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Global cell sorting is mediated by local cell–cell interactions in the *C. elegans* embryo

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Abstract

The *Caenorhabditis elegans* embryo achieves pattern formation by sorting cells into coherent regions before morphogenesis is initiated. The sorting of cells is coupled to their fate. Cells move extensively relative to each other to reach their correct position in the body plan. Analyzing the mechanism of cell sorting in in vitro culture experiments using 4D microscopy, we show that all AB-derived cells sort only according to their local neighbors, and that all cells are able to communicate with each other. The directions of cell movement do not depend on a cellular polarity but only on local cell–cell interactions; in experimental situations, this allows even the reversal of the polarity of whole regions of the embryo. The work defines a new mechanism of pattern formation we call "cell focusing".

Keywords: Cell–cell interactions; Cell movements; Pattern formation; Cell sorting; 4D microscopy; Cell polarity

Introduction

During embryogenesis, cells have to find their correct positions as they build patterns. Sorting of cells contributes to this patterning. Cell sorting processes have been the subject of intense research since Townes and Holtfreter first described aggregation experiments with amphibian neurula cells in 1955. Since then, many different models explaining cell sorting have been proposed. The most common hypothesis, the differential adhesion hypothesis (Steinberg, 1963), proposes that cells sort only because of their specific adhesive properties. This mechanism was, indeed, observed in vitro and in vivo (Cortes et al., 2003; Duguay et al., 2003; Godt and Tepass, 1998; Gonzalez-Reyes and St Johnston, 1998; Kostetskii et al., 2001). Another mechanism to guide cell sorting is based on active signaling events between cells as shown, for example, for the Ephrin/Eph-receptor system (reviewed in Xu et al., 2000). All analyses performed so far refer to sorting processes which were either limited to a specific part of the body or included only a few different cell types in cell culture experiments. Therefore, the process analyzed here represents the first example of cell sorting in the entire embryo.

We have described a global cell sorting process in the *Caenorhabditis elegans* embryo which guides cells to their terminal position at the premorphogenetic stage (Fig. 1A; Schnabel et al., 1997, 2006). Cell movements take the main part in this patterning process while the mitoses do rather little (Schnabel et al., 2006). Movements start after the initial specification of founder cells and bring the cells to their terminal positions at the premorphogenetic stage. At this stage, the descendants of the 12-cell stage embryo form coherent regions which do not mix (Schnabel et al., 1997). Alteration of the cell fates of the 8-AB-derived blastomeres present at the 12-cell stage results in a positioning of descendants according to their new fate, suggesting that the fate of a cell determines its terminal position in the embryo (Fig. 1B; Schnabel et al., 2006).

We proposed the "cell focusing" hypothesis (Schnabel et al., 2006) to explain the extensive cell sorting. According to this hypothesis, cells autonomously generate a positional value on their surface. As a result of comparing these values, cells move relative to each other until they find their "correct"

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position as defined by their neighbors at the premorphogenetic stage (Fig. 1C).

The "cell focusing" hypothesis raises testable predictions. Firstly, sorting should occur only locally since cells are provided with positional information by only their neighbors. Secondly, cells should be able to sort out independently of their position in the embryo and independently of the fate of neighboring cells. Previous experiments did not solve the question whether cells use their polarity to move in a certain direction or whether cells obtain directional information only from their local environment (Schnabel et al., 2006). In the latter case, cells might become trapped in a locally correct but globally wrong place. We refer to this situation as a "local minimum" (Fig. 1D). In contrast, cells which use a general polarity to sort should overcome this trapping by using this additional information (i.e., the polarity) to identify a right neighbor lying on the wrong side and to move accordingly (Fig. 1E). Therefore, the formation of "local minima" is inconsistent with an involvement of a general cell polarity in the cell sorting process.

The phenomenon of cellular polarity is studied extensively in *C. elegans* (reviewed in Gotta and Ahringer, 2001; Labbe and Goldstein, 2002; Lyczak et al., 2002; Pellettieri and Seydoux, 2002). Cells generally divide in anterior–posterior (a–p) direction and cell fates are specified according to the cleavage direction (Sulston et al., 1983). However, it is not known to which extent this cellular polarity contributes to pattern formation.

We removed cells from the eggshell and combined blastomeres in vitro. These manipulations alter the cell fates and the neighborhoods of cells—this challenges cells to sort in environments they normally never face. Analyzing the manipulated embryos by 4D microscopy, we show that cells sort locally "correctly" according to their fate, only relying on the local neighborhood. We show that the direction of cell movement does not depend on a cellular polarity but only on local cell–cell interactions in the embryo.

