Single-cell internalization during zebrafish gastrulation

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During gastrulation, germ layers are formed as prospective mesodermal and endodermal cells internalize and come to underlie the ectoderm [1–9]. Despite the pivotal role of gastrulation in animal development, the cellular interactions underlying this process are poorly understood. In zebrafish, mesoderm and endoderm formation requires the Nodal signals Cyclops and Squint and their cofactor One-eyed pinhead (Oep) [10–14]. We found that marginal cells in maternal-zygotic *oep* (MZ*oep*) mutants do not internalize during gastrulation and acquire neural and tail fates at the expense of head and trunk mesendoderm. The lack of internalization in MZ*oep* embryos and the cell-autonomous requirement for *oep* in Nodal signaling enabled us to test whether internalization can be achieved by individual cells or whether it depends on interactions within a group of cells. We found that individual MZ*oep* mutant cells transplanted to the margin of wild-type blastula embryos initially internalize with their neighbors but are unable to contribute to the mesendoderm. In the reciprocal experiment, single wild-type cells transplanted to the margin of MZ*oep* mutant embryos autonomously internalize and can express the mesendodermal markers *axial/foxA2* and *sox17*. These results suggest that internalization and mesendoderm formation in zebrafish can be attained autonomously by single cells.

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Results and discussion

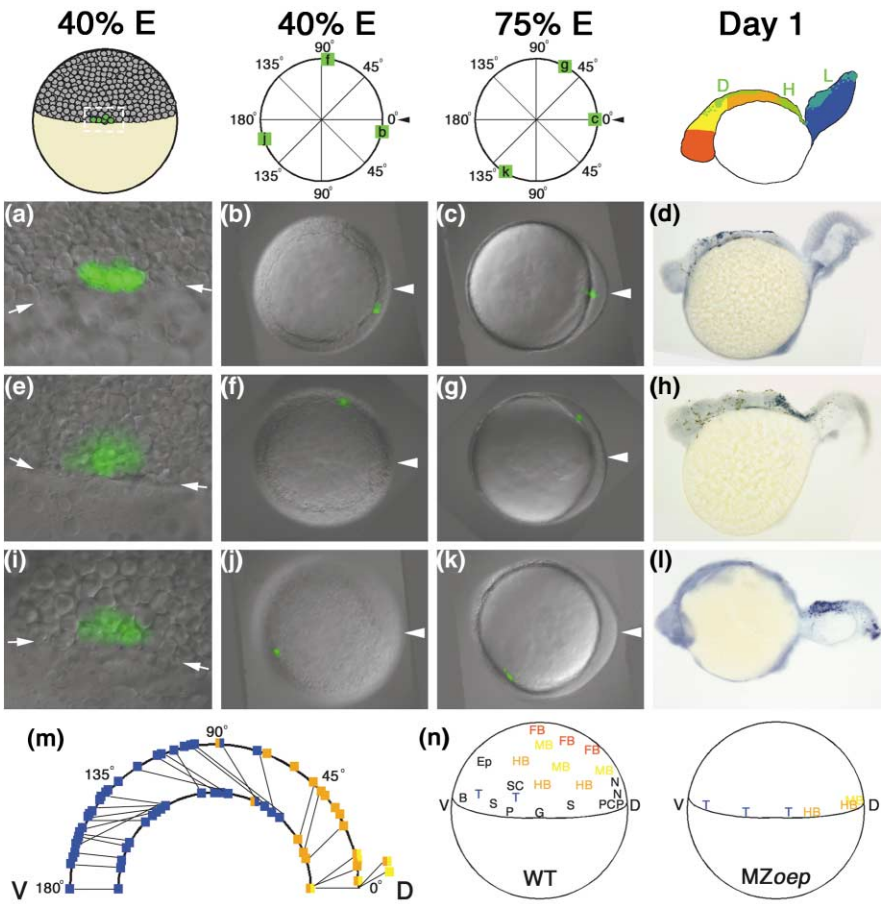
Marginal cells adopt neural and tail fates in MZ*oep* mutant embryos

