# Live analysis of endodermal layer formation identifies random walk as a novel gastrulation movement.

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**Running title:** A random walk for endoderm formation

**Key words:** zebrafish, gastrulation, endoderm, cell migration, random walk, Nodal, casanova.

**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 margin of the embryo. Expansion of the mesodermal layer is achieved through the combination of two directed migrations. The outer ring of precursors moves vegetalwards and continuously seeds mesodermal cells inside the embryo, which, once internalised, reverse their movement and adopt an animalwards motion [3-6]. In contrast to mesoderm, endodermal cells all internalise at the onset of gastrulation and must therefore adopt a different strategy to expand over the embryo [7, 8]. Using live imaging of transgenic zebrafish expressing YFP in endodermal cells, we demonstrate that, in contrast to mesoderm, once internalised, endodermal cells display a non-oriented / non-coordinated movement fit by a random walk, that rapidly disperses them over the surface of the yolk. Transplantation experiments reveal that this behaviour is largely cell autonomous, that it is induced by TGF-β/Nodal and dependent on the downstream effector Casanova. At mid-gastrulation, 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 localised group of cells into a layer expanded over the embryo.**

## Results and Discussion

### Identification of a transgenic line to monitor endodermal cell migration.

To examine endodermal cell behaviour *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-B). This pattern was highly reminiscent of the expression patterns of the endoderm specific genes, *casanova/sox32* and *sox17* (Figures S1D-E) [8, 10-12]. At 24 hours post fertilisation (hpf), YFP was detected in all endodermal derivatives (pharynx, stomach, 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 behaviour of hypoblastic cells (mesoderm and endoderm) revealed two phases during gastrulation. Once internalised, hypoblastic cells move towards 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 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 Experimental Procedures) identified two phases of different behaviours corresponding to the first and second halves of gastrulation (hereafter referred to as early and late gastrulation). During late gastrulation, similar to mesoderm, endodermal cells undergo convergence and extension movements (Figures 1E-G). However, during early gastrulation, endodermal cell movement differed from mesoderm in that they did not migrate towards the animal pole (Figures 1A-C, n=25 embryos; Figure S2 and Movie S1). Once on the yolk, endodermal cells move rapidly, with an average speed of 2.51 microns/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 mid-gastrulation 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 and how they participate in cell movements. Our data showed that all endodermal cells regularly produced large membrane extensions at this time point (>5μm; Movie S2). These cytoplasmic processes were short lived and almost always prefigured cell movement (91%, n=60 protrusions from 20 cells in 3 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-K ).

These non-oriented movements suggested that endodermal cell behaviour 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 characterise 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 characterised 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 non-directed 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 mid-gastrulation, at which time convergence started. Finally, consistent with the observation that each cell has a random behaviour, we could not detect any coordination in the movement of neighbouring 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.

**The random walk behaviour is induced by Nodal and requires Casanova**

Many studies have implicated Nodal signalling in endodermal induction (see [17] for review). In particular, the activation of the Nodal signalling pathway using a constitutively activated form of the Nodal receptor TARAM-A (Tar*) is sufficient to induce an endodermal identity and final differentiation, but the behaviour of activated cells during gastrulation was not established [18]. We thus first verified that activation of Nodal signalling also confers random walk behaviour. 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-D 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 micron/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 24hpf (Figure 2E). Thus, Nodal signalling appears sufficient to induce the same random walk behaviour 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 behaviour. Nodal-activated cells co-injected with a *casanova*-morpholino and transplanted into wild-type embryos did not follow a random walk, but instead migrated with an oriented animalwards movement and at 24hpf contributed to the hatching gland (parabolic MSD, R=0.999; Figure 2A-H and Movie S3). This result demonstrates that, not only the fate, but also the behaviour is dependent on *casanova*, suggesting that factors controlling this behaviour lie downstream of *casanova*. Interestingly, inhibition of *casanova*, even though it completely changed cell behaviour, did not affect cell morphology. Cells developed pseudopods prefiguring cell movement (Figures 2I). The difference with randomly migrating cells was in the orientation of protrusions which, in the absence of *casanova*, predominantly pointed towards the animal pole (compare Figures 2J and 1J; 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 towards 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]. One intriguing possibility is thus 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 behaviour.**