Materials and methods

Nematode strains and culture

Methods for culturing and handling of worms have been described elsewhere (Brenner, 1974). The following mutant alleles and strains were used: N2 Bristol (Brenner, 1974) and *glp-1 (e2144)* LG III (Priess et al., 1987).

In vitro culture of embryos and blastomeres

Preparation of embryos was carried out in a humidity chamber with 99% relative humidity at 25°C. Eggshell and vitelline membrane were removed according to Edgar (1995). Embryos and blastomeres were handled and dissociated using a drawn-out capillary needle (Biomedical Instruments, Zöllnitz, Germany) (Edgar, 1995). P_2 blastomeres were obtained by sequentially dividing AB and P_1 and then EMS and P_2. Cells were cultured in embryonic growth medium (EGM) supplemented with egg yolk (Edgar, 1995). To culture embryos under the 4D microscope, two different techniques were used. Cells were either put in a small hole in a 1% agarose pad on a microscope slide made by removing Sephadex beads (G50 super fine, Amersham Pharmacia) with an eyelash or cells were placed on a microscope slide equipped with spacers out of two layers of cling film. Microscope slides and cover slips were coated with 3.6 mg/ml Poly(2-Hydroxyethylmethacrylate) (Sigma) in 95% ethanol to prevent adhesion of cells to surfaces. Cover slips (24 × 60 mm, 1 mm) were sealed with pure white Vaseline to avoid evaporation.

Micromanipulation of embryos

P_1 was removed by sucking it with the mouth pipette through a hole in the eggshell, made by a thin glass needle (Gendreau et al., 1994). The manipulation was performed in a drop of EGM covered with mineral oil to avoid evaporation under an inverted microscope.

4D microscopy

The methods for 4D microscopy were described previously (Hutter and Schnabel, 1994; Schnabel et al., 1997). Modifications of the 4D microscope system are described in (Schnabel et al., 2006). Embryos were recorded at 25°C.

Lineage analysis

The 4D recordings were analyzed using the database SIMI©Biocell (Schnabel et al., 1997; http://www.simi.com). By following every cell in the recording, the 3D coordinates of the cells can be assigned to the cell lineage. Thus, data of cell descent, cell position, cell cleavage, and cell morphology (cell fate) are collected. These data can be used to generate 3D representations of all nuclear positions at any given time point of development and, thus, 3D movies. In this study, cell sorting is visualized by such movies as well as by showing the "starting point" of our analysis (the 12-cell stage with 8-AB blastomeres) and the "end point" of this process (the premorphogenetic stage with 256-AB blastomeres). Although we analyzed most embryos up to the premorphogenetic stage, cell positions of earlier generations are shown in some figures since 3D representations of the premorphogenetic stage tend to be confusing due to the large number of cells.

