate noise in the subcellular distribution of activated Rac1, ensuring that endodermal cells do not inappropriately respond to weak directional cues that may be present at these stages (perhaps guiding mesodermal cell migration). Our observations that loss of Nodal or Rac1 signaling during early gastrulation stages leads to increased directional persistence could be a result of the unmasking of these weak polarization signals that would normally be overwhelmed by the global Rac1 activity induced by high Nodal signaling at these early stages. This model is also consistent with our cell transplantation results in which precociously inducing persistent migration by DN Rac1 expression results in the mistargeting of endodermal cells to mesodermal tissues. Notably, our observations differ from cell culture studies in which decreasing Rac1 activity was sufficient to switch cells from random to persistent migration (Pankov et al., 2005).
Although such a simple signaling mechanism may indeed be sufficient to regulate migratory behaviors under basic cell culture conditions, our results illustrate the complexity of regulating cell migration in the dynamic environment of the developing embryo.

The best-characterized role for Nodal signaling during endoderm development has been the induction of endoderm-specific transcription factor genes. Although it has been previously suggested that Nodal may regulate cell movement (Yokota et al., 2003; Pézeron et al., 2008), the mechanisms by which Nodal could affect cell motility were unknown. Here, we have shown that inhibition of Nodal signaling not only slowed cell migration velocity and increased migration persistence but also suppressed actin dynamics and Rac1 activity. We have further identified the Rac-GEF Prex1 as a downstream target of Nodal signaling. Rac1 is a well-known regulator of actin polymerization and cell migration both in vitro (Gardiner et al., 2002; Srinivasan et al., 2003; Pankov et al., 2005; Woo and Gomez, 2006) and in vivo (Li et al., 2002; Kardash et al., 2010; Yoo et al., 2010), and it has also recently been shown to be crucial for the cell movements underlying gastrulation in mouse (Migeotte et al., 2011). Although our results suggest that the Nodal-dependent Rac1 activity we observed is a result of increased expression of Prex1, Rac1 may be activated via a transcription-independent pathway as well. We observed that acute SB-505124 treatment lasting as little as 15 min was sufficient to alter cell migration behavior (Fig. S5). Indeed, other TGF-β ligands have been shown to induce both rapid Rho GTPase activation that is Smad independent as well as sustained increases in Rho activity that involve gene transcription (Kardassis et al., 2009). It is also very likely that other cytoskeletal regulatory proteins besides Rac1 are involved in endoderm morphogenesis. Indeed, in our microarray analysis, we identified several genes associated with cell migration and cytoskeletal dynamics as potential targets of Nodal signaling (Fig. S3 A). In addition, a study using a proteomics-based approach identified at least four cytoskeleton-associated proteins that are differentially regulated between mesendodermal and ectodermal cells (Link et al., 2006); one of these proteins, Ezrin, was demonstrated to function during the migration of prechordal plate progenitor cells by regulating membrane protrusion (Diz-Muñoz et al., 2010). Future studies will no doubt identify additional cytoskeletal regulators important for tissue morphogenesis and organ development.

In this study, we provide evidence that prex1 is transcriptionally regulated by Nodal signaling. However, GEFs are also subject to posttranscriptional regulation. Although most GEFs are regulated by phosphorylation (Rossman et al., 2005), Prex1 is synergistically activated by PIP3 and Gβγ (Welch et al., 2002; Barber et al., 2007; Zhao et al., 2007). In neutrophils, Prex1 is thought to act as a coincidence detector that allows for high levels of Rac activation when both second messengers are generated (Weiner, 2002), as occurs when G-protein–coupled chemokine receptors are activated (Stephens et al., 1997). Zebrafish endodermal cells also express chemokine receptors, primarily Cxcr4a (Mizoguchi et al., 2008; Nair and Schilling, 2008). SDF-1–Cxcr4 signaling in primordial germ cells was recently shown to activate Rac1 in a Gβγ-dependent manner (Xu et al., 2012), making it very likely that Prex1 lies directly in this signaling pathway. However, in terms of endoderm development, several questions remain about the role of Prex1. First, to what extent are both PIP3 and Gβγ necessary for Prex1 function in vivo? Mizoguchi et al. (2008) suggested that phosphoinositide signaling is not highly active in migrating endodermal cells, and it may be possible to activate Prex1 with Gβγ alone, especially under conditions of low PIP3 concentrations (Welch et al., 2002). If PIP3 and/or Gβγ are required for full Prex1 activity, are they generated downstream of receptors such as Cxcr4, and, if so, how do those signaling pathways interact with Nodal signaling? Given that most studies of Prex1 to date have used neutrophils in culture, the developing zebrafish endoderm may represent a useful system to probe important questions about Prex1 function in vivo.

