Endodermal cells use contact inhibition of locomotion to achieve uniform cell dispersal during zebrafish gastrulation

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Abstract

The endoderm is one of the three primary germ layers that ultimately gives rise to the gastrointestinal and respiratory epithelia and other tissues. In zebrafish and other vertebrates, endodermal cells are initially highly migratory with only transient interactions among one other, but later converge together to form an epithelial sheet. Here, we show that during their early, migratory phase, endodermal cells actively avoid each other through contact inhibition of locomotion (CIL), a characteristic response consisting of 1) actin depolymerization and membrane retraction at the site of contact, 2) preferential actin polymerization along a cell-free edge, and 3) reorientation of migration away from the other cell. We found that this response is dependent on the Rho GTPase RhoA and EphA/ephrin-A signaling — expression of dominant-negative (DN) RhoA or treatment with the EphA inhibitor dasatinib resulted in behaviors consistent with loss of CIL, including increased contact duration times and decreased likelihood of migration reorientation after contact. Computational modeling predicted that CIL is required to achieve the efficient and uniform dispersal characteristic of endodermal cells. Consistent with our model, we found that loss of CIL via DN RhoA expression resulted in irregular clustering of cells within the endoderm. Together, our results suggest that endodermal cells use EphA2- and RhoA-dependent CIL as a cell dispersal and spacing mechanism, demonstrating how local interactions can give rise to tissue-scale patterns.

Introduction

During embryonic development, tissues and organs are built through the actions of individual cells which must be precisely coordinated in both space and time. The endoderm is one of the three primary germ layers formed early in development that gives rise to the epithelial linings of the gastrointestinal and respiratory organs and other structures including the pharyngeal arches, the thymus, and the thyroid (Frisdal and Trainor, 2014; Zorn and Wells, 2009). Endoderm morphogenesis occurs during gastrulation and involves a series of carefully coordinated changes in cell migration. These changes can be described as an alternating cycle of EMT-like (epithelial-to-mesenchymal transition) and MET-like behaviors (mesenchymal-to-epithelial transition) — endodermal cells first undergo internalization via delamination and highly dynamic single-cell migration followed by re-establishment of cell-cell adhesion to form an epithelium (Nowotschin et al., 2019). This EMT/MET-like cycle is especially prominent in zebrafish embryos. Upon internalization, zebrafish endodermal cells quickly disperse across the embryo to form a regularly spaced but noncoherent cell layer (Kikuchi et al., 2001; Warga and Nüsslein-Volhard, 1999). Then, during mid-late gastrulation, endodermal cells converge together onto the future embryonic midline, forming a coherent endodermal sheet that will undergo additional morphogenesis to form mature epithelia (Ng et al., 2005). Previous studies suggested that the initial dispersal of endodermal cells is driven by their intrinsically low directional persistence, resulting in random walk-type migration (Pézeron et al., 2008; Woo et al., 2012). Here, we show that, in addition these intrinsic factors, the rapid dispersal of endodermal cells may also depend on extrinsic factors, namely collisions with other endodermal cells.

During cell migration, collisions with other cells may result in a change in migration velocity and/or direction — a process termed contact inhibition of locomotion (CIL) (Stramer and Mayor, 2016). First described by Abercrombie and Heaysman in 1954 (Abercrombie and Heaysman, 1954), CIL has since been observed in a variety of contexts including axon

guidance (Drescher et al., 1995; Nakamoto et al., 1996), neural crest migration (Carmona-Fontaine et al., 2008), and cancer metastasis (Astin et al., 2010; Batson et al., 2014). CIL can function as a short-range guidance cue to direct cell migration (Batlle et al., 2002; Knöll and Drescher, 2002) or to regulate spatial positioning (Davis et al., 2012; Reese and Keeley, 2015; Soba et al., 2007; Villar-Cerviño et al., 2013). CIL can be triggered by contact between cells of the same type ("homotypic") or between two different cell types ("heterotypic) and can be further classified as Type I or Type II (Stramer and Mayor, 2016). In Type I CIL, cells respond to collisions by undergoing active repulsion and migrating away from the contacting cell. Type II CIL is more passive with cells either responding to collisions by simply halting their migration or undergoing random deflection. In this study, we show that zebrafish endodermal cells undergo homotypic, active repulsion-type CIL. We further show that CIL drives the rapid and uniform cell dispersal characteristic of the endoderm.

Results

Endodermal cells exhibit RhoA-dependent contact inhibition of locomotion

Previous studies have shown that, during early zebrafish gastrulation, endodermal cells undergo "random walk" migration with low directional persistence, which appears to drive their dispersal (Pézeron et al., 2008; Woo et al., 2012). To better understand the molecular mechanisms underlying endodermal cell dispersal, we performed live imaging at high spatial and temporal resolution of *Tg(sox17:GFP-UTRN)* embryos, which labels the actin cytoskeleton in endodermal cells (Woo et al., 2012). Consistent with previous reports, we found that endodermal cells frequently changed their migration direction during early gastrula stages (7-8 hours post-fertilization, hpf). However, we further observed that these changes in direction were sometimes preceded by collisions with other endodermal cells. Time lapse analysis revealed that endodermal cells underwent a characteristic set of responses upon colliding with another endodermal cell — contacting edges exhibited a loss of actin content and plasma membrane retraction while actin polymerization reoriented to a contact-free site, leading to the

establishment of a new leading edge (Fig. 1A and Video 1). Cumulatively, these responses resulted in cells redirecting their migration trajectories and moving away from each other. To quantify these repulsive responses, we measured the angle of collision by comparing migration trajectories before and after collision (Fig. 1C). We found that these collision angles were significantly clustered in the opposite direction from the cells' initial trajectories ($p=0.00166$, $n=10$ collisions) (Fig. 1D).

