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

Stephanie Woo, Michael P. Housley, Orion D. Weiner, and Didier Y.R. Stainier

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 ingression 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.

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

marker genes sox17 and sox32 (Fig. S1, E–H), suggesting that the effects on endodermal motility were not a result of mispecification. 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 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 A from the cells in B, 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 (I), 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.

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) embryos 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.
early stages, whereas migration velocity was significantly slower, suggesting that Rac1 acts cell autonomously to regulate endoderm migration (Fig. S2, G–J).

Next, we determined whether Nodal signaling regulates Rac1 activity (Fig. 5). To visualize Rac1 activity, we expressed a fluorescent probe consisting of the Rac1-binding domain of p21-activated kinase tagged to an RFP (RFP–p21-binding domain [PBD]; Srinivasan et al., 2003; Miller and Bement, 2009). Because detection of RFP-PBD fluorescence is facilitated by mosaic expression, we transplanted small groups of RFP-PBD–expressing endodermal cells into unlabeled hosts. To control for variation in cell size or shape, donor cells were colabeled with Alexa Fluor 647–conjugated 10,000–molecular weight dextran (A647-dextran) as a volume marker. Rac1 activity was determined by generating ratiometric images between the RFP-PBD and dextran signals and was pseudocolored based on ratio value (C and F). Warmer colors indicate enrichment of PBD relative to dextran. Bars, 10 μm. (G) Quantification of the mean ratio of PBD to dextran indicates that Nodal inhibition reduces Rac1 activity. DMSO, n = 121 cells; SB-505124, n = 125 cells. All error bars represent SEM. *P < 0.05. (H) Measurement of the size of cell regions where the PBD/dextran ratio is >1.0. Area of Rac1 activation is dramatically reduced upon Nodal inhibition.

Using the same RFP-PBD assay, we also investigated whether a drop in Rac1 activity accompanies the switch from random to persistent migration in wild-type gastrulae (Fig. S4 I). Surprisingly, we found that levels of Rac1 significantly increased during late gastrulation. One likely explanation is the onset of Cxcl12a–Cxcr4 chemokine signaling at this stage (Mizoguchi et al., 2008), which is known to activate Rac1 (Xu et al., 2012).

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 NETI, 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 (Fig. S3 A). 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 morpholo (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...
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:dsRed) expression were transplanted together with DN Rac1–expressing cells labeled by Tg(sox17:GFP) expression into unlabeled wild-type hosts before 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 effects of Prex1 on endodermal motility during early stages by injecting Prex1 MO into Tg(sox17:GFP-UTRN) embryos (Fig. 6, F–I). In these MO-injected embryos, we observed some GFP-UTRN–labeled cells positioned in the cell layers away from the yolk surface (Video 9), suggesting that reduction in Prex1 levels leads to defects in internalization or other epiboly movements. Notably, we did not observe these effects with DN Rac1 expression. As these superficial cells appeared rounded and immobile, we excluded them from subsequent analysis and restricted our measurements to the cells that were positioned at the yolk surface. Similar to the observations with both Nodal inhibition and DN Rac1 expression, we found that Prex1 knockdown significantly increased migration persistence (Fig. 6 H) and decreased migration velocity (Fig. 6 I).

Next, we examined whether Prex1 acts downstream of Nodal to promote random migration of endodermal cells by determining whether overexpressing Prex1 was able to rescue the effects of Nodal inhibition on cell motility (Fig. 6, J and K). Embryos injected with 500 pg Prex1 mRNA or an equivalent amount of mCherry mRNA as a control were treated with 50 µM SB-505124 at 5 h after fertilization, and cell motility was assessed at 7 h after fertilization. As we previously observed, control-injected embryos treated with Nodal inhibitor exhibited increased directional persistence and decreased migration velocity. 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 endodermally expressed Nodal target gene that activates Rac1 and mediates the Nodal-dependent dynamic motility of endodermal cells.
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 dorsoventrally (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–Cxc4 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 chemoattractants such as Cxcl12. Therefore, we propose a model in which, 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 overwhelms 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 in the adult 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.
prex1 in situ hybridization