During zebrafish development, mesoderm and endoderm are derived from cells at the blastula margin that internalize during germ-layer formation [7, 9, 15–17]. In zebrafish, the Nodal-related signals Cyclops (Cyc) and Squint (Sqt) and the EGF-CFC protein Oep are required prior to gastrulation for the specification of mesendodermal progenitors [10–14, 16, 18, 19]. Genetic and biochemical studies indicate that EGF-CFC proteins are an essential part of a receptor complex for Nodal signals [11, 20]. The absence of Nodal signaling in MZ*oep* embryos results in the failure of germ-layer formation and the absence of head and trunk mesendoderm [11]. Through fate map analysis with laser-assisted uncaging of caged fluorescein dextran (CFD), we asked whether, similar to cells in *cyc;sqt* embryos [16], marginal cells in MZ*oep* embryos acquire new fates and fail to internalize (Figure 1). We found that in MZ*oep* mutants, labeled cells remain at the margin and do not internalize between late blastula (40% epiboly) and mid-gastrula stages (70% epiboly; n = 26). Instead of forming axial and paraxial mesoderm and endoderm as in wild-type embryos, dorsal marginal cells in MZ*oep* embryos contribute to the midbrain and hindbrain; lateral and ventral marginal cells contribute to the tail and do not form involuting mesendoderm as in wild-type embryos (n = 40). Moreover, when the fate maps of the midbrain and hindbrain domains in MZ*oep* mutants are compared to those of the wild-type embryo (Figure 1), they are expanded not only toward the vegetal pole to encompass cells of the margin, but also dorsally. The dorsal shift of fate domains in MZ*oep* can be explained by the disruption of organizer activity in Nodal signaling mutants. When the activity of organizer genes is reduced, the embryo becomes ventralized and posteriorized, resulting in fate shifts such as that of the hindbrain and midbrain toward the dorsal side of the embryo [13, 14, 21]. Consistent with this idea, the expression of organizer genes such as *goosecoid* (*gsc*), *noggin*, and *dickkopf1* is reduced in the absence of Nodal signaling [13, 14].

Wild-type cells autonomously express mesendodermal markers in MZ*oep* hosts

Previous studies with zygotic *oep* mutants, which display only a partial loss of *oep* activity, suggested that *oep* acts cell-autonomously [11, 22, 23]. To determine if a cell autonomous response to Nodal signaling is sufficient to initiate mesendoderm formation, we tested whether small groups of cells transplanted from the margin of wild-type embryos to the margin of MZ*oep* host embryos could express the mesendodermal markers *axial/foxA2* and *sox17*. In wild-type embryos these genes are expressed when mesendodermal cells form the hypoblast at 50% epiboly [24, 25]. *Axial/foxA2* is expressed in endodermal and axial mesoderm cells, whereas *sox17* predominantly marks endodermal cells [22, 24, 25]. We found that transplanted wild-type cells autonomously expressed either