In the double transplantation experiments, we observed that some cells in which random migration was suppressed by DN Rac1 expression seemed unable to maintain endodermal identity and instead contributed to mesodermal tissues. Although we interpret these results as being a result of the suppression of random migration during early gastrulation, it is also possible that DN Rac1 impairs cell movements before gastrulation, such as epiboly and ingestion, which could aberrantly place cells in the mesodermal layer. However, although we did observe some endodermal cells that apparently failed to ingress in Prex1 MO–injected embryos, we did not see a similar effect with the low-level DN Rac expression used throughout this study, suggesting that pregastrulation movements are relatively unaffected. Thus, based on our time-lapse analyses, we propose that DN Rac1 expression precociously induces persistent migration, causing cells to more efficiently reach the dorsal side of the embryo. Once there, they may inappropriately interact with mesodermal cells or mesoderm differentiation signals. It is also possible that Rac1 is required for later aspects of endoderm morphogenesis, such as cell–cell adhesion during endodermal sheet formation, which may also affect the ability of Rac-deficient cells to remain within the endoderm.

The ability of cells to switch their migratory behavior has been observed in many different cell types and model systems (Bak and Fraser, 2003; Wolf et al., 2003; Pankov et al., 2005; Pézeron et al., 2008; Sanz-Moreno et al., 2008). In general, it is thought that random migration plays either a dispersive or exploratory role, whereas persistent migration promotes rapid and efficient translocation. The need for multiple modes of migration may be crucial not only during development but also in the adult cell as well. Most notably, processes such as wound healing and axon regeneration require cells to switch from a stationary state to a migratory one. Additionally, different types of invasive tumor cells are characterized by different migratory behaviors (Madsen and Sahai, 2010); some cells are even able to switch between multiple migration modes (Sanz-Moreno et al., 2008), which can impact the efficacy of drugs meant to block metastasis (Wolf et al., 2003; Micuda et al., 2010). Therefore, the findings presented in this study have clear implications beyond developmental processes.
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Materials and methods

Zebrafish strains and generation of Tg(sox17:GFP-UTRN)

Adult zebrafish were maintained under standard laboratory conditions. Tg(sox17:GFP) and Tg(sox17:dsRed) have been previously described (Chung and Stainier, 2008; Mizoguchi et al., 2008); a 5.0 kb region of the sox17 gene promoter drives expression of GFP or dsRed. Tg(sox17:GFP-UTRN) was generated using components from the Gateway Tol2 system (Invitrogen, Carlsbad, CA, USA), using the Entry vector p3E-CAT; synthesized by Gene Tools, LLC). The p5E-sox17 entry vector was generated by excising a 5.2 kb fragment of the sox17 promoter from pego-GFP-sox17 (Mizoguchi et al., 2008) with PstI and ApaI and blunt-end ligating this fragment into a Smal site of p3E-CAT. Ur recombination among p3E-sox17, pME-eGFP-no-stop, pME-eGFP, and pDEST-Tol2pA2 generated the construct pDEST-Tol2pA2-sox17:GFP-UTRN. This construct was used to generate the Tg(sox17:GFP-UTRN) line using standard transgenesis protocols.

Time-lapse microscopy

For time-lapse imaging, dechorionated embryos were embedded in 1% low-melting agarose within glass-bottom Petri dishes (MatTek Corporation). Fluorescent images were acquired on a microscope (Ti-E; Nikon) equipped with a spinning-disk confocal unit (CSU-22; Yokogawa Corporation of America), a charge-coupled device camera (Evolve; Photometrics), and running Micro-Manager software. The microscope stage was enclosed in a temperature-controlled case, and samples were kept at 28.5°C. Unless otherwise specified, time-lapse videos were acquired from the lateral marginal zone, with the embryonic shield oriented to the right. To image overall cell migration (persistence and velocity), z stacks of 4-µm intervals were acquired every 30 s with a 20×/0.75 NA objective. To image lamellipodial dynamics, z stacks of 4-µm intervals were acquired every 5 s with a 20×/0.75 NA objective with 1.5× zoom. To image retrograde flow, z stacks of 2-µm intervals were acquired every 5 s with a 40×/1.15 NA objective.