Results

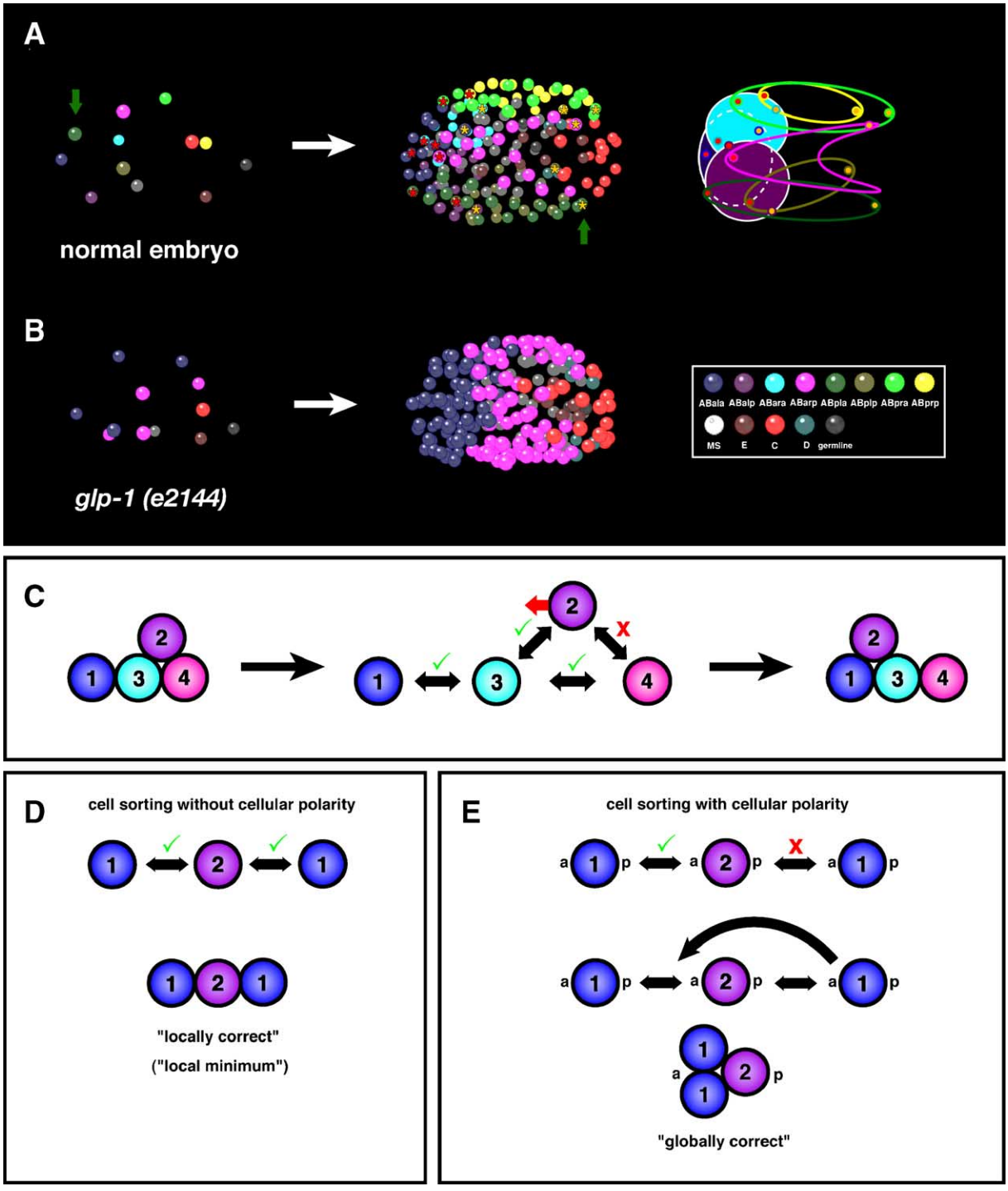
General polarity of the lineage

All conclusions of the manuscript are based on lineage analyses with the 4D microscope system. This tool enables the identification of the fates of the descendants of the 8-AB blastomeres present at the 12-cell stage by prominent features of the corresponding lineages (Hutter and Schnabel, 1994; Schnabel et al., 1997). Such an analysis is exemplified here, using the ABala- and ABarp-derived fates which are the most common fates in this study. ABala is the only AB-derived lineage producing four cell deaths in characteristic positions after the ninth cleavage. In contrast, ABarp produces only one early cell death in ABarpaaapp and 22 major hypodermal cells which can be distinguished by their size and morphology from the precursors of other cell types (Sulston et al., 1983). These features can be used to determine cell fates in cultured embryonic fragments which normally cease development after the first round of cell deaths and after the differentiation of the major hypodermis. In normal embryos with eggshells, the assignment of the a–p polarity after a cleavage—an important step in reconstructing the cell lineage—is never a problem since in a normal embryonic context the anterior daughter cell also executes the anterior cell fate. However, during the analysis of embryonic fragments, for example, of individual AB blastomeres, it is unknown whether the fragment has a "polarity" and,

if so, which is the anterior and which is the posterior daughter cell. Therefore, we generally "lineaged" all descendants until after the ninth cleavage to score cell deaths and hypodermal cells. Then, by using these cell fates as "landmarks", we reconstructed the original a–p polarity of the cultivated blastomere and, if necessary, swapped the preliminary a–p assignments of the cell fates after mitosis. Since we could always reconstruct the lineages, we conclude that an isolated AB blastomere has the autonomous capacity to produce the normal lineage pattern—however, in the absence of a general polarity, fate assignments may occasionally be reversed. During normal embryogenesis, this polarity of the lineage coincides with the general polarity of the embryo.

