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Report

Live Analysis of Endodermal Layer Formation Identifies Random Walk as a Novel Gastrulation Movement

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Summary

During gastrulation, dramatic movements rearrange cells into three germ layers expanded over the entire embryo [1–3]. In fish, both endoderm and mesoderm are specified as a belt at the embryo margin. Mesodermal layer expansion is achieved through the combination of two directed migrations. The outer ring of precursors moves toward the vegetal pole and continuously seeds mesodermal cells inside the embryo, which then reverse their movement in the direction of the animal pole [3–6]. Unlike mesoderm, endodermal cells internalize at once and must therefore adopt a different strategy to expand over the embryo [7, 8]. With live imaging of YFP-expressing zebrafish endodermal cells, we demonstrate that in contrast to mesoderm, internalized endodermal cells display a nonoriented/noncoordinated movement fit by a random walk that rapidly disperses them over the yolk surface. Transplantation experiments reveal that this behaviour is largely cell autonomous, induced by TGF-β/Nodal, and dependent on the downstream effector Casanova. At midgastrulation, endodermal cells switch to a convergence movement. We demonstrate that this switch is triggered by environmental cues. These results uncover random walk as a novel Nodal-induced gastrulation movement and as an efficient strategy to transform a localized cell group into a layer expanded over the embryo.

Results and Discussion

Identification of a Transgenic Line to Monitor Endodermal Cell Migration

To examine endodermal cell behavior in vivo, we screened for an enhancer trap line with specific expression in the endoderm during gastrulation [9]. Gastrulating *Et(CLG-YFP)smb602* embryos exhibited YFP expression in a population of deep, flattened, isolated hypoblastic cells and in forerunner cells (Figures S1A and S1B available online). This pattern was highly reminiscent of the expression patterns of the endoderm-specific genes *casanova/sox32* and *sox17* (Figures S1D and S1E) [8, 10–12]. At 24 hr postfertilisation (hpf), YFP was detected in all endodermal derivatives (pharynx, stomach, and gut; Figure S1C). Molecular analysis of *Et(CLG-YFP)smb602* revealed that the enhancer trap reporter is integrated 18 kb upstream from the *casanova* gene and 67 kb upstream from *sox17* (Figure S1H). Together, these data suggest that *Et(CLG-YFP)smb602* embryos express *yfp* under the control of endoderm-specific *casanova* and/or *sox17* *cis*-regulatory elements. We used a morpholino directed against *casanova* mRNA to specifically prevent endoderm formation in *Et(CLG-YFP)smb602* embryos [11, 13]. This completely abolished YFP expression (Figure S1G), confirming that *Et(CLG-YFP)smb602* is a specific endodermal-expressing line and represents an ideal reagent for live imaging of endodermal cells during gastrulation.

During Early Gastrulation, Endodermal Cells Disperse over the Yolk Cell with a Random Walk

In zebrafish, previous analyses of the behavior of hypoblastic cells (mesoderm and endoderm) revealed two phases during gastrulation. Once internalized, hypoblastic cells move toward the animal pole in a directed fashion [4, 6] and, during the second half of gastrulation, undergo convergence and extension movements to collect along the embryonic axis (see [5, 14] for a review). However, because the hypoblast is primarily composed of mesodermal cells with a small population of endodermal cells, prior studies most likely described mesodermal cell movements. Time-lapse analyses of *Et(CLG-YFP)smb602* embryos (see the Supplemental Experimental Procedures) identified two phases of different behaviors corresponding to the first and second halves of gastrulation (hereafter referred to as "early gastrulation" and "late gastrulation," respectively). During late gastrulation, similar to mesoderm, endodermal cells undergo convergence and extension movements (Figures 1E–1G). However, during early gastrulation, endodermal cell movement differed from mesoderm in that they did not migrate toward the animal pole (Figures 1A–1C, n = 25 embryos; Figure S2 and Movie S1). Once on the yolk, endodermal cells move rapidly, with an average speed of 2.51 μm/min (n = 164 cells on 14 embryos). But, in contrast to what happens during directed migrations, cells moved in all directions (Figures 1D and 1H). Moreover, each cell turned very frequently (Figure 1I), resulting in a low persistence (37% compared to 81% during late gastrulation, n = 164 and 68, $p < 0.001$). Previous analyses of fixed midgastrulation embryos have revealed that endodermal cells develop cytoplasmic extensions [15]. However, it was not clear whether these extensions also exist during early gastrulation, whether they are dynamic, or how they participate in cell movements. Our data showed that all endodermal cells regularly produced large membrane extensions at this time point (>5 μm; Movie S2). These cytoplasmic processes were short lived and almost always prefigured cell movement (91%, n = 60 protrusions from 20 cells in three