Taken collectively, the responses we observed in colliding endodermal cells are consistent with contact-dependent inhibition of locomotion (CIL), a process by which cells redirect their migration in response to contacting another cell (Stramer and Mayor, 2016). CIL is a multistep process triggered by activation of a contact-dependent signaling cascade that affects adhesion, membrane, and cytoskeletal dynamics. While several different cell surface receptors have been reported to mediate of CIL, almost all function by activating the Rho GTPase RhoA, which in turn can activate actomyosin-driven membrane retraction, regulate actin polymerization dynamics, and influence leading edge orientation (Ridley, 2015).

To determine if endodermal CIL similarly requires RhoA, we expressed a dominant-negative (DN) RhoA (Ruchhoeft et al., 1999) in zebrafish embryos and examined its effects on endodermal cell migration. Given the key role of RhoA in cell migration (Ridley, 2015), we first measured the effects of DN RhoA expression on migration speed and the confinement ratio, a measure of migration efficiency defined as the net displacement divided by the total distance traveled (Tinevez et al., 2017). While the confinement ratio was significantly lower in DN RhoA-expressing cells compared to control, there was no significant difference in the average instantaneous migration speed, suggesting that DN RhoA expression did not generally impair the ability of cells to migrate (Fig. S1). We then determined whether DN RhoA affected CIL-associated behaviors. In contrast to control cells, we found that cells from DN RhoA-expressing embryos did not change their migration trajectories in response to cell collisions (Fig. 1B and Video 2), and their collision angles were no longer clustered in the opposite direction to their

pre-collision trajectory (p = 0.098) (Fig. 1D). Consistent with an attenuated inhibitory response, we also found that DN RhoA expression significantly increased the duration of cell-cell contact during collision events (control, 181.1 ± 16.7 s; DN RhoA, 395.5 ± 46.49 s; p < 0.0001) (Fig. 1E). Together, these results suggest that RhoA is required for endodermal cells to undergo CIL.

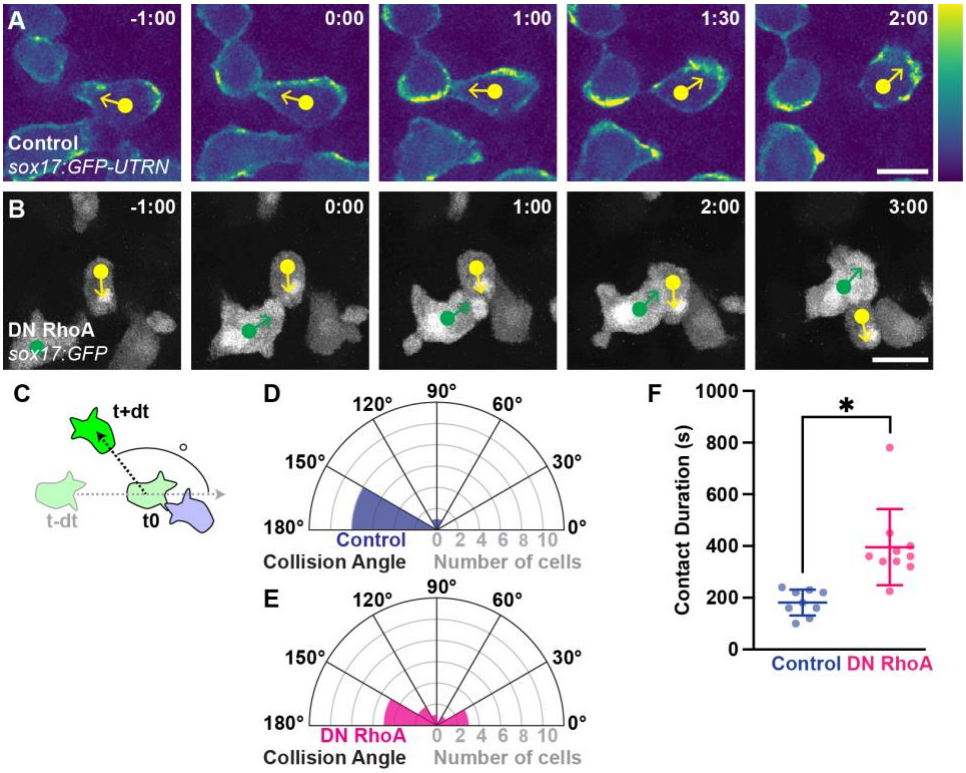

Figure 1. Endodermal cells undergo RhoA-dependent contact inhibition of locomotion. A. Endodermal cells labeled with *Tg(sox17:GFP-UTRN)* at the indicated time points before and after collision. Images have been pseudocolored based on fluorescence intensity to highlight changes in actin localization. Arrows indicate migration direction. **B.** Endodermal cells labeled with *Tg(sox17:GFP)* and expressing dominant-negative (DN) RhoA at the indicated time points before and after collision. Arrows indicate migration direction. **C.** Schematic of collision angle measurements. **D-E.** Polar plots of collision angles. They are clustered in control (D, p=0.00166) but not in DN RhoA-expressing (E, p = 0.098) cells. P values determined by Rayleigh test. **F.** Quantification of contact duration during collision events from control (blue) or DN RhoA-expressing (magenta) endodermal cells. *p = 0.0011 by Welch's t-test.