4D analysis of cell sorting

Since the lineage analysis of embryos using SIMI©Biocell enables the evaluation of cell fates at the level of single cells, their sorting can be analyzed in detail. Comparing different wild-type embryos shows that cells move to a specific position at the



premorphogenetic stage within little variation of neighborhood and form coherent regions in the embryo (Schnabel et al., 1997). Thus, the positioning of cells in manipulated embryos can be evaluated by comparing the shape of the formed regions and the neighborhoods of the terminal position of cells with the wild-type situation. For example, the ABala-derived region is positioned anteriorly in the embryo and the ABarp- and ABp-derived regions elongate in a–p direction (Fig. 1A). Furthermore, cells which exist several times in the embryo due to cell fate transformations sort into positions which lie in close proximity (Fig. 1B; Schnabel et al., 2006). These properties can be used to evaluate cell sorting—cells are considered to be "correctly" sorted when their terminal position resembles their position in a wild-type situation or when they are in close neighborhood to cells of the same fate. This is contrary to situations in which cells did not move (sort) at all or in which cells moved into a neighborhood which does not resemble the normal neighborhood.

In vitro combination of embryos and early blastomeres

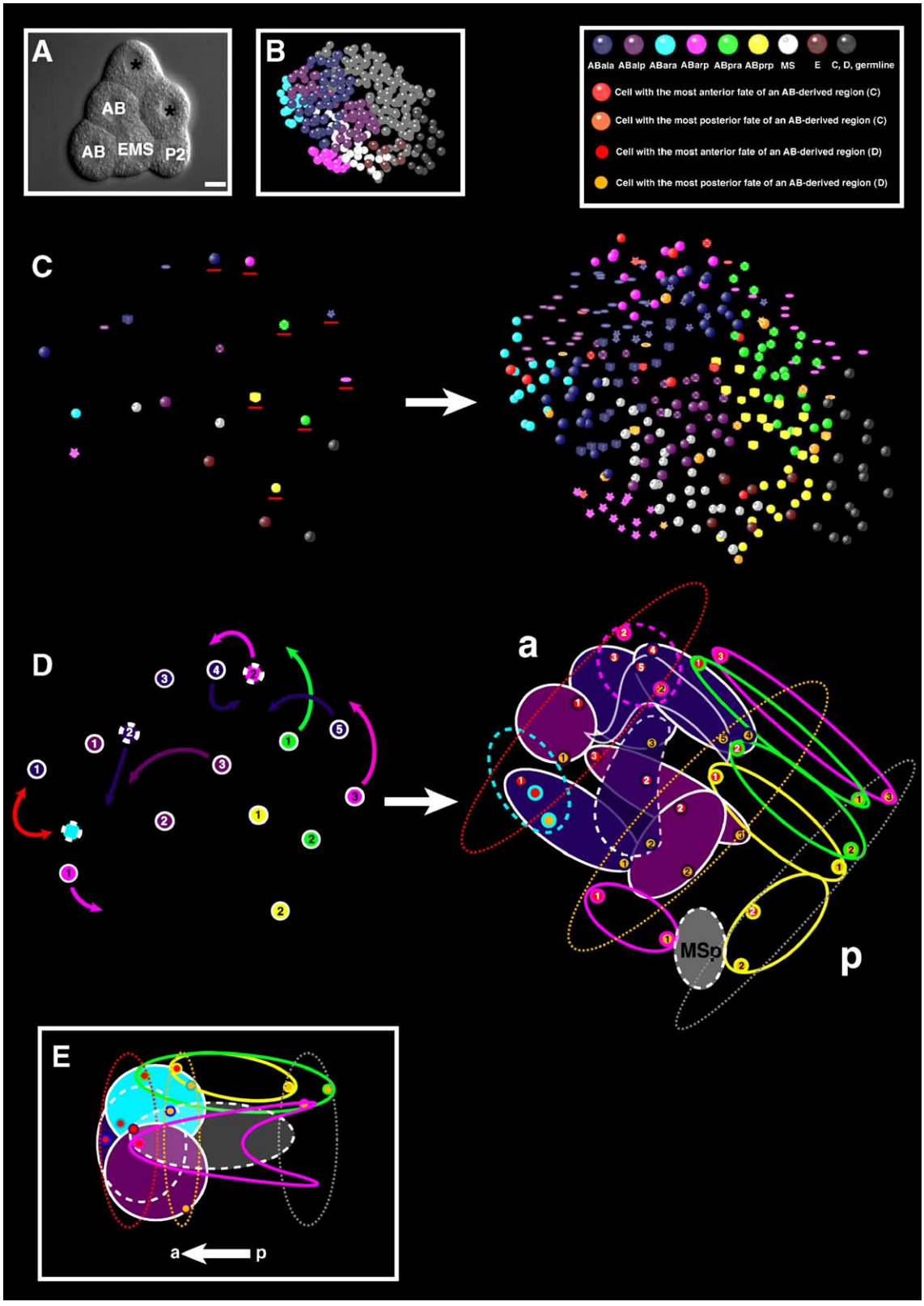
A stringent way of testing the "cell focusing" hypothesis is to combine blastomeres in vitro, placing cells in neighborhoods they normally never face. We combined two complete early embryos (12 to 24 cells). This does not result in a normal "embryo" with double the number of cells, which would indicate a global sorting mechanism, but cells intermingle only locally where the embryos touch (data not shown). This indicates that cell sorting occurs only locally.