Figure 1



Fate mapping of MZ*oep* embryos. The first row is a schematic representation of the data shown in the columns below. The position of uncaged cells is shown in green. In the schematic of a day 1 zebrafish embryo, the forebrain domain is depicted in red, the midbrain in yellow, the hindbrain in orange, and the tail in blue. Morphology and expression of *krox20* and *pax2* determined these domain boundaries. Using laser-assisted uncaging of caged fluorescein dextran (CFD), we labeled groups of 4–8 cells at the margin of MZ*oep* embryos at 40% epiboly (n = 40), the stage before involution occurs in wild-type embryos. Because the dorsal-ventral axis is not apparent in embryos at this stage, we immobilized the embryos and analyzed the position of labeled cells at 75% epiboly, when the dorsal side could be assigned in MZ*oep* embryos (see Supplementary materials and methods). This allowed us to retrospectively assign a dorsal-ventral position to cells labeled at 40% epiboly and construct fate maps of the marginal region in MZ*oep* embryos at both 40% and 75% epiboly. Due to convergence, a dorsal movement of the labeled cells between the two stages is observed. **(a–c,e–g,i–k)** Pseudocolored fluorescence/DIC overlays showing cells containing uncaged fluorescein dextran in green. (a,e,i) Frontal views of labeled marginal cells at 40% epiboly. White arrows indicate the margin. (b,f,j) View through the vegetal pole of embryos at 40% epiboly. White arrowheads indicate the dorsal side. (c,g,k) View through the vegetal pole at 75% epiboly. White arrowheads indicate the dorsal side. **(d,h,l)** Day 1 embryos. Labeled cells were detected with anti-fluorescein antibody coupled to alkaline phosphatase. (a–d) A group of marginal cells labeled (b) 12° from the dorsal side at 40% epiboly has converged to (c) 0° from the dorsal side at 75% epiboly. (d) The labeled cells give rise to the midbrain and hindbrain on day 1. (e–h) A group of marginal cells labeled (f) 88° from the dorsal side at 40% epiboly has converged to (g) 64° from the dorsal side at 75% epiboly. (h) The labeled cells contribute to the hindbrain at day 1. (i–l) Labeled cells (j) 159° from the dorsal side have converged to (k) 125° from the dorsal side at 75% epiboly. (l) The labeled cells contribute to the tail. **(m)** Fate map summary of marginal cells in MZ*oep* embryos. The outer arc represents the margin at 40% epiboly. The inner arc represents the margin at 75% epiboly. Data points from both sides of the embryos are represented. Dorsal is to the right, and ventral is to the left. Each box represents the position of labeled cells in an individual embryo. Lines connect the positions of labeled cells at the two time points. For some embryos, the position of labeled cells was only obtained at the earlier time point. The color of the boxes indicates the fate of the labeled cells at day 1 of development and corresponds to the diagram of the MZ*oep* embryo above. **(n)** Schematic representation of the gastrula fate map in wild-type and MZ*oep* embryos. Dorsal is to the right and ventral to the left; animal is up and vegetal down. The wild-type fate map is based on previous reports [15, 17, 18, 27]. The MZ*oep* fate map is based on the data represented in (m). Abbreviations are as follows: (FB) forebrain; (MB) midbrain; (HB) hindbrain; (N) notochord; (PCP) prechordal plate; (S) somite; (G) gut; (P) pronephros; (T) tail; (SC) spinal chord; (B) blood; and (Ep) epidermis.

axial/foxA2 in 92% (n = 24) or *sox17* in 88% (n = 8) of MZ*oep* host embryos (see Figure S1 in the Supplementary material available with this article on the internet). Mutant cells were not recruited by the transplanted wild-type cells to express these markers. These results demonstrate that *oep* acts autonomously in the specification of mesendodermal progenitors.

Rationale for single-cell transplantation experiments

Embryological studies have described two major types of movements associated with the internalization of mesoderm and endoderm progenitors [1–4]. In *Drosophila* and *Xenopus*, mesoderm and endoderm appear to internalize by involution, the movement of cells as a cohesive sheet. In contrast, in chicks and mice, mesendoderm cells are thought to internalize by ingression, the delamination of individual cells as they undergo an epithelial-to-mesenchymal transition. In zebrafish, three major steps of germ-layer formation can be distinguished [7, 9]; first, cells move toward the margin; second, cells internalize to form a marginal group of deep cells; and third, the deep cells contribute to the hypoblast (the mesendodermal germ

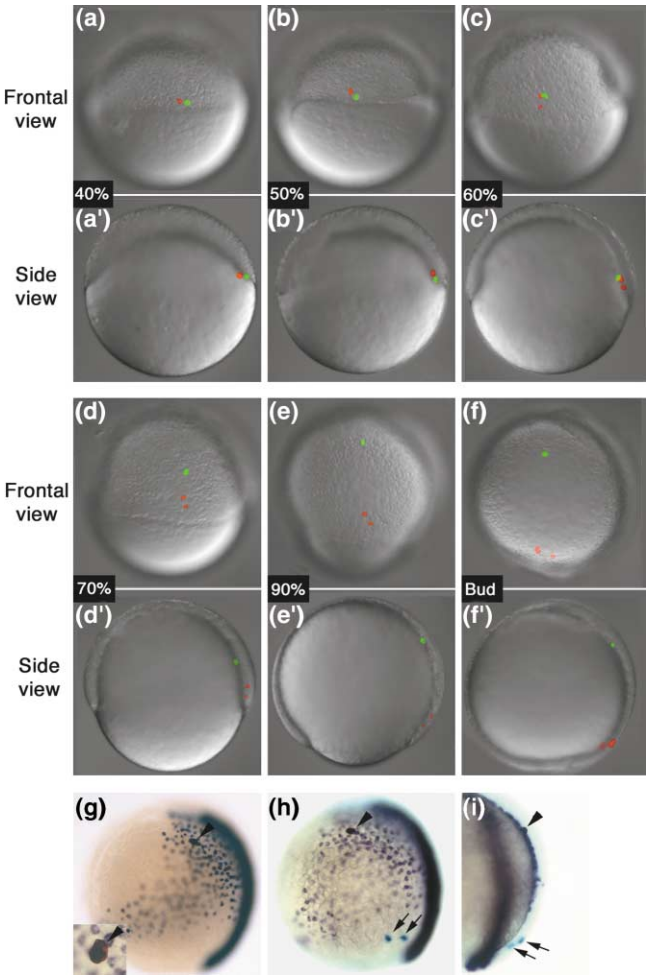
layer), which underlies the epiblast (the ectodermal germ layer). Since marginal cells appear to undergo a coordinated movement to form the hypoblast, zebrafish germ-layer formation has generally been considered to be mediated by a process resembling involution [7, 9]. However, it has been suggested that ingression movements also contribute to the internalization of mesendodermal cells in zebrafish [26].