Control, n = 10 collisions; DN RhoA, n = 12 collisions. Scale bars, 10 µm. Time shown as minutes:seconds.

Endodermal CIL is mediated by EphA signaling

To identify the signaling pathway(s) upstream of RhoA that endodermal cells may use to trigger CIL, we performed transcriptional profiling by RNA sequencing of endodermal cells at three different time points representing different morphogenetic movements — 8 hpf (CIL), 12 hpf (convergence), and 24 hpf (mature adhesion) (Table S1). Candidate genes were chosen based on the following criteria: 1) moderate to high expression levels at 8 and 12 hpf that decreased at 24 hpf, as CIL behaviors are likely no longer active at this stage, and 2) receptor-ligand pairs that appeared to be co-expressed, as endodermal cells appear to undergo homotypic CIL. Based on these criteria, we identified EphA/ephrin-a signaling as a strong candidate (Fig. 2A). We found that the receptors *epha2a* and *epha4* were expressed at 8-12 hpf and downregulated by 24 hpf, and our sequencing data showed co-expression of the ephrin-a ligands *efna1a* and *efna5a*. A-type Eph receptors and their ligands have been extensively characterized as contact-dependent repulsive cues in a wide range of cell types and conditions (Astin et al., 2010; Drescher et al., 1995; Nakamoto et al., 1996; Villar-Cerviño et al., 2013) and have been demonstrated to activate RhoA (Batson et al., 2014; Shamah et al., 2001).

To determine if EphA signaling is required for endodermal CIL, we treated embryos with dasatinib, a small molecular inhibitor of A-type Eph receptors (Chang et al., 2008). Similar to DN RhoA expression, we found that dasatinib-treated cells did not significantly change their migration direction in response to collision ($p = 0.142$) (Fig. 2B, D and Video 3) in contrast to control, DMSO-treated cells ($p < 0.0001$) (Fig. 2C). We also found that dasatinib treatment significantly increased contact duration times compared to DMSO (dasatinib-treated, 11.82 ± 32.31 s; DMSO-treated, 15.45 ± 41.97 s; $p = 0.0471$) (Fig. 2E). These effects are likely not due to general cell migration impairment as migration speed and confinement ratio were not

significantly affected by dasatinib treatment (Fig. S2). Taken together, these results strongly suggest that EphA signaling mediates CIL in endodermal cells.

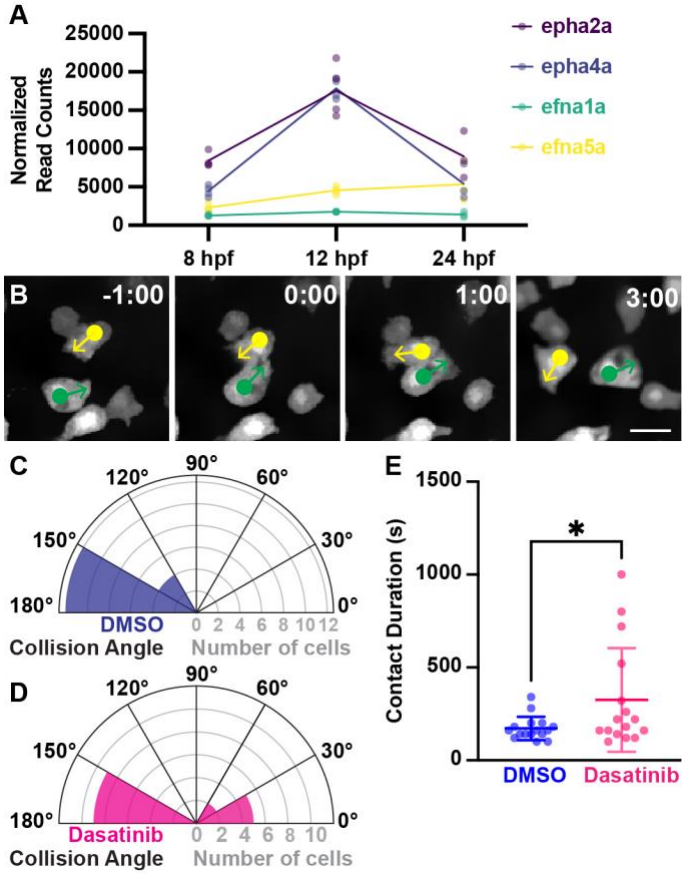

Figure 2. Endodermal CIL is dependent on EphA/ephrin-A signaling. A. Normalized RNA sequencing read counts of Eph receptors *epha2a* and *epha4a* and ephrin ligands *efna1a* and *efna5a* at the indicated time points. hpf, hours post-fertilization. **B.** Endodermal cells in *Tg(sox17:GFP)* embryos treated with 5 µM dasatinib at the indicated time points before and after collision. Time shown as minutes:seconds. Arrows indicate migration direction. Scale bar, 10 µm. **C-D.** Polar plots of collision angles. They are significantly clustered under DMSO (C, $p < 0.0001$) but not dasatinib treatment (D, $p = 0.142$). p values determined by Rayleigh test. **E.** Quantification of contact duration during endodermal collision events from DMSO- (blue) or dasatinib-treated (magenta) embryos. *$p = 0.0471$ by Welch's t-test. DMSO, n = 16 cells. Dasatinib, n =16 cells.