To generate the prex1 in situ probe, the prex1 ORF was cloned into pCR-Blunt II-TOPO (Invitrogen). For probe synthesis, pCR-Blunt II-TOPO-prex1 was digested by SfiI and in vitro transcribed with T7. For in situ hybridization, embryos at 70% epiboly were dechorionated and fixed in 4% PFA overnight at 4°C. Embryos were sunk in 30% sucrose, embedded in optimal cutting temperature medium, and cryosectioned (12 µm thick). After drying sections were fixed in 4% PFA for 10 min at room temperature. Sections were then acetylated with 0.1 M triethanolamine, 2.1 mM HCl, and 0.25% acetic anhydride for 10 min at room temperature. Sections were permeabilized with 1% Triton X-100 in 1x PBS for 30 min at room temperature. Nonspecific binding was blocked by incubating sections in hybridization buffer (50% farmamide, 5 µSC, 0.1% tween 20, 50 mg/ml heparin, and 500 ng/ml RNA, pH 6.5) for 4 h at room temperature in a humidified chamber. The prex1 probe solution was diluted to 200 ng/ml in hybridization buffer, and sections were incubated overnight at 65°C. Sections were then washed once with 5x SSC at 65°C, twice with 0.2x SSC at 65°C, and then transferred to room-temperature TBS (100 mM Tris HCl, pH 7.5, and 150 mM NaCl). Sections were blocked for 1 h at room temperature in 2% blocking reagent (Roche). Antidigoxigenin antibody (Roche) was diluted 1:5,000 in 2% blocking reagent, and sections were incubated overnight at room temperature. Sections were washed every 30 min for 4 h with TBS and then equilibrated for 5 min in NTM buffer (100 mM Tris HCl, pH 9.5, 100 mM NaCl, and 5 mM MgCl2). Sections were stained with NBT/BCIP solution (1:50 in NTM buffer; Roche) overnight at room temperature. For double labeling, sections were briefly fixed with 4% PFA, sections were permeabilized, washed, and washed twice with xylene. Sections were mounted in Permount (Thermo Fisher Scientific) and imaged on a microscope (Axioplan; Carl Zeiss) with a 20x/0.75 NA objective lens.

Rac1 activity assay

pCS2-TagRFP-PBD was generated by replacing the GFP coding sequence of pCS2-GFP-PBD (Miller and Bement, 2009) with TagRFP (Evrogen). Mosaic expression of TagRFP-PBD was accomplished using established cell transplantation techniques (Stafford et al., 2006; Chung and Stainier, 2008). Tg(sox17:GFP-UTRN) donor embryos were injected with 200 pg TagRFP-PBD mRNA, 300 pg sox32 mRNA, and 2 µg A647-dextran (10,000 molecular weight; Invitrogen). At sphere stage, donor cells were transplanted to the marginal zone of an unlabeled wild-type host embryo of the same sex. Rac1-deficient donor cells were generated by injecting Tg(sox17:dsRed) embryos with 5 µg sox32 and 2.5 pg DN Rac1 mRNA. At 4–5 h after fertilization, cells from both control and DN Rac1–expressing donor embryos were transplanted simultaneously to the marginal zone of an unlabeled wild-type host embryo of the same stage. At 22–24 h after fertilization, embryos were imaged on a confocal microscope (LSM 5; Carl Zeiss) with a 20x/0.75 NA objective lens, and z stacks were acquired at 2-µm intervals. Maximum projections were generated and analyzed using ImageJ software. Volumes were restricted to the trunk region along the yolk extension to exclude the sox17 positive dorsal forerunner derivatives. User-defined ROIs were drawn around the gut tube (endodermal), the rest of the trunk dorsal to the gut tube (nonendodermal), and the entire trunk region (total). We measured the area occupied by GFP- or dsRed-positive cells within each ROI and then divided the endodermal area by the total area measured within the total ROI to calculate the percentage of contribution to endodermal or nonendodermal tissues.