At mid-gastrulation 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 mid-blastula (4hpf) or late blastula (5hpf) embryos were transplanted together into late blastula (5hpf) hosts. During the first half of gastrulation, the two populations of endodermal cells dispersed randomly over the yolk. When the host embryo reached mid-gastrulation, the two cell populations stopped their random walk and simultaneously initiated convergence movements towards the embryonic axis (Figures 2K-O' and Movie S4), strongly suggesting that the switch in endodermal cell behaviour is controlled by extrinsic cues. To confirm this, Nodal-activated cells from early gastrulae (6hpf) were transplanted into late gastrula hosts (8hpf). These cells immediately assumed a directed dorsalwards movement (Figure S5). In the converse experiment, late converging cells transplanted into a 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 behaviour does not depend on interactions between hypoblastic cells.**

Random walk behaviour, 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 were often 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 behaviour 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 behaviour as in wild-type embryos. They migrated at the same average speed (2.2 microns/min, n=11 cells on 3 embryos), moved in all directions (Figures 3A-B, Movie S5), and followed a random walk (linear MSD, R=0.992; Figure 3C). Importantly, even though deprived of neighbouring endodermal cells, they frequently changed direction (Figure 3D), resulting in the same persistence as in wild-type embryos (37%, n=11 cells on 3 embryos). We

conclude that collisions between endodermal cells in wild-type embryos do not cause the random walk.

Within the hypoblast, endodermal cells are also mixed with mesodermal cells. Endodermal behaviour could therefore result from collisions with mesodermal neighbours which are not labelled 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-D 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 towards 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, endodermal cells converging either autonomously or, more likely, through interactions with their mesodermal neighbours [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. We thus wondered if a simple individual behaviour as random walk could account for the formation of the germ layer when considered collectively, at the level of the cell population. Mathematical modelling of the embryo was used to address

this issue (see Experimental Procedures). In contrast to the previously proposed animalwards movement, random migration leads to a diffusive spreading of cells that generates a pattern strikingly similar to the one observed *in vivo* (Figure 4, 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 neighbouring 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, Movie S7).

**Conclusion**

Through our characterisation of endodermal cell behaviour *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 behaviour appears specific to endodermal cells, does not depend on cell interactions in the hypoblast and is controlled by Nodal signalling. At mid-gastrulation endodermal cells switch to convergence and extension movements upon perception of environmental cues.

Random walk is a simple individual cellular behaviour, but, when considered collectively, appears as a very effective strategy for a spatially restricted group of cells to colonise a new territory. This process may therefore be widely used during development. This may be the case for endoderm formation in other species, like in chick where fate map analyses have shown that precursors restricted to the rostral tip of the primitive streak colonise 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 colonise the cortex through a non oriented dispersion [26]. The formation of the endodermal layer may represent a convenient model to better characterise the mechanisms controlling this spreading strategy.

## Experimental Procedures

### Zebrafish strains

The enhancer trap line *Et(CLG-YFP)smb602* was generated as previously described [9]. MZ*oep* embryos were obtained from natural spawns of homozygous $oep^{tz57}$ fish [27].

### Time-Lapse Imaging and Analysis.

Dechorionated embryos were mounted in 0.4% agarose in embryo medium. Images were collected at one-minute intervals with an Axioplan 2 microscope (Zeiss), a coolsnap-cf digital camera (Photometrics) and MetaVue software (Molecular Devices). Cell tracking was performed manually in ImageJ and automatically in Matlab (The MathWorks). Both approaches gave similar tracks. MSD were calculated using the Cell_motility software [28], and curve fitting and Pearson's R calculations were generated in Kaleidagraph (Synergy Software). Statistical analyses were performed in R (R-project) by Anova tests on Linear Mixed-Effects Models or Chi2 tests when appropriate. In all rose diagrams, the area of each sector is proportional to the observed frequencies. On Figure 2R, neighbouring cells were defined as cells separated by less than 40 microns (changing this threshold did not change the conclusion).