Computational modeling predicts CIL promotes efficient and uniform dispersal of endodermal cells

Previous reports demonstrated that endodermal cells migrate in a “random walk” manner with low directional persistence during early gastrulation stages (Pézeron et al., 2008; Woo et al., 2012). Our results presented here suggest that gastrulation-stage endodermal cells are also undergoing CIL. To test how these behaviors contribute to the observed patterns of endodermal cell migration, we developed a computational model in which we could vary different migration parameters at the cellular level and assess spatial and temporal distributions of cells at the whole embryo level (Fig. 3).

We developed an agent-based model, in which different migration rules were implemented in cells as they migrated over the surface of the 3-dimensional embryo. The embryo was modelled as a sphere and cells were modelled as discs moving on the sphere’s surface. At the start of each simulation, cells are evenly distributed along the margin, similar to in the embryo at 50% epiboly, with an initial direction of migration pointing towards the animal pole (Fig. 3A). Key parameters, such as the embryo’s radius, average cell radius, cell velocity and the estimated number and division rate of cells, were set based on measurements taken from time lapse imaging of control *Tg(sox17:GFP)* embryos (Table S2). Two aspects of migratory behavior where then implemented and varied in the model — persistence and CIL.

To control the persistence of migration, cells were set to travel in straight lines with small, random turns added at each step, so as to produce a characteristic ‘persistence length’ — the average distance traveled before turning by 90 degrees. Simulations with long persistence lengths therefore contained cells migrating in straighter lines whereas short persistence lengths result in frequent and random turning. To control CIL behavior in the model, we first noted that cells in the embryo were generally not observed to pass through each other, so cells in the model stop migrating upon collision with another cell. When CIL is implemented, a new migration direction opposite to the initial direction is assigned. When CIL is not implemented, the collisions are resolved once the migration angle has been changed enough by the random directional changes defined in the persistence behavior.

We first used the model to investigate how persistence length alone (i.e., in the absence of CIL) affects the distribution of endodermal cells (Fig. 3B-D). Simulations were initiated as described above and run for 25 hours of embryo time. At regular time intervals, we quantified cell distributions using a grid-based measure of spatial heterogeneity (Fig. 3B) in which 0 represents a maximally homogenous distribution and 1 is maximally heterogeneous (i.e., all cells in the same location) (Schilcher et al., 2017). In simulations with a long persistence length (256 µm), heterogeneity at first rapidly decreased as cells rapidly migrated away from the margin. Over time however, cells aggregated into large clusters as collisions lead to cells becoming jammed (Fig. 3C). In contrast, simulations with short persistence length (16 µm) resulted in a less rapid loss of heterogeneity initially, as frequent turning slowed the progression of cells away from the midline. However, by the end of the simulation, heterogeneity was overall lower compared to simulations with long persistence length. Clustering still occurred but was less severe, because the cells' propensity to change direction more frequently allowed collisions to be resolved more quickly (Fig. 3D).

We then compared these simulations with the case where contact inhibition is implemented (Fig. 3E-G). As before, simulations with long persistence lengths showed the most rapid initial decrease in heterogeneity (Fig. 3E). However, these simulations revealed that CIL entirely prevented the formation of aggregates (Fig. 3F), as collisions resolved immediately due to the immediate change in direction, which allowed the embryo to reach a much lower heterogeneity overall (0.23 ± 0.01 compared with 0.39 ± 0.02). In simulations with a short persistence length and CIL implemented, heterogeneity again declined at a slower rate as cells were slower to disperse across the embryo although an equivalent level of heterogeneity was eventually reached. And again, no cell clusters or aggregates were formed (Fig. 3G).

Overall, these simulations suggest that, in contrast to previous reports, low directional persistence alone does not explain the dispersed distribution characteristic of endodermal cells; at all persistence lengths tested, the absence of CIL resulted in cell clustering that is rarely

observed in actual zebrafish embryos. Instead, our computational model predicts that uniform dispersal of endodermal cells is primarily driven by CIL.

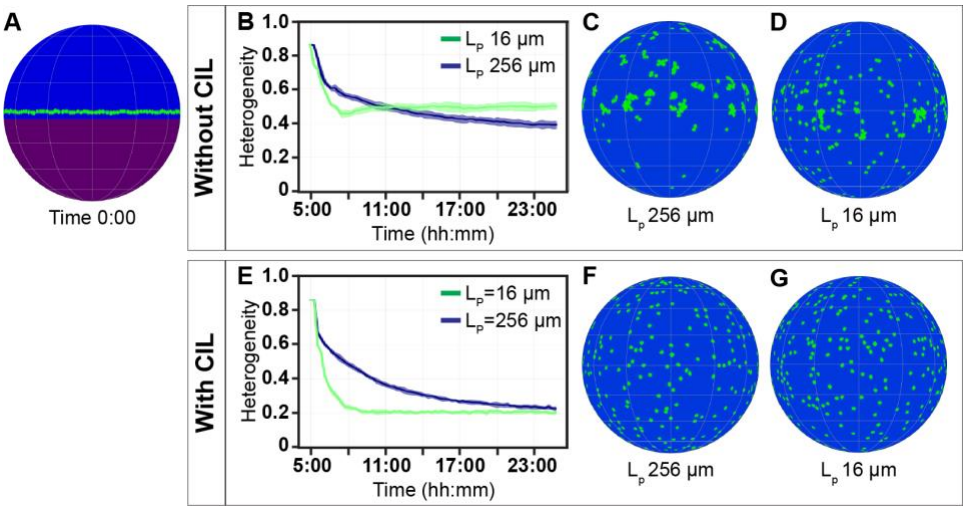

Figure 3. Computational modeling predicts CIL is required for uniform and efficient dispersal of endodermal cells. A. Arrangement of cells at the start of each simulation. Green, endodermal cells. Purple, yolk. Blue, extent of epiboly. **B-D.** Simulations run without CIL. B, quantification of heterogeneity over time. C-D, arrangement of cells at the end of simulations with long (L_p 256 µm, C) or short (L_p 16 µm, D) persistence lengths. **E-F.** Simulations run with CIL. E, quantification of heterogeneity over time. F-G, Arrangement of cells at the end of simulations with long (L_p 256 µm, F) or short (L_p 16 µm, G) persistence lengths.