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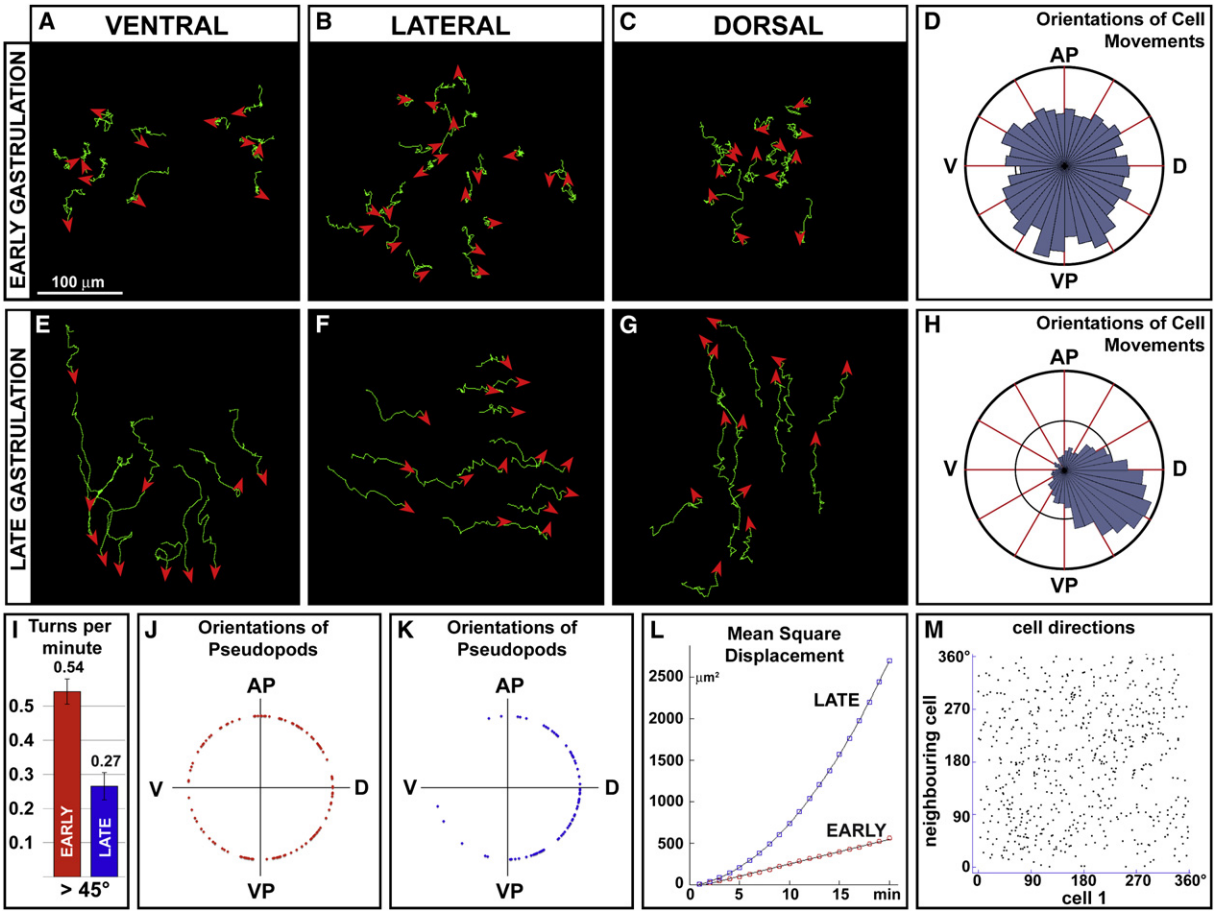