Despite these extensive descriptive studies, it is unclear if internalization requires the coordinated movement of a group of cells or if it can be achieved through the autonomous action of individual cells. In order to distinguish between community-dependent or cell-intrinsic mechanisms driving morphogenetic movements, one must analyze the behavior of single cells. The lack of internalization in MZ*oep* mutant embryos and the cell-autonomous requirement for *oep* enabled us to test whether internalization during gastrulation can be achieved by single cells or whether a group of cells is required. We reasoned that transplanting a single MZ*oep* mutant cell into a wild-type embryo would test if a group of internalizing wild-type cells could carry the mutant cell inside to contribute to the hypoblast. In the converse experiment, we expected that transplanting a single wild-type cell into a MZ*oep* mutant embryo would test if a single cell is capable of autonomous internalization and mesendoderm formation.

Single MZ*oep* cells can internalize but do not contribute to the hypoblast in wild-type hosts

We first asked if single marginal MZ*oep* mutant cells transplanted to the margin of wild-type embryos could be internalized along with movements of their wild-type neighbors and then contribute to the hypoblast. Single MZ*oep* cells, labeled with rhodamine-biotin-dextran, and single wild-type cells, labeled with fluorescein-dextran, were cotransplanted to the margins of wild-type host embryos at mid-blastula (sphere) stage and then followed through gastrulation (n = 21; Figures 2 and S2). Like their wild-type host neighbors, transplanted wild-type cells were able to internalize, contribute to the hypoblast, move toward the animal pole and express *axial/foxA2* (Figures 2 and S2 and Table S1). The *axial/foxA2*-expressing wild-type cells were always found abutting the yolk (Figure 2i and data not shown). When a MZ*oep* cell was transplanted superficially into the marginal region, the cell moved to the margin and internalized to contribute to the group of marginal deep cells. However, unlike their wild-type neighbors, mutant cells did not move toward the animal pole or contribute to the hypoblast (see Figure S2 in the Supplementary material). Instead, the MZ*oep* mutant cells moved more superficially and proceeded toward the vegetal pole with the vegetal movement of the margin during epiboly (Figure S2c–d). Even when an individual MZ*oep* cell was transplanted deep in the blastoderm and positioned close to the yolk in the region of