Image analysis, statistics, and image processing

Image analysis was performed using ImageJ software (National Institutes of Health). All measurements were made from maximum projections of spinning-disk confocal z stacks. Instantaneous velocity and cell position were measured using the Manual Tracking plugin. The persistence index was calculated by dividing the total distance traveled by the net distance traveled. Circularities were determined using the built-in circularity calculations in ImageJ, which uses this formula: circularity = 4π(area/perimeter²). Lamellipodial lifetime (area/perimeter²) was measured using the MTrack2 plugin. Lamellipodia were classified as oriented in the direction of cell migration.

RNA expression constructs and MOS

mRNAs and MOs were injected at the one- or two-cell stage. Capped messenger RNA was synthesized using the mMESSAGE mMACHINE kit (Ambion). The following expression plasmids were used in this study: N-terminal myc-tagged human DN (pCS2-Myc-DN; Rac1 in pCS2-pcs2-sox32; Chung and Stainier, 2008), and TagRFP-PBD in pCS2 (Miller and Bement, 2009). pCS2-mCherry-Prex1 was generated by PCR amplification of the prex1 ORF, which was cloned into pCS2-mCherry (Burkel et al., 2007). The prex1 MO was designed to target the translation initiation site and was synthesized at 1 mg/ml (5'-CCCTCCTCCTATTATTATCGCT-3'; synthesized by Gene Tools, LLC).
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For in situ hybridization, DN Rac1–expressing Tg(sox17:GFP) cells were transplanted into wild-type host embryos at 4–5 h after fertilization, which were then fixed at 22–24 h after fertilization. Whole-mount in situ hybridization for myoD and mfnb was performed as previously described (Thistle and Thissen, 2008). In brief, fixed embryos were dehydrated in methanol at –20°C overnight. After rehydration, embryos were hybridized with 100 ng/µl riboprobe in hybridization buffer overnight at 65°C. Excess probe was removed with graded SSC washes. Antidigoxigenin antibody was used at 1:10,000 (Roche) and developed with Fast Red (Roche). Embryos were imaged by confocal microscopy, as described above.

For time-lapse imaging, wild-type donor embryos were injected with 300 pg sox32 mRNA and 2 µg tetramethylrhodamine-dextran (10,000 molecular weight; Sigma-Aldrich) as a control or with 300 pg sox32 mRNA, 2.5 pg DN Rac1 mRNA, and 2 µg FITC-dextran (10,000 molecular weight; Sigma-Aldrich). Cells were transplanted at stage 10 as described in the previous section. At shield stage, embryos were embedded in 1% low-melting agarose and imaged on a widefield fluorescence microscope (Zeiss). Images were acquired every 5 min. Analysis was restricted to host embryos containing laterally incorporated donor cells.

Microarray analysis
To identify endodermally enriched transcripts, endodermal cells were isolated at 70% epiboly by transferring Tg(sox17:GFP) embryos to Ca2+-free Ringer’s solution followed by mechanical disruption with a 200 µtip pipette tip. Dissociated cells were collected by centrifugation and resuspended in Ca2+-free Ringer’s, and GFP-positive endodermal cells were separated from nonfluorescent nonendodermal cells by FACS. mRNAs were extracted from both populations using the RNAqueous-Micro Kit (Ambion). cDNAs were amplified, labeled with Cy3 (DMSO or mCherry) or Cy5 (sox32 mRNA, and 2 µg FITC-dextran) and hybridized to the Zebrafish Gene Expression Microarray (V2; microarray services were performed by MOgene, LC using a preprinted Agilent Technologies array). To examine gene expression under Nodal-inhibited conditions, Tg(sox17:GFP) embryos were treated at 4 h after fertilization with 50 µM SB-505124 (Sigma-Aldrich) or 0.5% DMSO. For Nodal-activated conditions, Tg(sox17:GFP) embryos were injected at the one-cell stage with 2 ng taram-a* mRNA or 2 ng mCherry mRNA as a control. GFP-positive endodermal cells were isolated by FACS at 70% epiboly, and total RNA was extracted using the RNAqueous-Micro Kit. cDNAs were amplified, labeled with Cy3 (DMSO or mCherry) or Cy5 (SO-505124 or taram-a*), and hybridized to the Agilent Zebrafish Gene Expression Microarray (V2). The extracted data were normalized and quality controlled using GeneSpring GX software (Agilent Technologies).