Figure 1. Endodermal Cells Disperse with a Random Walk Movement during Early Gastrulation

(A–C and E–G) Representative examples of 30 min tracks with 1 min intervals of endodermal cells in *Et(CLG-YFP)smb602* embryos during early (55%–70% epiboly [A–C]) or late (75%–90% epiboly [E–G]) gastrulation (similar tracks were obtained for more than 400 cells on about five embryos per position and stage). During early gastrulation cells move in a nonoriented fashion, whereas during late gastrulation they undergo convergence-extension movements (i.e., dorsal cells migrate anteriorly, lateral cells converge toward the embryonic axis, and ventral cells migrate toward the vegetal pole). Red arrowheads indicate the direction of the last tracked movement. The animal pole is to the top, and for lateral views, dorsal is to the right.

(D and H) Rose diagrams representing the directions of endodermal cell movements. During early gastrulation cells migrate in all directions (D) compared to the oriented migration of converging cells during late gastrulation (H). Early gastrulation data were obtained from four time lapses on lateral views (D) and late gastrulation data from four time-lapses on lateral views (H).

(I) The graph indicates the average number of turns per minute per cell during early gastrulation (n = 164 cells) or during late gastrulation (n = 68 cells). On average, cells maintained the same direction (angle of turn < 45°) for only 2.21 min compared to 4.95 min for converging cells ($p < 0.0001$). Error bars represent standard errors.

(J and K) Polar plots of the distribution of the outgrowth positions of pseudopods relative to the cell center for lateral cells during early (J) and late (K) gastrulation. Each pseudopod was counted only once, even though pseudopods often persisted for more than one frame. For each diagram, 20 cells from four embryos were analyzed over a 30 min period.

(L) Plot (dot and square) and curve fit (line) of the MSD of cells during early (red) and late (blue) gastrulation, showing that whereas converging cells have an oriented migration (parabolic fit, R = 0.999, n = 164 cells), cells move in a random walk during early gastrulation (linear fit, R = 0.998, n = 58 cells).

(M) Scatter plot of the direction of a cell and of its closest neighbor, showing that cell movements are not coordinated (R = 0.12, n = 589).

embryos). Consistent with this idea, protrusions formed in all directions during early gastrulation, whereas most of them pointed dorsally for converging lateral cells (Figures 1J and 1K).

These nonoriented movements suggested that endodermal cell behavior may correspond to a random walk. To confirm this hypothesis, we calculated the mean square displacement (MSD) of endodermal cells during both early and late gastrulation. MSD is a measure of the average distance a cell travels over time and is used to characterize cell movement: An oriented movement leads to a parabolic MSD, whereas a linear MSD identifies a random walk [16]. During late gastrulation, converging cells were indeed characterized by a MSD with a parabolic fit (R = 0.999, Figure 1L). However, during early gastrulation, endodermal cells exhibited a MSD with a linear fit (R = 0.998, Figure 1L), demonstrating that this nondirected movement is a random walk. This random walk, which appeared as a general feature of endodermal cells during early gastrulation (Figure S3), was initiated readily after involution and continued until midgastrulation, at which time convergence started. Finally, consistent with the observation that each cell has a random behavior, we could not detect any coordination in the movement of neighboring cells (Figure 1M). Together, these results demonstrate that during the first half of gastrulation, endodermal cells undergo a random walk over the surface of the yolk.