CIL is required for uniform dispersal of endodermal cells

To experimentally validate the predictions made by our computational model, we took advantage of our observation that DN RhoA expression appears to abolish CIL responses (Fig. 1B-F). At 10 hpf, endodermal cells in control embryos are maximally dispersed across the embryo in a strikingly regular and evenly spaced array (Fig. 4A). In contrast, endodermal cells were irregularly spaced in embryos expressing DN RhoA, creating cell clusters interspersed with large cell-free gaps as predicted by our model (Fig. 4B). We quantified this effect on spacing regularity using Voronoi diagrams and Delaunay triangulation (Lau et al., 2021; Reese

and Keeley, 2015), related techniques that mathematically describe spatial patterns. In Voronoi diagrams, Voronoi domains are computed as the area surrounding a point of interest that lies closer to that point than any other points in a defined space (Fig 4C). With Delaunay triangulation, each point is connected to three neighbors in such a way that non-overlapping triangles are formed. Variability in either Voronoi domain areas or triangle edge lengths would indicate irregularity in the spatial distribution of points. In our analysis, we found DN RhoA-expressing embryos exhibited a significantly wider (i.e., more variable) distribution of Voronoi domain areas compared to control ($p = 0.008$) (Fig. 4D). When we performed Delaunay triangulation, we similarly observed a significantly wider distribution of edge lengths in DN RhoA-expressing embryos when compared to control ($p < 0.0001$) (Fig. 4F). When considered together with our data showing that RhoA is required for endodermal cells to undergo CIL (Fig. 1), these data support the prediction of our model, that CIL functions to drive the uniform, regularly spaced dispersal of endodermal cells.

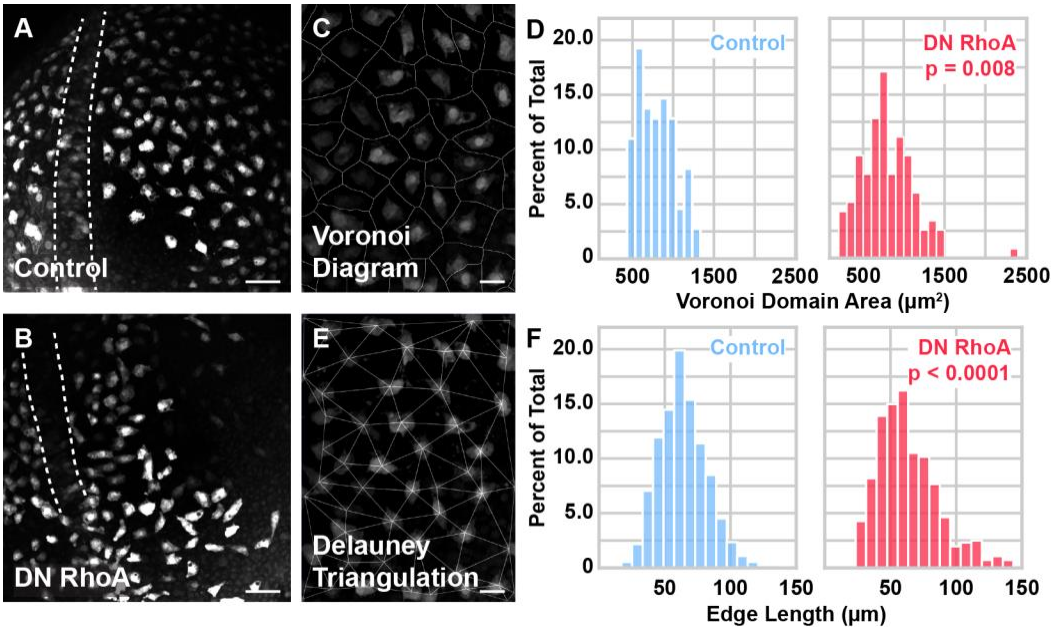

Figure 4. Voronoi diagram and Delauney triangulation analysis show CIL is required for uniform dispersal of endodermal cells. A-B. Representative images of control (A) and DN RhoA-expressing (B)

Tg(sox17:GFP) embryos at 10 hours post-fertilization. Dashed lines, notochord. Scale bars, 25 µm. **C, E.** Representative Voronoi diagram (C) and Delaunay triangulation (E) computed from a control *Tg(sox17:GFP)* embryo at 10 hpf. Scale bars, 10 µm. **D, F.** Distributions of Voronoi domain areas (D) and triangle edge lengths (F) of control (blue) and DN RhoA-expressing (magenta) embryos. p values determined by Levene test for equality of variance. Control, n = 217 cells from 4 embryos. DN RhoA, n = 216 cells from 4 embryos.