Cell transplantations

Cell transplantations were performed as previously described (Stafford et al., 2006; Chung and Stainier, 2008). For double transplantation experiments (Fig. 7), control endodermal donor cells were generated by injecting Tg(sox17:dsRed) embryos with 30 pg sox32 mRNA. Rac1-deficient donor cells were generated by injecting Tg(sox17:GFP-UTRN) embryos with 1 µg sox32 and 2.5 pg DN Rac1 mRNA. At 4–5 h after fertilization, cells from both control and DN Rac1–expressing donor embryos were transplanted simultaneously to the marginal zone of an unlabeled wild-type host embryo of the same stage. At 22–24 h after fertilization, embryos were imaged on a confocal microscope (LSM 5; Carl Zeiss) with a 20x/0.75 NA objective lens, and z stacks were acquired at 2-µm intervals. Maximum projections were generated and analyzed using ImageJ software. Volumes were restricted to the trunk region along the yolk extension to exclude the sox17 positive dorsal forerunner derivatives. User-defined ROIs were drawn around the gut tube (endodermal), the rest of the trunk dorsal to the gut tube (nonendodermal), and the entire trunk region (total). We measured the area occupied by GFP- or dsRed-positive cells within each ROI and then divided the endodermal or nonendodermal area by the area measured within the total ROI to calculate the percentage of contribution to endodermal or nonendodermal tissues.
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 nla was performed as previously described (Thibeau and Thibes, 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 sphere stage, as described in the previous section. At shield stage, embryos were embedded in 1% lowmelting agarose and imaged on a widefield fluorescence microscope (Z.1; Carl Zeiss) with a 5x objective lens. 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 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. mRNA was extracted from both populations using the RNAqueous-Micro Kit (Ambion). cDNAs were amplified, labeled with Cy3 (from endodermal cells) or Cy5 (from nonendodermal cells), and hybridized to the Zebrfish 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 transplanted at 6 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 RNeasy Micro Kit (Ambion). cDNAs were amplified, labeled with Cy3 (Cy5) or Cy5 (Cy3) and hybridized to the Agilent Zebrfish 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 6 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 lefty2 is known as a nodal target gene, was used to confirm Nodal inhibition and activation (Fig. S3 B). At 70% epiboly, total RNA was extracted using the RNeasy Micro Kit. cDNAs were amplified, labeled with Cy3 (Cy5) or Cy5 (SB-505124 or taram-a*), and hybridized to the Agilent Zebrfish Gene Expression Microarray (V2). The extracted data were normalized and quality controlled using GeneSpring GX software (Agilent Technologies).


dfn1  NM_131263
lif2  NM_130961
prex1 XM_694535
tiam1 XM_001924009
arhgel25b XM_692957

Table 1. List of primers used for qPCR
GenBank/EMBL/DDBJ accession no. | Sequence
--- | ---
XM_001924009 | 5′-CAAGAAGAGTAGTGACCGCTAGCAT-3′
XM_694535 | 5′-ACCGTGACACATTGCTGGAG-3′
XM_001924009 | 5′-TGGAGATTACAGTACTGGCATGAT-3′
XM_694535 | 5′-ACAGATCCAAAAGGCCAACCC-3′
XM_001924009 | 5′-TAAAGGGCGCATCTGGTCCT-3′
XM_694535 | 5′-ITGATGACGACCATGACCTAC-3′
XM_001924009 | 5′-TCCCTCAAGATGATGAAGAACGAT-3′
XM_694535 | 5′-GAGGTGCAATTGGAGAGGAGACC-3′
XM_694535 | 5′-CGGAGAATGAAATGTTGCAACCTCCAT-3′
XM_694535 | 5′-GCTTTTCCCTGCCAAGGATA-3′

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 candidates cytoskeletal and migration-related Nodal target genes identified by microarray analysis and shows changes in expression of lefty2, tiam1, and arhgel25b in response to Nodal signaling. Fig. S4 shows the effects of Nodal or Rac1 inhibition on endodermal 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 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.

We thank A. Ayala and M. Alva for fish care, K. Thorn and A. Thwin at the Nikon Imaging Center at University of California San Francisco for access to and assistance with the spinning-disc confocal microscope and deconvolution software, and J. Tollsruhn and R. Amato for help with in situ hybridization on embryo sections. We also thank H. Bourne and A. Read for critical comments on the manuscript.

S. Woo was supported by National Institutes of Health grants T32HL007731 and K10DK092312. M.P. Housley was supported by the California Institute for Regenerative Medicine (grant no. TG2-01153). O.D. Weiner was supported by National Institutes of Health grant GM084040. This work was supported in part by grants from the National Institutes of Health (DK60322) and the Packard Foundation to D.Y.R. Stainier.

Submitted: 5 March 2012
Accepted: 1 August 2012

References