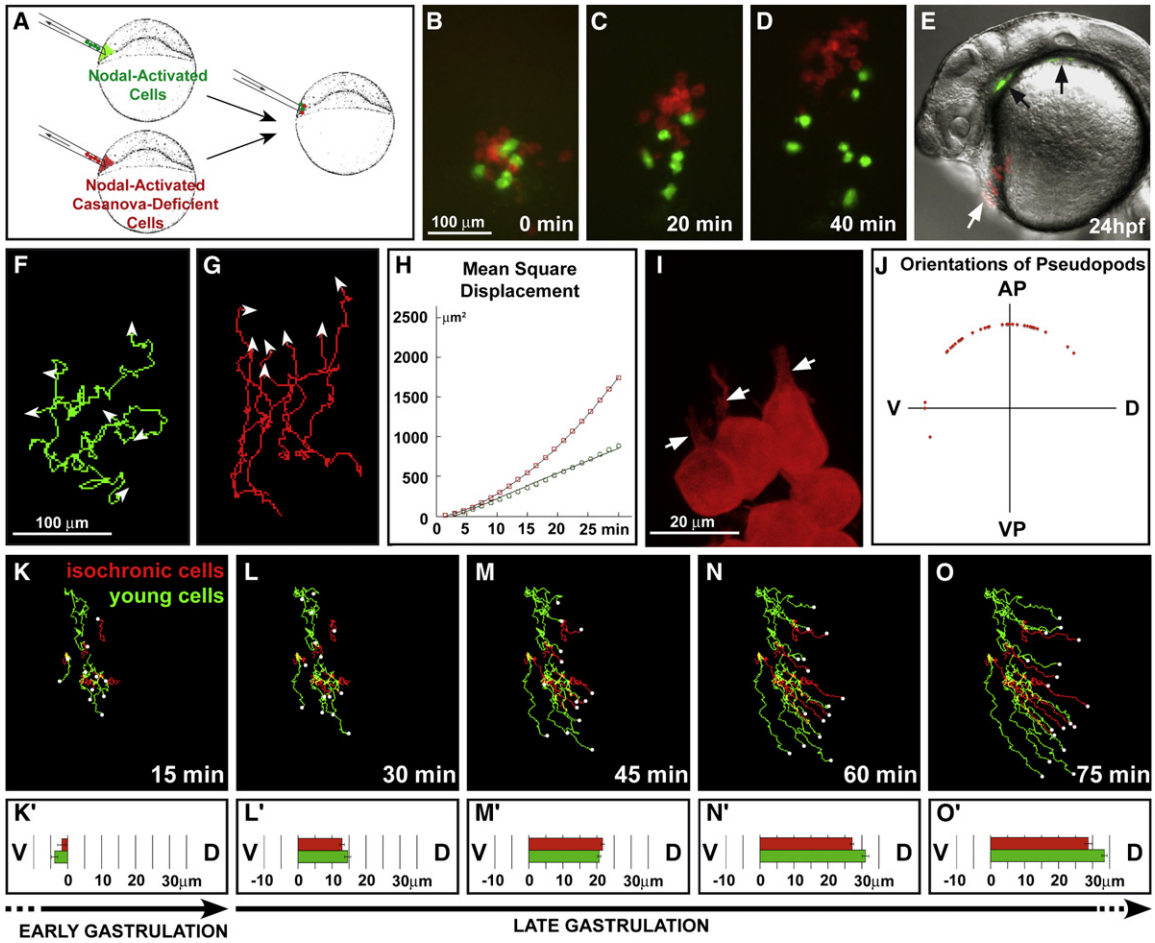

Figure 2. Control of Random Walk Behavior

(A–J) Random walk is inducible by Nodal and depends on *casanova*. In (A), a schematic of the experimental procedure is illustrated. (B–D) Nodal-activated cells (green, Tar* cells) and Nodal-activated cells coinjected with a morpholino against *casanova* (red, Tar*-MO*casanova* cells) were transplanted into wild-type host embryos and monitored during early gastrulation. (E) At 24 hpf, Tar* cells are found within the endoderm (pharynx, black arrows), whereas Tar*-MO*casanova* cells contribute to the hatching gland (white arrow). (F and G) Shown are representive examples of 40 min tracks with 1 min intervals of Tar* and Tar*-MO*casanova* cells after transplantation. (H) A MSD plot reveals that Tar* cells (green) migrate in a random walk (linear fit, R = 0.996), whereas Tar*-MO-*casanova* cells (red) display an oriented migration (parabolic fit, R = 0.999). For each population, 30 cells from four embryos were analyzed. (I) Shown is a representive example of Tar*-MO*casanova* cell morphology during early gastrulation. Cells develop large cytoplasmic processes (white arrows). In (J), a polar plot illustrates the distribution of the outgrowth positions of pseudopods relative to the cell center of Tar*-MO*casanova* cells during early gastrulation. Seventeen cells from three embryos were analyzed over 20 min.