Discussion

In this study, we demonstrated that collisions between migrating endodermal cells elicited a response consistent with active repulsion-type contact inhibition of locomotion (CIL). We observed that contact between endodermal cells quickly led to membrane retraction and decreased actin polymerization at the site of contact while new actin polymerization initiated at a contact-free site, resulting in reorientation of the leading edge and allowing the cells to move away from each other. We then demonstrated that endodermal CIL requires RhoA and EphA/ephrin-A signaling — expression of dominant-negative RhoA or treatment with the EphA inhibitor dasatinib attenuated migration reorientation after collision and increased the duration of cell-cell contact. Computational modeling predicted a role for CIL in driving efficient dispersal of endodermal cells. We experimentally validated this prediction by using DN RhoA expression to block CIL, which, consistent with our model, resulted in irregular cell clustering and disrupted the uniform distribution of endodermal cells.

Our results suggest that the uniform dispersal characteristic of endodermal cells is driven primarily by CIL. However, our computational modeling suggested there may be a role for directional persistence, in cooperation with CIL, in setting the rate of dispersal. Our model predicted that the most efficient strategy for dispersing cells uniformly over the entire embryonic surface was to combine CIL with a long persistence length. However, this mechanism would apparently be at odds with previous reports demonstrating endodermal cells migrate with low

directional persistence (Pézeron et al., 2008; Woo et al., 2012). One possibility is that the low directional persistence previously observed was the result of CIL that was unappreciated at the time, and endodermal cells may intrinsically possess high directional persistence consistent with our model. However, single-cell transplantation experiments have shown that isolated endodermal cells can migrate in a random walk pattern (Pézeron et al., 2008). In our model, short persistence lengths decreased the severity of cell clustering in the absence of CIL, suggesting that low directional persistence may provide robustness at the expense of efficiency. Clearly, additional work is needed to resolve contributions of directional persistence and CIL to endoderm dispersal.

CIL has been observed in a broad range of cell types and conditions. Although CIL can function to guide cell migration (Batlle et al., 2002; Knöll and Drescher, 2002), the repulsive interactions we observed between endodermal cells occur during a stage where their migration is directionally random (Woo et al., 2012). Instead, endodermal CIL is more reminiscent of cells that use mutual repulsion to regulate spatial positioning — for example, the tiled spacing of axons and dendrites (Soba et al., 2007), the uniform distribution of Cajal-Retzius cells in cerebellar cortex (Villar-Cerviño et al., 2013), cell bodies in the retina (Reese and Keeley, 2015), and hemocytes in the Drosophila embryo (Davis et al., 2015, 2012). For neurons and sensory organs, spacing via homotypic repulsion is thought to be important for maximizing coverage while minimizing sensory overlap (Grueber and Sagasti, 2010), but the function of self-avoidance in other tissue types is unclear. Our results suggest that in the endoderm, cells use CIL to achieve rapid and uniform dispersal. Achieving this uniform spacing, and especially achieving it in a timely manner, may be important to ensure cells are equally distributed across the entire length of the embryonic axis prior to major morphogenetic movements such as convergence and extension. In fact, early mispositioning of endodermal cells is known to produce major defects such as organ duplication (Nair and Schilling, 2008). One implication of

CIL as a positioning mechanism is that possibility that tissue- or even embryo-wide patterns can arise based only on local, cell-cell interactions.

During zebrafish gastrulation, internalized endodermal cells undergo widespread dispersal followed by convergence (Ng et al., 2005; Warga and Kimmel, 1990; Warga and Nüsslein-Volhard, 1999; Woo et al., 2012). In contrast, endodermal cell migration appears more collective and physically confined in other organisms such as in mouse (Kwon et al., 2008; Viotti et al., 2014), chick (Kimura et al., 2006), and *Xenopus* (Wen and Winklbauer, 2017). CIL has been shown to promote collective migration in addition to cell dispersal. For example, CIL mediated by non-canonical Wnt signaling was shown to impart directional information to migrating streams of neural crest cells in vivo (Carmona-Fontaine et al., 2008). It would be interesting to examine other organisms and determine if CIL is a conserved feature of endodermal morphogenesis.

Our results suggest that CIL drives the dispersal of zebrafish endodermal cells. However, this dispersal is transient, and cells will eventually converge together, first forming a coherent endodermal sheet that then differentiates into mature epithelia. Future studies will be needed to determine the role of CIL in the transition from dispersal to convergence movements. One possibility is that endodermal cells undergo a developmentally regulated downregulation of CIL signaling pathways. However, it is also possible that CIL remains active but is "overpowered" by increased cell-cell adhesion during the sheet formation process. Abercrombie used the phrase "negative taxis" to describe cells' use of CIL to sense and migrate into cell-free spaces (Abercrombie and Ambrose, 1962). This concept of negative taxis could apply not only to endodermal cell dispersal but also to subsequent epithelial sheet formation, as it would allow cells to sense and fill in any cell-free gaps in the newly forming sheet. Thus, CIL could function as a single mechanism operating at multiple stages of endodermal development.

Materials and methods

Zebrafish strains

Adult zebrafish were maintained under standard laboratory conditions. Zebrafish in an outbred *AB*, *TL*, or *EKW* background were used as wild-type strains. *Tg(sox17:GFP)*^{s870} and *Tg(sox17:EGFP-Hsa.UTRN)*^{s944} have been previously described (Mizoguchi et al., 2008; Woo et al., 2012). This study was performed with the approval of the Institutional Animal Care and Use Committee (IACUC) of the University of California Merced (Protocol #2023-1144).