To try to force a stronger interaction, we combined a 4-cell stage embryo and an isolated AB blastomere. Indeed, a stronger interaction of the two parts can be observed which also alters the early cell–cell contacts considerably. For example, P_2 of the whole embryo now contacts the descendants of the added AB blastomere and not its own AB blastomere (Fig. 2A), and this changes the induction of cell fates (Hutter and Schnabel, 1994). The descendants of the additional AB blastomere are now influenced by an entire embryonic field derived from the 4-cell embryo (Fig. 2; $n = 3$). Cells of both parts move significantly to reach their terminal position. Cells of the two times AB-derived regions tend to stay together, even though the regions move relative to each other and, within the regions, cells rearrange as well. If cells of the same regional identity meet, they may intermingle and align, corresponding to their fates (Figs. 2D, S1D; Movie S1, Supplementary material; Schnabel et al., 2006). The analysis of neighborhoods at the terminal stage shows that the arrangement of cells partly resembles the normal situation, and that cells with the same fate lie in close proximity as observed earlier (Schnabel et al., 2006). The global pattern, however, is aberrant.

How cells move to optimize their neighborhood can be seen in Fig. 2D. For example, in the anterior left part of the "embryo", the blastomeres ABala #1, ABalp #1, ABara, and ABarp #1 reposition to establish the same neighborhoods as in normal development. During development, descendants of ABala #1 move as in wild-type embryos slightly posteriorly but then come under the influence of ABala #2-derived cells which leads to a posterior displacement of the region—this does not take place in normal development. During these first cleavages, the descendants of ABara gain contact to ABalp #1 descendants and move together anteriorly (Movie S1, Supplementary material) to optimize the contact between an ABalp- and an ABara-derived region (Fig. 2D). At the same time, ABarp #1 moves posteriorly and, thus, avoids contact with ABala #1 descendants. The anterior part of ABala #1 touches ABara as in normal development (Schnabel et al., 2006). The described cell movements lead to a configuration in which the local pattern corresponds to the arrangement in normal embryos, however, the global pattern is not "correct". Our analysis suggests that most cells sort "correctly" according to their fate, although they may be trapped in "local minima"—like the descendants of ABarp #2 which are finally trapped in an anterior environment (consisting of descendants of ABala #3, #4, #5, and the anterior descendants of ABpra #1) that prevents their movement and elongation in the posterior direction (see also Fig. S1A, Supplementary material). In contrast, ABarp #3 elongates in the anterior