(K–O′) Transition to a convergence movement is induced by the embryonic environment. Nodal-activated cells from midblastula (4 hpf) embryos (young cells, green) and late-blastula (5 hpf) embryos (isochronic cells, red) were transplanted into late-blastula (5 hpf) host embryos. Shown is a representative example of four independent experiments. (K–O) Shown are tracks of both cell populations after 15, 30, 45, 60, or 75 min of monitoring. Host midgastrulation (70% epiboly) corresponds to t = 15 min. The white dots indicate the end position of each track. (K′)–(O′) illustrates the mean net displacement toward the dorsal side for each 15 min interval. During host early gastrulation, both cell populations first migrate randomly without any dorsal bias (K′). They simultaneously start to converge dorsally after host midgastrulation (L′–O′). Error bars represent standard errors.

The Random Walk Behavior Is Induced by Nodal and Requires Casanova

Many studies have implicated Nodal signaling in endodermal induction (see [17] for a review). In particular, the activation of the Nodal signaling pathway with a constitutively activated form of the Nodal receptor TARAM-A (Tar*) is sufficient to induce an endodermal identity and final differentiation, but the behavior of activated cells during gastrulation was not established [18]. We thus first verified that activation of Nodal signaling also confers random walk behavior. To do so, Nodal-activated cells were transplanted into wild-type embryos (Figure 2A). Nodal-activated cell movements were indistinguishable from those of endogenous endodermal cells (Figures 2B–2D and Movie S3), corresponding to a random walk (MSD with a linear fit, R = 0.996; Figure 2H), with the same average speed (2.6 μm/min, p = 0.87, n = 30) and the same persistence. Furthermore, cellular morphology and pseudopod dynamics of Nodal-activated and endogenous endodermal cells were very similar. During the second half of gastrulation, Nodal-activated cells converged normally (Figure S4A) and ultimately populated endodermal derivatives at 24 hpf (Figure 2E). Thus, Nodal signaling appears sufficient to induce the same random walk behavior as observed in endogenous endodermal cells.

Downstream of Nodal, the transcription factor *casanova/sox32* is required for the acquisition of the endodermal fate [13]. We therefore tested whether *casanova* was necessary for random walk behavior. Nodal-activated cells coinjected

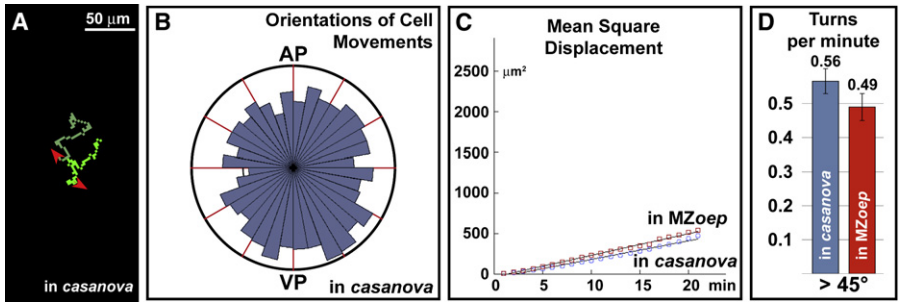

Figure 3. Random Walk Does Not Depend on Cell Interactions

One or a few Nodal-activated cells were transplanted into endoderm-deficient *casanova* morphant embryos and into endoderm- and dorsal mesoderm-deficient MZ*oep* mutant embryos.