Figure 2



A single MZ*oep* cell does not contribute to mesendoderm in a wild-type embryo. A single wild-type host embryo is shown in all panels. The animal pole is up. **(a–f,a′–f′)** Fluorescence overlays of the host embryo at the noted stages. The top panels, (a–f), show a frontal view of the transplanted cells, while in the bottom panels, (a′–f′), the embryo is oriented so that the cells are viewed from the side and dorsal is to the right. The wild-type donor cell is marked in green; the MZ*oep* donor cell and its daughters are marked in red. (a–f) The wild-type cell moves toward the animal pole, while the MZ*oep* cells move toward the vegetal pole with the progression of the margin. (a′) Both the MZ*oep* and the wild-type transplanted cells are located at the margin, and the wild-type cell is more superficial than the MZ*oep* transplanted cell. (b′) The wild-type cell begins to move deeper, while the MZ*oep* cell remains approximately at the same depth. (c′–f′) The wild-type cell is adjacent to the yolk and moves toward the animal pole. The MZ*oep* cells move toward the vegetal pole with the progression of the margin during epiboly. **(g)** *axial/foxA2* expression in the wild-type host embryo. Immunostaining for the lineage tracer marks the transplanted wild-type cell in brown. The black arrowhead is pointing to the transplanted wild-type cell. The inset shows a close-up of the wild-type cell. The wild-type cell is also expressing *axial/foxA2*. **(h)** The same view as in (g). The lineage tracer marks the transplanted MZ*oep* cells in light blue. Black arrows point to MZ*oep* cells. (i) Side view showing that the *axial/foxA2* expressing cells, including the wild-type transplanted cell indicated by the black arrowhead, are located adjacent to the yolk. The MZ*oep* cells, indicated by black arrows, do not contact the yolk or express *axial/foxA2*.

the nascent hypoblast, the MZ*oep* cell was unable to contribute to the hypoblast (Figure 2). The MZ*oep* cell remained in approximately the same position until 70% epiboly, when the MZ*oep* cells moved away from the yolk to a more superficial position (Figure 2c′–f′). Similar results were obtained when a group of MZ*oep* cells was transplanted and followed (data not shown). Moreover, the transplanted MZ*oep* cells never expressed *axial/foxA2* (Figure 2 and Table S1). These results indicate that while *oep* mutant cells are able to contribute to the group of deep cells at the margin, they are unable to contribute to the hypoblast or to move toward the animal pole. This suggests that community-dependent mechanisms can contribute to internalization but are not sufficient for hypoblast and mesendoderm formation.

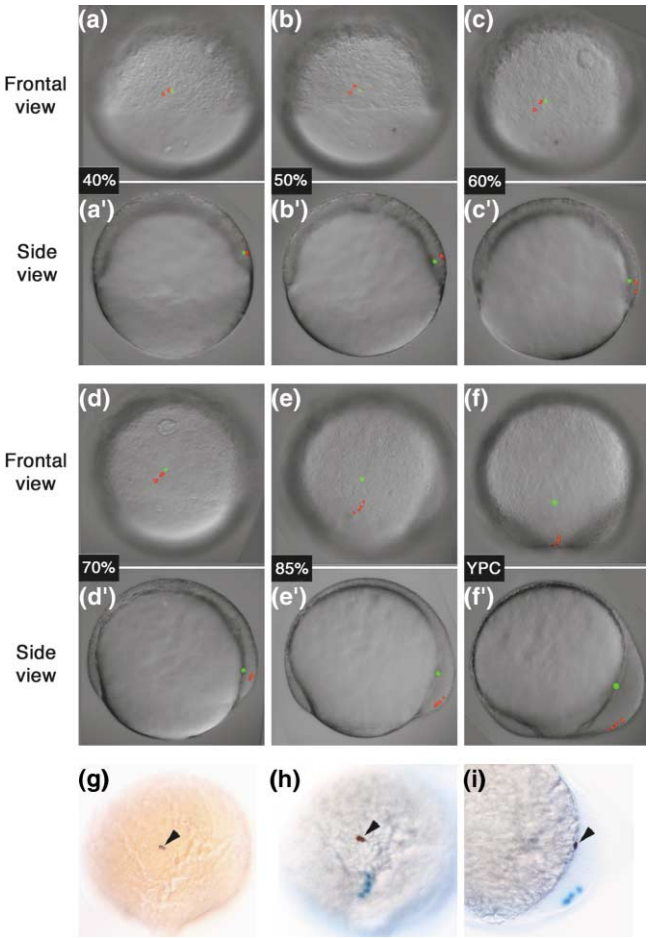
Single wild-type cells can internalize and form mesendoderm in MZ*oep* mutant hosts