Microscopy

For time-lapse imaging, dechorionated embryos were embedded in 1% low-melting agarose within glass-bottom Petri dishes (MatTek Corporation) and submerged in 0.3X Ringer's solution. For all imaging, the microscope stage was enclosed in a temperature-controlled case, and samples were kept at 28.5°C. For fluorescent images in Fig. 1A and Video 1, Z-stacks of 4-µm intervals were acquired every 5 s with a 20x/0.75 NA objective 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 MicroManager software. All other fluorescent images were acquired with a 30x/1.05 NA objective on a microscope (IX83; Olympus) equipped with a spinning-disk confocal unit (CSU-W1; Andor), a scientific complementary metal–oxide–semiconductor (sCMOS) camera (Prime 95b; Teledyne Photometrics), and MicroManager software. For time lapse imaging (Fig. 1B, Fig. 2B, Video 2, and Video 3), Z-stacks of 4 µm intervals were acquired every 10-20 s. For still images in Fig. 4, 10 hpf embryos were fixed in 4% paraformaldehyde overnight at 4°C, dechorionated, embedded in 1% low-melting agarose within glass-bottom Petri dishes (MatTek Corporation), and submerged in 1X phosphate-buffered saline (PBS); Z-stacks intervals were acquired at 2 µm intervals.

Image analysis, image processing, and statistics

Image analysis was performed using ImageJ software (National Institutes of Health). All measurements were made from maximum projections of spinning-disk confocal Z-stacks that

were then segmented with the Trainable Weka Segmentation plugin (Arganda-Carreras et al., 2017). For all figures and videos, images were denoised using the Non-local Means Denoised plugin in ImageJ and processed in Photoshop (Adobe) as follows: brightness levels were adjusted, converted to 8-bit depth, and cropped.

Average instantaneous migration speed, confinement ratio, and cell position measurements used to calculate collision angle and cell contact duration were measured using the TrackMate plugin for ImageJ (Tinevez et al., 2017); at least 3 embryos were analyzed per condition. Statistical analysis for collision angles was performed using Astropy package in Python (The Astropy Collaboration et al., 2022). p-values were calculated using the Rayleigh test for uniformity of circular distribution. $p<0.05$ rejected the null hypothesis (i.e., uniform distribution). Polar plots were generated with Matplotlib (Caswell et al., 2023)

Spacing regularity analysis (Fig. 4) was performed from maximum projections of spinning-disk confocal z stacks that were cropped to 358.36 x 482.75 µm regions on interest (ROIs) centered approximately 80 µm to the left or right of the notochord then segmented with the Trainable Weka Segmentation plugin. Voronoi diagrams and Delaunay triangulation were generated using the Voronoi Delaunay plugin in ImageJ. To minimize artifacts, we excluded cells along the edges of the ROIs from analysis. Statistical analysis of the distributions of Voronoi domain areas and edge lengths were performed using SciPy package in Python (Virtanen et al., 2020). p-values were calculated using the Levene test for equality of variance; $p<0.05$ rejected the null hypothesis (i.e., equal variance). Histograms were generated with Matplotlib (Caswell et al., 2023).

DN RhoA Expression

For DN RhoA expression, we generated the expression plasmid pµTol2-tagRFP-DN-RhoA by PCR amplification of tagRFP and human dominant-negative (N19) RhoA (Ruchhoeft et al., 1999), which were cloned into pµTol2 (LaBelle et al., 2021) by enzymatic assembly (Gibson et al., 2009). As a control, we generated the expression plasmid pCS2-palm-TagRFP by PCR

amplification of TagRFP fused to the membrane-targeting palmitoylation sequence of Gap43 (Poulain et al., 2010) and cloned into pCS2+. Capped messenger RNA was synthesized using the mMESSAGE mMACHINE kit (Ambion) and 200 pg mRNA was injected into embryos at the one-cell stage.

RNA Sequencing

To identify endodermally enriched transcripts, GFP-positive cells were isolated from *Tg(sox17:GFP)* embryos at 8, 12, and 24 hpf by fluorescence-activated cell sorting as previously described (Woo et al., 2012). RNA was extracted from GFP-positive cells using the RNAqueous-Micro Kit (Ambion). Library construction was performed using the Illumina TruSeq mRNA stranded kit. Single-end sequencing was performed on an Illumina HiSeq 4000 machine. Sequencing yielded approximately 1.4 billion reads with an average read depth of 62 million reads per sample. Reads were then normalized and aligned to the zebrafish genome (GRCz10.87) using the STAR_2.5.2b aligner. Reads that mapped uniquely to known mRNAs were used to assess differential expression.

Dasatinib treatment

Embryos were treated with 5 µM dasatinib (Sigma-Aldrich SML2589) from the one-cell stage until bud stage. Control embryos were treated with dimethyl sulfoxide (DMSO; Sigma-Aldrich D2650) only.

Computational modeling

An agent-based model of the embryo and endoderm cells was written in python, using the NumPy (Harris et al., 2020) and SciPy (Virtanen et al., 2020) libraries. The embryo was modelled as a sphere and cells were modelled as circular discs which travelled across the surface of the sphere in discrete time steps at constant velocity. Changes in the direction of cell migration were governed by the persistence and collision behavior (see below). The model was parameterized from live imaging data of embryos (Table S2).

Persistence. Cells travel in straight lines over the sphere's surface (termed great circles in geometry) unless a persistence length **P** is defined. In that case the great circle is rotated at every step by an angle $\boldsymbol{\theta}$ which is chosen from a normal distribution with standard deviation given by:

$$\sigma = \frac{\pi}{2}\sqrt{\frac{S}{P}}$$

where S is the size of the step being taken.