Real-time quantitative PCR
To examine gene expression under Nodal-inhibited conditions, wild-type embryos were treated at 5 h after fertilization with 50 µM SB-505124 (Sigma-Aldrich) or 0.5% DMSO. For Nodal-activated conditions, wild-type embryos were injected at the one-cell stage with 2 ng taram-a* mRNA or 2 ng mCherry mRNA as a control. Expression of left2, a known Nodal target gene, was used to confirm Nodal inhibition and activation (Fig. S3 B). At 70% epiboly, total RNA was extracted using the RNAqueous-Micro Kit, and 1 ng was used for reverse transcription with the SuperScript VILO cDNA Synthesis Kit (Invitrogen). The quantitative PCR reaction mixture contained 2 µl of 10-fold diluted cDNA, 12.5 µl SYBR green PCR master mix (Applied Biosystems), 714 nM of each primer, and nuclease-free water to a total volume of 25 µl in 48-well plates (Ilumina). Reactions were performed in the Eco Real-Time PCR System (Ilumina, Inc.) as follows: initial activation at 95°C for 10 min followed by 40 cycles of 30 s at 95°C, 30 s at 60°C, and 30 s at 68°C. Once the PCR was completed, a melt-curve analysis was performed to determine reaction specificity. Samples were run in duplicate, and data presented in Figs 5 A and 5 B represent means from three independent reactions. The housekeeping gene elf1a was used as a reference. Table 1 lists the primers used in this study.

Online supplemental material
Fig. S1 shows sox17 and sox32 expression in SB-505124–treated embryos and embryos expressing DN Rac1. Fig. S2 shows analysis of migratory behaviors of transplanted endodermal cells as well as the migratory parameters of transplanted DN Rac1 cells. Fig. S3 lists candidate cytoskeletal and migration-related Nodal target genes identified by microarray analysis and shows changes in expression of elf2, tiam1, and arhgef25b in response to Nodal signaling. Fig. S4 shows the effects of Nodal or Rac1 inhibition on endoderm migration at late gastrulation and compares levels of Rac1 activity between early and late gastrulation. Fig. S5 shows the effects of acute Nodal inhibition on endoderm migration. Videos 1 and 2 depict actin dynamics at early and late gastrulation, respectively. Video 3 depicts the switch from random to oriented migration during gastrulation. Video 4 shows the initiation of collective migration and endodermal sheet formation. Video 5 shows the effects of Nodal inhibition on endodermal cell migration. Video 6 shows dynamic Rac1 activity in migrating endodermal cells. Video 7 shows the migration of transplanted control and DN Rac1–expressing cells from 75% epiboly to early somitogenesis. Videos 8 and 9 are 2 stacks through control and Prex1 MO–injected embryos, respectively. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201203012/DC1.

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Table 1. List of primers used for qPCR

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References


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Figure S1.  *sox17* and *sox32* expression in SB-505124–treated embryos and embryos expressing DN Rac1. (A–H) Analysis of *sox17* (A, B, E, and F) and *sox32* (C, D, G, and H) expression at 70% epiboly by in situ hybridization suggests that initial induction of endodermal fate is not affected by treatment with SB-505124 (SB; B and D) or DN Rac1 expression (F and H) compared with control (A, C, E, and G). In lateral views, dorsal is to the right. Bars, 100 µm.