### mRNA synthesis and Morpholino.

Capped mRNA of Tar* [18], membrane-bound GFP (mGFP) and membrane-bound mCherry (mbCherry) [29] were synthesized from pSP64T and pCS2+ constructs with

the mMessage mMachine Kit (Ambion). *Casanova* morpholino has been described previously [11].

**Cell Transplantation**

Donor embryos were injected with 80 pg of mGFP or 80 pg of mbCherry mRNAs and with either 1.2 pg of tar* mRNA or with tar* and cas-MO (0.3mM). At dome stage, one or two marginal cells were transplanted into wild-type, *casanova* morphants or MZ*oep* mutant embryos. For heterochronic graft experiments, cells were transplanted from sphere and 40% epiboly stage embryos into 40% epiboly stage embryos, from shield stage embryos into 80% epiboly stage embryos and from 80% epiboly stage embryos into shield stage embryos.

**Whole-Mount In Situ Hybridization**

In situ hybridisation was done following standard protocols [30]. Probes used were: *cas* [11], *sox17* [8], *fkd7* [31].

**Modelling of random walk**

A simulated lateral view of the embryo was represented as a disc (diameter=700 μm) and cells were represented as 15μm wide dots. According to observations, 100 cells were distributed at the margin (50 μm wide) along a dorso-ventral gradient at the start of the simulation. At each time step (t) and for each cell (c), a displacement vector ($\vec{d}$) was generated and the new position vector ($\vec{p}$) was evaluated by the following equation:

$$\vec{p}_t^{\,c} = \vec{p}_{t-1}^{\,c} + \vec{d}_t^{\,c}$$

To generate ($\vec{d}$), a displacement distance was randomly picked from a table of observed values, and a direction was picked either in a Gaussian distribution centred towards the animal pole (oriented movement) or in a normal distribution (random walk). To prevent cells from overlapping, each time a displacement would lead a cell

to overlap with one of its neighbours, a new displacement was generated. To reproduce the exclusion zone observed *in vivo*, cells were prevented from entering the ventro-animal most fifth of the embryo. Each cell was allowed to divide once during gastrulation. The daughter cell was then randomly positioned close to the mother cell. Modelling was performed using MatLab software.

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**Figure Legends**

Figure 1. Endodermal cells disperse with a random walk movement during early gastrulation.

(A-C and E-G) Representative examples of 30 minutes tracks with 1 minute 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 5 embryos per position and stage). During early gastrulation cells move in a non-oriented fashion, whereas during late gastrulation they undergo convergence-extension movements (i.e. dorsal cells migrate anteriorly, lateral cells converge towards the embryonic axis and ventral cells migrate towards the vegetal pole). Red arrowheads indicate the direction of the last tracked movement. Animal pole to the top and for lateral views, dorsal 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 4 time-lapses on lateral views (D) and late gastrulation data from 4 time-lapses on lateral view (H).

(I) 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 indicate standard errors.

(J-K) Polar plots of the distribution of the outgrowth positions of pseudopods relative to the cell centre 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 4 embryos were analysed over a 30 minutes 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 neighbour, showing that cell movements are not coordinated (r=0.12, n=589).

Figure 2 Control of random walk behaviour.

(A-J) . Random walk is inducible by Nodal and depends on *casanova*.

(A) Schematic of the experimental procedure.

(B-D) Nodal-activated cells (green, Tar* cells) and Nodal-activated cells co-injected with a morpholino against *casanova* (red, Tar*-MO*casanova* cells) were transplanted into wild-type host embryos and monitored during early gastrulation.

(E) At 24hpf, Tar* cells are found within the endoderm (pharynx, black arrows) whereas Tar*-MO*casanova* cells contribute to the hatching gland (white arrow).