In the converse experiment, we asked whether a single marginal wild-type cell could internalize in a MZ*oep* mutant, in which marginal cells do not internalize. Single wild-type cells, labeled with fluorescein-dextran, and single MZ*oep* cells, labeled with rhodamine-biotin-dextran, were taken from the margin of donor embryos at the sphere stage, transplanted into the margin of MZ*oep* host embryos, and followed through gastrulation (Figures 3 and S3). The transplanted MZ*oep* cells moved toward the vegetal pole with the advancement of the margin and did not internalize or express *axial/foxA2* (Figures 3 and S3; Table S1). By contrast, the individual wild-type cells autonomously internalized and came to lie adjacent to the yolk (n = 16 out of 19 transplanted cells; Figures 3 and S3). Interestingly, in MZ*oep* embryos the wild-type cells internalized by directly moving deep toward the yolk without first moving to the most vegetal region of the margin, as in wild-type embryos (Figures 3 and S3). The transplanted wild-type cells juxtaposed to the yolk can express *axial/foxA2* (Figure 3g–i and Table S1), indicating that they can develop into endoderm and/or axial mesoderm despite the failure of MZ*oep* mutant cells to do so. These results indicate that an individual cell is capable of autonomously internalizing to form mesendoderm during gastrulation.

Conclusions

Our results suggest that both single-cell and community-based mechanisms contribute to germ-layer formation. Single wild-type cells can internalize in the region of the blastula margin in MZ*oep* mutants, and this observation supports autonomous ingression models of internalization (Figures 3 and S3). Moreover, these cells express markers for a subset of mesendodermal cells. Interestingly, wild-type cells transplanted into MZ*oep* mutant embryos move directly across the blastoderm to a position adjacent to the yolk, without first moving toward the vegetal pole around the margin, as in wild-type embryos. This observa-

Figure 3



A single wild-type cell internalizes and forms mesendoderm in a MZ*oep* mutant embryo. **(a–i)** The same MZ*oep* host embryo is shown in all panels. The animal pole is up. **(a–f,a′–f′)** Fluorescence overlays of the host embryo at noted stages. The top panels, (a–f), show a frontal view of the transplanted cells, while in the bottom panels, (a′–f′), the embryo is oriented so that the cells are viewed from the side and dorsal is to the right. The wild-type donor cell is marked in green; the MZ*oep* donor cells and their daughters are marked in red. (a–f) The wild-type cell remains in approximately the same animal-vegetal position, while the MZ*oep* cells move toward the vegetal pole with the progression of the margin. (a′) Both the MZ*oep* and wild-type transplanted cells are located superficially. (b′) The wild-type cell begins to move deeper, while the MZ*oep* cells remain superficial. (c′–f′) The wild-type cell is adjacent to the yolk, while the MZ*oep* cells are more superficial. (f′) Note that in MZ*oep* embryos the forebrain forms more toward the vegetal pole than in wild-type embryos [11] and that the wild-type transplanted cell comes to underlie the prospective forebrain even though its animal-vegetal position does not change. **(g,h,i)** The black arrowhead is pointing to the *axial/foxA2*-expressing cell. (g) Frontal view of *axial/foxA2* expression; the embryo is positioned the same as in (f). (h) Immunostaining of the lineage tracers for the transplanted cells. The wild-type transplanted cell is brown and overlaps with *axial/foxA2* expression. The transplanted MZ*oep* cells are light blue. The embryo is positioned the same as in (f) and (g). (i) Side view. Dorsal is to the right, the same as in (f′). The *axial/foxA2*-expressing wild-type cell is adjacent to the yolk, while the blue MZ*oep* cells are more superficial.

tion suggests that in wild-type embryos cell-cell interactions prevent cells from internalizing directly through the blastoderm. Further supporting cell-cell interaction models of internalization is the finding that single *oep* mutant cells can be carried inside with their wild-type neighbors. Germ-layer formation could thus be viewed as the locally coordinated but autonomous ingression of single cells. This view may reconcile seemingly conflicting descriptions of gastrulation movements in fish. In Fundulus, cells in the region of the margin appear to ingress to form the hypoblast [8], consistent with single-cell internalization. In zebrafish, marginal cells internalize locally in a movement interpreted as involution [7], but it has also been proposed that ingression contributes to internalization [26]. Our observations suggest that the localized ingression of individual cells at the margin could generate the involution-like movement observed at the onset of gastrulation. Involution would thus simply be the consequence of the coordinated ingression movements of single cells.

Supplementary material

Three supplementary figures, a table, and materials and methods are available with the electronic version of this article at http://images.cellpress.com/supmat/supmatin.htm.

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