Figure S2. Analysis of endodermal cell motility by cell transplantation. (A–F) Donor endodermal cells were generated by overexpression of *sox32* and transplanted into host embryos at 50% epiboly. (A and B) Representative migration tracks over a 1-h period from transplanted *sox32*-overexpressing cells during early (70% epiboly; A) and late gastrulation (90% epiboly; B). Dorsal is to the right. (C and D) Quantification of migration persistence (C) and instantaneous velocity (D) of donor cells at early (70% epiboly) and late gastrulation (90% epiboly). Similar to endogenous endodermal cells, transplanted cells underwent a switch from random to persistent migration during late gastrulation (C), although their migration velocity did not change significantly (D). Early, *n* = 73 cells; late, *n* = 72 cells. (E and F) Transplanted *sox32*-overexpressing cells also underwent cell shape changes between early (70% epiboly; E) and late gastrulation stages (90% epiboly; F). (G–J) Effects of DN Rac1 expression on cell migration during early gastrulation are cell autonomous. Endodermal cells from control (Ctrl) embryos or embryos injected with DN Rac1 mRNA were transplanted into wild-type hosts at 50% epiboly, and migration parameters of transplanted cells were measured at 70% epiboly. (G and H) Representative migration tracks over a 1-h period from control cells (G) and cells expressing DN Rac1 (H). Dorsal is to the right. (I and J) Quantification of migration persistence (I) and instantaneous velocity (J) from control and DN Rac1–expressing cells. Loss of Rac1 activity significantly increased migration persistence and decreased migration velocity. Control, *n* = 106 cells; DN Rac, *n* = 135 cells. Bars, 25 µm. All error bars represent SEM. *, *P* < 0.05.
Figure S3. List of migration and cytoskeleton-associated genes potentially regulated by Nodal signaling and validation of Nodal-dependent tiam1 and arhgef25b expression. (A) Candidate migration and cytoskeleton-associated genes identified by microarray analysis of Nodal-responsive transcripts. (B) Quantification of changes in gene expression in response to Nodal signaling by real-time quantitative PCR. Gene expression in SB-505124–treated (SB) embryos was normalized to DMSO-treated controls, and expression in taram-a*–expressing embryos (TA*) was normalized to control embryos expressing mCherry. Changes in expression of the known Nodal target lefty2 (ltf2) confirmed Nodal inhibition or activation. Although tiam1 and arhgef25b appear to be induced by Nodal activation, neither gene was consistently down-regulated by SB-505124 treatment. The data shown are mean fold changes from six independent experiments. Error bars represent SEM.
Figure S4. Effects of Nodal or Rac1 inhibition on endoderm migration at late gastrulation and comparison of levels of Rac1 activity between early and late gastrulation. (A–H) Effects of Nodal receptor inhibition and DN Rac1 expression on cell migration during late gastrulation (90% epiboly). (A and B) Representative migration tracks over a 1-h period from embryos treated with DMSO carrier (A) or 50 µM Nodal receptor inhibitor SB-505124 (SB; B). Dorsal is to the right. (C and D) Quantification of migration persistence (C) and instantaneous velocity (D) shows that Nodal inhibition led to modestly increased migration persistence but did not affect migration velocity. DMSO, n = 100 cells; SB-505124, n = 51 cells. (E and F) Representative migration tracks over a 1-h period from control embryos (E) and embryos expressing DN Rac1 (F). Dorsal is to the right. (A, B, E, and F) Bars, 25 µm. (G and H) Quantification of migration persistence (G) and instantaneous velocity (H) from control (Ctrl) embryos and embryos expressing DN Rac1. Loss of Rac1 activity slightly increased migration persistence but did not affect migration velocity. (I) Rac1 activity increases during late gastrulation. Rac1 activity was determined by measuring the ratio between RFP-PBD and fluorescent dextran signals. Early, n = 95 cells; late, n = 33 cells. Error bars represent SEM. *, P < 0.05.

Figure S5. Acute SB-505124 treatment can alter cell migration behavior. (A and B) Representative migration tracks from embryos 15 min before (A) and 15 min after (B) a 15-min incubation with 100 µM SB-505124 (SB). Dorsal is to the right. Bars, 25 µm. (C and D) Quantification of migration persistence (C) and instantaneous velocity (D) from early and mid-gastrulation embryos before and after treatment with DMSO or SB-505124. Acute treatment with SB-505124 significantly increased migration persistence but did not significantly affect migration velocity. DMSO, n = 20 cells; SB-505124, n = 100 cells. All error bars represent SEM. *, P < 0.05.
Video 1. **Actin dynamics during early gastrulation.** A migrating endodermal cell from a Tg(sox17:GFP-UTRN) embryo 7 h after fertilization. Images were analyzed by time-lapse confocal microscopy using a microscope (Ti-E) equipped with a spinning-disk confocal unit (CSU-22). The arrow points to a protrusion in which retrograde flow is especially prominent. Frames were acquired every 5 s for 15 min. Playback is 10 frames/s.