Fig. 1. Cell movements and the "cell focusing" hypothesis. (A) 3D representation of a wild-type embryo at the 8-AB cell and the 128-AB cell stages (see Schnabel et al., 1997 and the accompanying paper (Schnabel et al., 2006) for a detailed analysis). Anterior is to the left. Colors in the spheres in the 3D representations refer to cell fates as shown on the right side of panel B. The green arrows point at ABpla (left) and its most posterior descendants (right) to indicate the long distance the cell and its precursors moved during development. The right scheme illustrates the form and relative arrangement of the AB-derived regions in the 128-AB cell stage. Each AB-derived region is represented by an ellipsoid structure; the colors refer to cell fates as shown in panel B. The position of the most anterior cell (ABxxxaaaa) and the most posterior cell (ABxxxpppp) of a region is indicated by red and orange spheres, respectively. The color of their margins represent their origin at the 8-AB cell stage (ABxxx fate); the numbers indicate to which region the corresponding cell belongs if there is more than one region of the same fate. The positions of the cells are adopted from the original data (indicated by the red and orange asterisks in the 3D representation). To make the scheme more clear, the ABp ellipsoids are not filled. Schemes using the same abstractions are used in Figs. 2, 3, 5, S1 to help the interpretation of embryos and fragments. (B) 3D representation of a *glp-1 (e2144)* embryo in the 8-AB cell and the 128-AB cell stage (see accompanying paper (Schnabel et al., 2006) for a detailed analysis). Only ABala- and ABarp-derived cell fates are present. The ABala-derived regions stay anteriorly, and the ABarp-derived regions elongate in a–p direction. This shows that cells sort according to their fate. Cells with the same fate position in similar positions in the embryo (Schnabel et al., 2006). (C) Cartoon illustrating the "cell focusing" hypothesis (Schnabel et al., 2006). Cells in a "wrong" constellation (left) compare their positional values—autonomously generated according to their fate (here indicated by the numbers; middle)—and move relative to each other (red arrow). Cells move until they find their "correct" position defined by proper neighbors (right). (D, E) Cartoons illustrating two possible ways of how cells locally compare their positional values with that of their neighbors (read from top to bottom). In panel D, cells do not use a cellular polarity for cell sorting, whereas in panel E, cells use such a polarity. (D) Looking at their neighbors, cells become trapped in a locally correct but globally wrong place since they miss information about the "more correct" position. A cell with a "lower" positional value should only be located on the anterior of the more posterior cell. We refer to this situation as a "local minimum". (E) Cells which use a cellular polarity can overcome this trapping by using this additional information of polarity to identify a right neighbor lying on the wrong side (red cross) and to move accordingly (black arrow). A "globally correct" pattern forms.

direction by active movements because the neighboring ABpra-derived cells provide the full range of a–p information (Fig. 2D and also Fig. S1D, Supplementary material). This indicates that cells only navigate according to their direct neighborhood and have no far-ranging global polarity information. It also shows that the different shapes of region ABarp #2—which is almost circular—and region ABarp #3—which is nicely elongated—depend on their different local



environments. Another example of "correct" cell sorting is provided by the interactions of the five ABala-derived regions. Many descendants of the five ABala blastomeres move extensively and arrange in such a way that they either touch their normal neighbors (although derived from different mothers) or cells of identical fate (Fig. S1C, Supplementary material). For other examples of "correct" cell sorting see Fig. S1 (Supplementary material).

This experiment is consistent with the "cell focusing" hypothesis and demonstrates that cells do not stay put in completely abnormal neighborhood situations. They rather adapt to the local situation in different parts of the embryo by moving to a terminal position which corresponds to their locally "correct" position in a normal embryo. However, the global topology of the embryo is significantly altered.

Isolation of AB by removal of P_1 and in vitro culture of AB

The behavior of a large number of cells with many different fates appears to be too complex to deduce the rules by which cells sort. Therefore, we followed the opposite strategy and analyzed the behavior of isolated AB-derived blastomeres in vitro. As a control for the complete isolation of AB in vitro, we first completely removed P_1 from the eggshell by microsurgery. Both manipulations cause embryos to execute only ABa-derived fates (ABala, ABarp).

The removal of P_1 ($n = 5$) as well as the in vitro culture of AB ($n = 14$) leads to different phenotypes compared to non-manipulated *glp-1 (e2144)* embryos (Schnabel et al., 2006) which also only execute ABa-derived fates (Hutter and Schnabel, 1994). In *glp-1 (e2144)* embryos, ABala- and ABarp-derived cells sort out along the a–p axis, generating a pattern in which ABala-derived cells are positioned anteriorly and the ABarp-derived regions elongate in a–p direction (Fig. 1B; Schnabel et al., 2006). In both AB isolation experiments, however, the ABarp-derived regions do not elongate, and ABala-derived regions are not confined to the anterior part of the embryonic fragment (Fig. 3). The terminal patterns are variable and appear to correlate with the starting conformation at the 12-cell stage. Although cells move less than in normal embryos, they are not only placed by mitoses but still move actively to their terminal positions (Movie S2, Supplementary material). A detailed analysis shows that many cells of similar fate are placed in a close neighborhood, demonstrating that cells sort in "correct" positions. In isolation, the AB-derived blastomeres show a new cleavage pattern. After each cleavage, the direction of mitoses rotates by approximately 90° (Fig. 5C), whereas in normal embryos cells divide mainly in the a–p axis (Sulston et al., 1983; Schnabel et al., 2006).

The P_1 removal experiment shown in Fig. 3A provides an extreme example for globally abnormal but locally "correct" cell sorting. All ABala-derived regions spread out radially from the center of the embryo. This experiment suggests that there is no global polarity in the AB blastomere, but that polarity is established locally depending on the neighborhood relations of the cells.