(A) Representative example of 50 min tracks (with 1 min intervals) of two cells derived from one cell transplanted into a *casanova* morphant embryo.

(B) Rose diagram of the directions of cell movements shows that in *casanova* morphant, transplanted cells migrate in all directions (n = 11 cells from three embryos).

(C) Plot (dot) and curve fit (line) of the MSD showing that these cells move in a random walk during early gastrulation (in *casanova*: linear fit, R = 0.992, n = 11 cells from three embryos; in MZ*oep*: linear fit, R = 0.997, n = 14 cell from four embryos).

(D) The graph illustrates the average number of turns per minute per Nodal-activated cell. Even in the absence of other endodermal cells (in *casanova*) or of hypoblastic cells (in MZ*oep*), Nodal-activated cells frequently change their direction. Error bars represent standard errors.

with a *casanova* morpholino and transplanted into wild-type embryos did not follow a random walk but, instead, migrated with an oriented movement toward the animal pole and at 24 hpf contributed to the hatching gland (parabolic MSD, R = 0.999; Figures 2A–2H and Movie S3). This result demonstrates that not only the fate but also the behavior is dependent on *casanova*, suggesting that factors controlling this behavior lie downstream of *casanova*. Interestingly, inhibition of *casanova*, even though it completely changed cell behavior, did not affect cell morphology. Cells developed pseudopods prefiguring cell movement (Figure 2I). The difference with randomly migrating cells was in the orientation of protrusions that, in the absence of *casanova*, predominantly pointed toward the animal pole (compare Figures 1J and 2J; n = 20). This suggests that the switch between random walk and oriented migration does not require modification of the cell motility per se but, rather, implies an ability to bias protrusion formation toward one direction. Consistent with this idea, blocking guidance receptor activity in *Drosophila* border cells results in protrusions forming in all directions instead of pointing in the direction of the oriented movement [19]. Thus one intriguing possibility is that *casanova* prevents endodermal cells from sensing environmental cues present in the early gastrula, resulting in protrusions forming in all directions and cells moving with a random walk.

An Inductive Cue Transitions Endodermal Cells from Random Walk to Convergent Behavior

At midgastrulation, however, endodermal cells switch from a random walk to a convergence movement. This transition could result from an intrinsic maturation process that allows cells to sense their environment or alternatively from the appearance of new environmental cues that cells can respond to. To discriminate between these two possibilities, heterochronic transplant experiments were performed. Nodal-activated cells from either midblastula (4 hpf) or late blastula (5 hpf) embryos were transplanted together into late blastula (5 hpf) hosts. During the first half of gastrulation, the two populations of endodermal cells dispersed randomly over the yolk. When the host embryo reached midgastrulation, the two cell populations stopped their random walk and simultaneously initiated convergence movements toward the embryonic axis (Figures 2K–2O′ and Movie S4), strongly suggesting that the switch in endodermal cell behavior is controlled by extrinsic cues. To confirm this, Nodal-activated cells from early gastrulae (6 hpf) were transplanted into late gastrula hosts (8 hpf). These cells immediately assumed a directed movement toward the dorsal side (Figure S5). In the converse experiment, late-converging cells transplanted into young gastrula hosts initiated a random walk (Figure S5). These results demonstrate that the switch between random walk and convergence is not controlled by an intrinsic process but, rather, by cues provided by their environment.

The Random Walk Behavior Does Not Depend on Interactions between Hypoblastic Cells

Random walk behavior, the result of cells changing direction frequently, can be achieved through two distinct mechanisms. First, similar to the Brownian motion of particles, cells may move along straight paths and only change direction when they collide into one another. Such a mechanism has been proposed to explain the erratic motion of T cells in lymph nodes [20]. Alternatively, cells might change direction independent of extrinsic signals, as with fibroblasts in vitro [21]. No correlation could be found between cell collisions and cell changes in direction (p = 0.7, n = 1245 cell movements), suggesting that even though collisions often were observed between endodermal cells, changes in direction do not rely on such collisions. To ascertain this conclusion, we directly assessed the importance of the cellular environment on the migratory behavior of endodermal cells.