Video 2. **Actin dynamics during late gastrulation.** A migrating endodermal cell from a Tg(sox17:GFP-UTRN) embryo 9 h after fertilization. Images were analyzed by time-lapse confocal microscopy using a microscope (Ti-E) equipped with a spinning-disk confocal unit (CSU-22). Frames were acquired every 5 s for 15 min. Playback is 10 frames/s.

Video 3. **Tracking of endodermal cell migration during gastrulation.** The lateral marginal region of a Tg(sox17:GFP-UTRN) embryo was imaged from 6–9 h after fertilization. Dorsal is to the right. Images were analyzed by time-lapse confocal microscopy using a microscope (Ti-E) equipped with a spinning-disk confocal unit (CSU-22). To illustrate the switch from random to oriented migration, one cell was tracked for the duration of the video using the Manual Tracking plugin in ImageJ. Playback is 10 frames/s.

Video 4. **Dorsal view of a Tg(sox17:GFP-UTRN) embryo at 10 h after fertilization showing initiation of endodermal sheet formation.** Images were analyzed by time-lapse confocal microscopy using a microscope (Ti-E) equipped with a spinning-disk confocal unit (CSU-22). Frames were acquired every minute for 3 h. Playback is 10 frames/s.

Video 5. **Effects of Nodal inhibition on endodermal cell migration.** A Tg(sox17:GFP-UTRN) embryo treated with 50 µM SB-505124 starting at 5 h after fertilization was imaged at 7 h after fertilization. Images were analyzed by time-lapse confocal microscopy using a microscope (Ti-E) equipped with a spinning-disk confocal unit (CSU-22). Frames were acquired every minute for 1 h. Playback is 10 frames/s.

Video 6. **Rac1 activity in migrating endodermal cells.** Endodermal cells expressing RFP-PBD and colabeled with A647-dextran were imaged at 7 h after fertilization. Images were analyzed by time-lapse confocal microscopy using a microscope (Ti-E) equipped with a spinning-disk confocal unit (CSU-22). Frames were acquired every 15 s for 7.5 min. Rac1 activity is presented as a color-coded ratiometric image (generated in ImageJ) between the RFP and Cy5 channels. Warmer colors indicate enrichment of PBD relative to dextran. Playback is 10 frames/s.
Video 7. **Migration of transplanted control and DN Rac1–expressing endodermal cells in a wild-type host.** Control cells (red) were labeled with tetramethylrhodamine-dextran (10,000 molecular weight; Sigma-Aldrich), and DN Rac1–expressing cells (green) were labeled with FITC-dextran (10,000 molecular weight). Images were analyzed by time-lapse widefield fluorescence microscopy using an inverted microscope (Z.1). Frames were acquired every 5 min for 9 h, starting at ~75% epiboly and continuing until early somitogenesis. Playback is 10 frames/s.

Video 8. **Z stack through a control Tg(sox17:GFP-UTRN) embryo at 75% epiboly followed by a maximum projection.** Images were acquired every 4 µm on a microscope (Ti-E) equipped with a spinning-disk confocal unit (CSU-22). Maximum projection was generated in ImageJ. Because of the curvature of the embryo, individual endodermal cells appear in different z planes, but a maximum projection shows that they lie in one cell layer (at the yolk surface).

Video 9. **Z stack through a Prex1 MO–injected Tg(sox17:GFP-UTRN) embryo at 75% epiboly followed by a maximum projection.** Images were acquired every 4 µm on a microscope (Ti-E) equipped with a spinning-disk confocal unit (CSU-22). Maximum projection was generated in ImageJ. Unlike in control embryos, endodermal cells in Prex1 MO–injected embryos are found in multiple cell layers between the yolk surface (0 µm) and enveloping layer, with cells sometimes appearing to be stacked on top of one another (asterisks), suggesting a defect in ingression.