In summary, the absence of P_1 impairs pattern formation, implying that P_1 or its descendants play a role in this process. However, the experiments show that cells sort "correctly" on a local scale. This suggests that P_1 is not essential for cell sorting per se, but that it influences cell sorting indirectly.

Combination of two AB blastomeres in vitro

The altered sorting behavior of cells observed in embryonic fragments derived from isolated AB blastomeres could be caused, among other things, by the low number of cells present. In the checkered pattern of cells with ABala- and ABarp-derived fates occurring in the early development of these

Fig. 2. In vitro combination of an early embryo and an AB blastomere. Colors in the 3D representations refer to cell fates as shown in the legend. Scale bar: 10 μm. (A) DIC image shortly after combination. Two AB daughters were added to a 4-cell stage embryo. The cells of the whole embryo are named, the added AB daughters are marked with asterisks. (B) 3D representation of the embryo with two times 128-AB descendants. The descendants of the added AB blastomere are colored in grey. A single a–p axis is formed and the cells of the added AB blastomere join the whole embryo. (C) 3D representations of the "embryo" with two times 8-AB (left)- and two times 128-AB-derived blastomeres (right). The eight descendants of the added AB blastomere are underlined with red bars. The composition of cell fates is altered compared to two normal 8-AB cell stages. Since the left–right inducing cells (MSp and ABalap) are not present or do not touch the ABp descendants at the right time, two ABpra- and two ABprp-derived regions but not the left bilateral homologs are present (Hutter and Schnabel, 1995b). In the two times 128-AB cell stage, the most anterior (ABxxxaaaa) and the most posterior cell (ABxxxpppp) of a region are colored in red and orange, respectively. To allow a distinction between the different regions with the same cell fate, the spheres of the 3D representation are replaced by five different geometrical structures. The formed pattern is very complex. It is globally abnormal but shows many features of locally "correctly" sorted patterns. This is explained in panel D using a schematic representation which is provided to assist the evaluation of cell sorting. (D) Schematic representation of panel C; for details, refer to legend of Fig. 1A. Arrows in the two times 8-AB cell stage (left) indicate the movements of their descendants during development. For instance, the most anterior cells of the ABarp (#3)- and ABpra (#1)-derived regions at the right side of the embryo move anteriorly leading to an elongation of these regions. The ABarpaaaa cell (ABarp#3) catches up with the ABpraaaaa cell (ABpra#1) which shows that cells move relative to each other. On the left side, the descendants of the ABara- and an ABala-derived region (#1) exchange their position (red arrow; Movie S1, Supplementary material). During these movements, cells change positions illustrating that also individual cells and not only whole regions move (Movie S1, Supplementary material). Descendants of three other ABala-derived regions also move during development so that all five ABala-derived regions touch at the terminal stage although not all of their founder cells touched each other at the beginning of development. Many movements guide cells in positions that appear to be "correct" if compared to normal embryos. For example, three domains can be found in which cells are positioned similar to normal embryos (red, orange, and grey hatched ellipsoid; compare with the normal embryo shown in panel E). Also on the single cell level, many cells are positioned "correctly". Some examples for that are illustrated in Fig. S1 (Supplementary material). (E) Schematic representation of the AB-derived regions of a normal embryo (same embryo as in Fig. 1A). In the scheme, only those regions are shown, which also occur in the experiment shown in panels C and D (ABpla, ABplp and all regions of the P_1 lineage but MSp are left out for clarity). The ABxxxaaaa and ABxxxpppp cells can be assigned to three domains in the a–p axis indicated by red, orange, and grey hatched ellipsoids, respectively.