(F-G) Representive examples of 40 minutes tracks with 1 minute intervals of Tar* and Tar*-MO*casanova* cells after transplantation.

(H) 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 4 embryos were analysed.

(I) Representive example of Tar*-MO*casanova* cell morphology during early gastrulation. Cells develop large cytoplasmic processes (white arrows).

(J) Polar plot of the distribution of the outgrowth positions of pseudopods relative to the cell centre of Tar*-MO*casanova* cells during early gastrulation. 17 cells from 3 embryos were analysed over 20 minutes.

(K-O') Transition to a convergence movement is induced by the embryonic environment.

Nodal-activated cells from mid-blastula (4hpf) embryos (young cells, green) and late-blastula (5hpf) embryos (isochronic cells, red) were transplanted into late-blastula (5hpf) host embryos. Repeated in four independent experiments.

(K-O) Tracks of both cell populations after 15, 30, 45, 60 or 75 minutes of monitoring. Host mid-gastrulation (70% epiboly) corresponds to t=15min. White dots indicate the end position of each track.

(K'-O') Mean net displacement towards the dorsal side for each 15 minute-interval. During host early gastrulation, both cell populations first migrate randomly without any dorsal bias (K'). They simultaneously start to converge dorsally after host mid-gastrulation (L'-O'). Error bars indicate standard errors.

Figure 3. Random walk does not depend on cell interactions.

One or a few Nodal-activated cells were transplanted into *casanova* morphant embryos that are completely deprived of endoderm and into MZ*oep* mutant embryos that are deprived of endoderm and most mesoderm.

(A) Representative example of 50 minutes tracks (with one-minute 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 3 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 3 embryos; in MZ*oep*: linear fit, R= 0.997*,* n=14 cell from 4 embryos).

(D) 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.

Figure 4. Random walk is sufficient for endodermal cells to colonise the yolk surface.

(A) Cell migration was mathematically simulated to test how the observed pattern of endodermal cells at mid-gastrulation can be achieved. At the beginning of gastrulation, 100 cells were localised at the margin of the blastoderm (50% epiboly). Depending on their position along the dorso-ventral axis, cells were marked in red (dorsal), blue (lateral) or green (ventral). An oriented motion towards 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, the expansion of the layer being limited both by cell speed and by the margin. At mid-gastrulation (75% epiboly), cells have reached a position similar to the expression pattern of endodermal markers and have partially mixed.

(B) Distribution of endodermal cells at the beginning and at mid-gastrulation as assayed by *sox17* expression.

Figure S1. The *Et(CLG-YFP)smb602* line specifically expresses YFP in endodermal cells.

(A, B) Live imaging of *Et(CLG-YFP)smb602* embryos reveals YFP expression in isolated hypoblastic cells located on the yolk surface and in the forerunner cells (white arrows) during gastrulation.

(C) At 20hpf, YFP is detected in all endodermal derivatives (arrowheads) and in derivatives of the forerunner cells (arrows).

(D-F) Location of endodermal cells during gastrulation (D and E) and of endodermal derivatives at 24hpf (F, arrowheads) revealed by the expression of the endodermal markers *sox17* and *fkd7*.

(G) Live imaging of *Et(CLG-YFP)smb602* embryos injected with *casanova*-Morpholino.

(H) Organisation of the *casanova* and *sox17* genomic locus and CLGY reporter integration site in *Et(CLG-YFP)smb602*.

(A, D and H) Dorsal view, animal pole to the top. (B and E) Lateral view, animal pole to the top and dorsal to the right. (C and F) Lateral view, anterior to the left.

Figure S2. Endodermal cells do not behave like mesodermal cells during early gastrulation.

(A, C) Evolution of a clone of mesodermal cells (labelled in red) transplanted at the margin of an *Et(CLG-YFP)smb602* embryo (in green) from 60% to 70% epiboly (lateral view, animal pole to the top, dorsal to the right).

(D) Net path of internalised mesodermal cells (red) and endodermal cells (green) from time-lapse in A-C. Whereas mesodermal cells move towards the animal pole, endodermal cells do not show any preferred direction.