First, one or two Nodal-activated cells were transplanted into *casanova* morphants, which are completely and specifically devoid of endoderm [11, 13]. These isolated endodermal cells displayed the same behavior as in wild-type embryos. They migrated at the same average speed (2.2 μm/min, n = 11 cells on three embryos), moved in all directions (Figures 3A and 3B and Movie S5), and followed a random walk (linear MSD, R = 0.992; Figure 3C). Importantly, even though deprived of neighboring endodermal cells, they frequently changed direction (Figure 3D), resulting in the same persistence as in wild-type embryos (37%, n = 11 cells on three embryos). We conclude that collisions between endodermal cells in wild-type embryos do not cause the random walk.

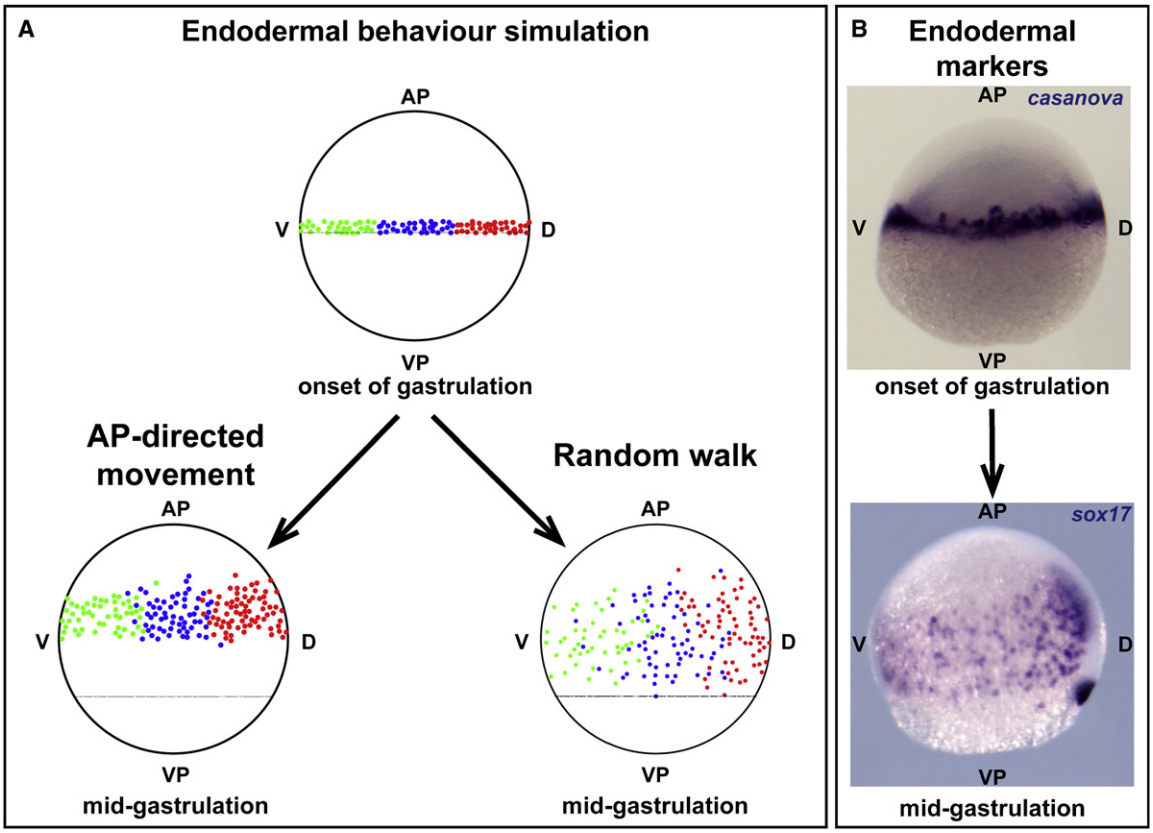

Figure 4. Random Walk Is Sufficient for Endodermal Cells to Colonize the Yolk Surface