Contact inhibition. Behavior upon collision depends on whether we implement contact inhibition or not. In both cases the definition of collision is any move which results in overlapping cells. In the case of no contact inhibition, a move which results in a collision is rejected - the cells can't pass through each other. Therefore, the cells will stop until their direction changes randomly, according to the persistence length, to produce a move which doesn't cause overlap. In the case where we do implement contact inhibition, the direction of motion of the cells after collision is given by the cross product of their position vectors. Therefore, they move in opposite directions after collision and those directions are along the line connecting their centers.

Epiboly. During the simulated time frame, the enveloping layer (EVL) progresses from 50% to 100% epiboly. Simulated cells are restricted to move only in regions where the EVL has reached. When a simulated cell attempts to move beyond the boundary, the move is rejected and the cell's direction of motion is randomized. A velocity of 0.004 %/s is chosen for the progression of the EVL edge as taken from previous reports (Kimmel et al., 1995).

Heterogeneity measure. To quantify the regularity of cell spacing patterns during cell dispersal in the model we used a grid-based measure of heterogeneity (Schilcher, 2017). We first divide the surface of the sphere into S^2 equal sections. To do that, we initially divide the

surface into S equal longitudinal segments. We then calculate the S polar angles θ_i which divide these segments into sections of equal area, given by:

$$cos\theta_i = 2i/S$$

For a perfectly homogenous distribution of cells, each segment would contain N/S^2 cells, where N is the number of cells. So, for each segment we define a deviation:

$$\partial_i = |N_i - N/S^2|$$

Where N_i is the number of cells found in region i. Then for the whole sphere we can quantify the heterogeneity as:

$$\partial = \frac{1}{2N} \sum_{i}^{sphere} |N_i - N/S^2|$$

Supplemental Material

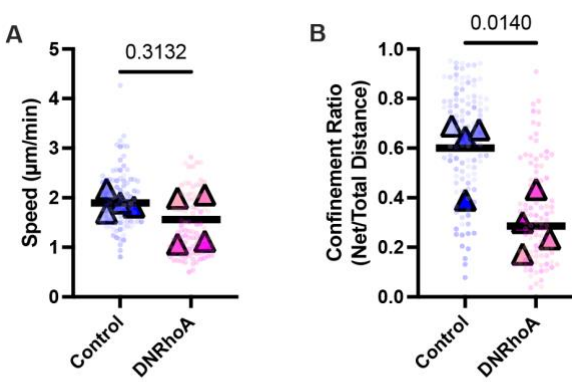

Figure S1. DN RhoA expression does not significantly affect the average instantaneous migration speed (A) although the confinement ratio is significantly lower (B). Data is color-coded per embryo. Dots represent individual cell tracks. Triangles represent the mean of all tracks per embryo. Horizontal bars represent the mean of all embryos. P values were calculated by Welch's t-test.

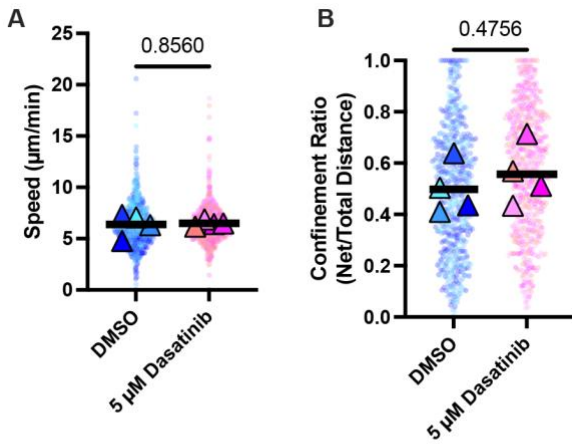

Figure S2. Dasatinib treatment does not significantly affect the average instantaneous migration speed (A) or confinement ratio (B). Data is color-coded per embryo. Dots represent individual cell tracks. Triangles represent the mean of all tracks per embryo. Horizontal bars represent the mean of all embryos. P values were calculated by Welch's t-test.

Table S1. RNA sequencing data. Contains all statistics for all pairwise comparisons as well as read counts for each sample (in counts per million reads). Column headings defined below:

a) FC – unlogged fold change for relevant comparison

b) Log2FC – log2 fold change for the relevant comparison

c) RawP – unadjusted p value

d) FDR – p value adjusted for multiple comparisons

e) Sample values – normalized sample values

Table S2. Parameters used for computational modeling.

Video 1. Endodermal cells exhibit contact inhibition of locomotion. Migrating endodermal cells from a *Tg(sox17:GFP-UTRN)* embryo. Images were acquired by spinning disk time-lapse confocal microscopy starting at 7 hours post-fertilization. Frames were acquired every 5 s for 15 min. Playback is 10 frames/s.

Video 2. DN RhoA expression abolishes endodermal CIL. Migrating endodermal cells from a *Tg(sox17:GFP)* embryo injected with DN RhoA mRNA. Images were acquired by spinning disk

time-lapse confocal microscopy starting at 7 hours post-fertilization. Frames were acquired every 5 s for 15 min. Playback is 10 frames/s.

Video 3. Inhibition of EphA receptors abolishes endodermal CIL. Migrating endodermal cells from a *Tg(sox17:GFP)* embryo treated with 5 µM dasatinib. Images were acquired by spinning disk time-lapse confocal microscopy starting at 7 hours post-fertilization. Frames were acquired every 5 s for 15 min. Playback is 10 frames/s.

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