Figure S3. Random walk is observed all over the embryo.

(A) Plot (dot) and curve fit (black line) of the MSD of ventral, lateral and dorsal cells showing that, regardless of their position within the embryo, endodermal cells move in a random walk during early gastrulation (linear fit, ventral: R=0.993, 33 cells on 3 embryos; Lateral: R=0.997, 41 cells on 4 embryos; Dorsal: R=0.998, 90 cells on 6 embryos). For comparison, the MSD of converging cells (lateral cells during late gastrulation) is presented.

(B-D) Directions of endodermal cell movements during early gastrulation depending on ventral, lateral or dorsal position. At all positions, cells move in all directions. Nevertheless, lateral and ventral cells show a tendency to move more frequently towards the vegetal pole. This bias probably reflects the existence of a ventro-animal most region which is deprived of endodermal cells (Figure 4B). The origin of this exclusion zone remains to be established, but one possibility is that this region expresses cues preventing endodermal cells from colonising it.

Figure S4. Nodal-activated cells converge during the second half of gastrulation, in wild-type and in *casanova* embryos.

(A) In wild-type embryos, Nodal-activated cells converge during the second half of gastrulation, as endogenous endodermal cells do.

(B) In *casanova* mutants, activated cells converge and form endodermal derivatives at 24hpf. However, their migration is less directed and less persistent (64%, p=0.01, n=10 cells from 3 embryos) than in wild-type embryos.

Figure S5. The embryonic environment controls random versus directed migration.

(A) Early (green) Nodal-activated cells were transplanted into late gastrula hosts. Conversely, late (red) activated cells were transplanted into young gastrula hosts.

(B) Representative examples of 45 minutes tracks of the two cell populations.

(C) Mean net displacement towards the dorsal side for each cell population, during 45 minutes (n=16 and n=40 cells). Error bars indicate standard errors.

(D) Plot (square) and curve fit (line) of the MSD of the two cell populations.

Supplemental Movie S1:

Time lapse of an *Et(CLG-YFP)smb602* embryo during gastrulation (from 60% epiboly to 90% epiboly). Lateral view, animal pole is to the top and dorsal to the right. Arrow in first frame indicates the initial position of the margin.

Supplemental Movie S2:

Time lapse of an *Et(CLG-YFP)smb602* embryo at high magnification during early gastrulation. Lateral view, animal pole is to the top and dorsal to the right.

Supplemental Movie S3:

Nodal-activated cells (in green) and Nodal-activated cell injected with MO*casanova* (in red) were transplanted into a wild-type embryo and monitored during gastrulation. Dorsal view, animal pole is to the top. Dashed line delineates the embryo while an arrow indicates the initial position of the margin.

Supplemental Movie S4:

Nodal-activated cells from a mid-blastula embryo (in green) and a late blastula embryo (in red) were transplanted into a late blastula host and monitored during gastrulation. Lateral view, animal pole is to the top and dorsal to the right. Dashed line delineates the embryo while an arrow indicates the initial position of the margin.

Supplemental Movie S5:

Nodal-activated cells (in green) were transplanted into a *casanova* morphant embryo and monitored during gastrulation. Dorsal view, animal pole is to the top. Dashed line delineates the embryo while an arrow indicates the initial position of the margin.

Supplemental Movies S6 and S7:

Cell migration was mathematically simulated to test what cellular movements can account for the observed pattern of endodermal cells at mid-gastrulation. At the beginning of gastrulation, 100 cells were localised at the margin of the blastoderm (50% epiboly). Depending on their position along the dorso-ventral axis, cells were marked in red (dorsal), blue (lateral) or green (ventral). (Movie S6) An oriented motion towards the animal pole cannot account for the pattern observed *in vivo* at 75% epiboly. (Movie S7) In contrast, a random walk efficiently spreads cells over the yolk surface. At 75% epiboly, cells have reached a position similar to the expression pattern of endodermal markers and have partially mixed.