(A) Cell migration was mathematically simulated to test how the observed pattern of endodermal cells at midgastrulation can be achieved. At the beginning of gastrulation, 100 cells were localized at the margin of the blastoderm (50% epiboly). Depending on their position along the dorsoventral axis, cells were marked in red (dorsal), blue (lateral), or green (ventral). An oriented motion toward the animal pole cannot account for the pattern observed in vivo at 75% epiboly. In contrast, a random walk efficiently spreads cells over the yolk, with the expansion of the layer being limited both by cell speed and by the margin. At midgastrulation (75% epiboly), cells have reached a position similar to the expression pattern of endodermal markers and have partially mixed.

(B) Shown is the distribution of endodermal cells at the onset of gastrulation and at midgastrulation as assayed by *sox17* expression.

Within the hypoblast, endodermal cells also are mixed with mesodermal cells. Endodermal behavior could, therefore, result from collisions with mesodermal neighbors that are not labeled in the *Et(CLG-YFP)smb602* line. To test this possibility, the same experiments were performed in MZ*oep* embryos, which are devoid of endoderm and of most mesoderm [22]. Again, cells behaved exactly as in wild-type embryos (Figures 3C and 3D and data not shown), demonstrating that random walk of endodermal cells does not require hypoblastic cell interactions.

During the second half of gastrulation, cells transplanted into *casanova* morphants converged and joined the midline, but their migration toward the dorsal side was less directed and less persistent (64%, p = 0.01) than in wild-type embryos (Figure S4). This result shows that endoderm-endoderm interactions influence the normal convergence of the layer but that they are not absolutely required for convergence. Rather endodermal cells are converging either autonomously or, more likely, through interactions with their mesodermal neighbors [23]. Consistent with this latter possibility, cells transplanted into MZ*oep* embryos failed to converge (data not shown).

Random Walk Can Account for the Observed Dispersion of Endodermal Cells

This cell autonomy of the random walk contrasts with the classical idea that gastrulation movements are tightly controlled migrations oriented by environmental cues. Thus, we wondered if a simple individual behavior as random walk could account for the formation of the germ layer when considered collectively at the level of the cell population. Mathematical modeling of the embryo was used to address this issue (see the Supplemental Experimental Procedures). In contrast to the previously proposed movement toward the animal pole, random migration leads to a diffusive spreading of cells that generates a pattern strikingly similar to the one observed in vivo (Figure 4 and Movies S6 and S7). Therefore, random walk appears as a simple and effective strategy to transform the narrow marginal ring of endodermal cells into a sheet of cells dispersed over the embryo. Interestingly, precise fate map analyses had previously shown that neighboring cells at the onset of gastrulation may end up in divergent endodermal derivatives [15]. The existence of a random migration phase, which induces cell mixing, could account for this outcome (Figure 4A and Movie S7).

Conclusions

Through our characterization of endodermal cell behavior in vivo, we have identified a novel and unexpected step in endoderm formation corresponding to a period of active migration with a random walk movement, which serves to expand the layer during the first half of gastrulation. This random walk behavior appears specific to endodermal cells, does not depend

on cell interactions in the hypoblast, and is controlled by Nodal signaling. At midgastrulation, endodermal cells switch to convergence and extension movements upon perception of environmental cues.

Random walk is a simple individual cellular behavior but, when considered collectively, appears as a very effective strategy for a spatially restricted group of cells to colonize a new territory. This process, therefore, may be used widely during development. This may be the case for endoderm formation in other species, like in chicks where fate map analyses have shown that precursors restricted to the rostral tip of the primitive streak colonize most of the rostrocaudal extent of the gut [24, 25]. This appears to be the case in cerebral cortex development, where recent studies established that Cajal-Retzius cells colonize the cortex through a nonoriented dispersion [26]. The formation of the endodermal layer may represent a convenient model to better characterize the mechanisms controlling this spreading strategy.

Supplemental Data

Supplemental Experimental Procedures, five figures, and seven movies are available at http://www.current-biology.com/cgi/content/full/18/4/276/DC1/.

Acknowledgments

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