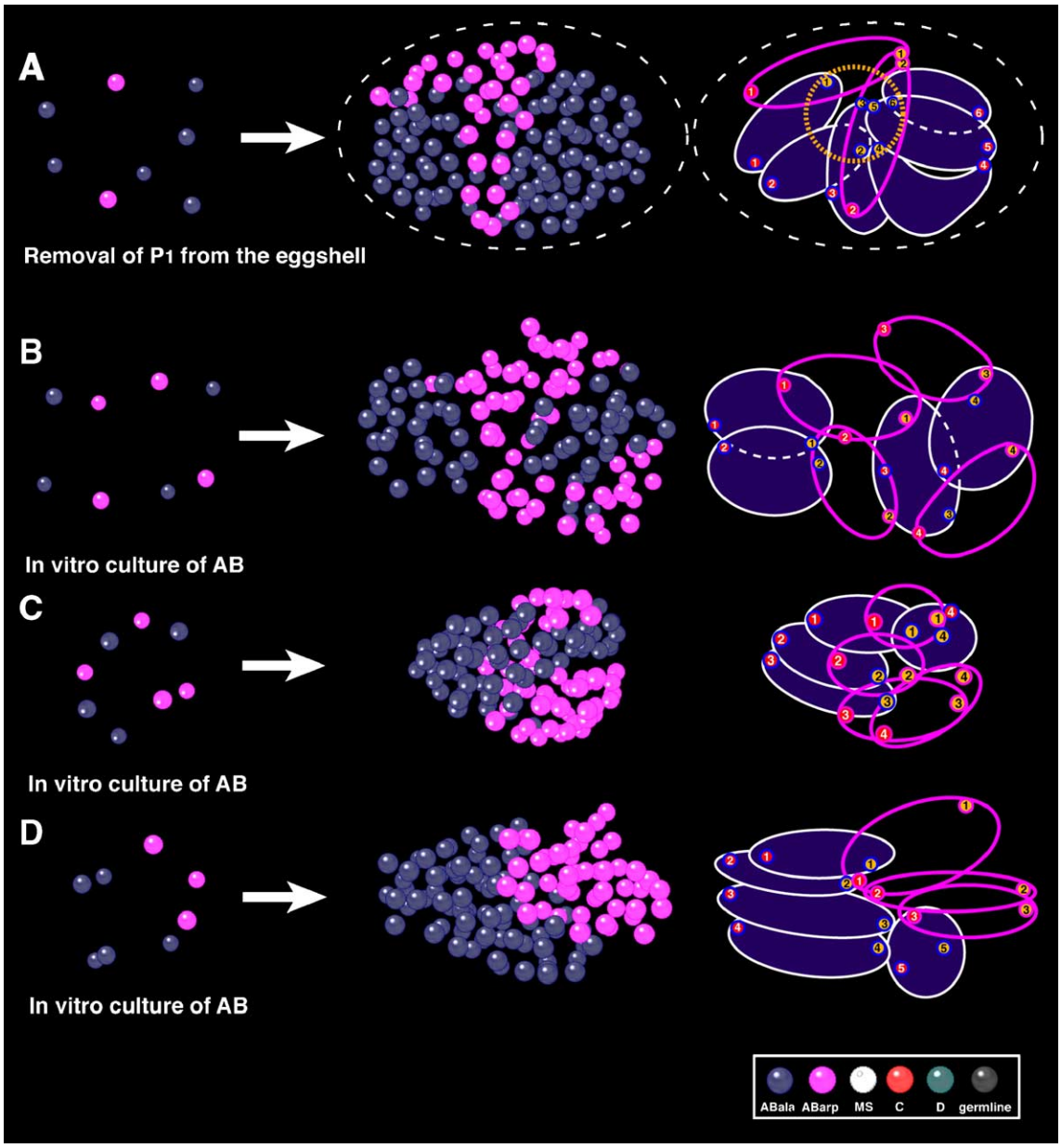


Fig. 3. Development of isolated AB blastomeres. 3D representations of 8-AB and 128-AB cell stages. Colors refer to cell fates as shown in the legend. If present, the eggshell is indicated by a hatched line. A schematic representation is provided to assist the evaluation of cell sorting; for details refer to legend of Fig. 1A. Like in a *glp-1 (e2144)* embryo (Fig. 1B) only ABala- and ABarp-derived fates are present since the inducing P1 descendants are missing. (A) Wild-type embryo from which P1 was removed by microsurgery. Anterior is to the left. Comparing the two stages shows that cells remain in their original environment and do not move very much. ABala-derived cells do not only lie at the anterior but also at the posterior pole. One ABarp-derived region is even oriented perpendicular to the a–p axis. Thus, it appears that normal pattern formation is impaired. However, the scheme shows that anyway a sorted pattern is formed. The ABala-derived cells elongate radially from a center in the middle of the embryo in which all ABalapppp cells are positioned (orange circle). (B–D) In vitro culture of isolated wild-type AB blastomeres. Three examples are shown. Like in panel A, cells do not move very much, and the early topology correlates with the terminal pattern. The development of isolated AB blastomeres is variable, but in all embryonic fragments, the ABarp-derived regions do not elongate in a–p direction like in normal embryos. The fragment in panel B is barely sorted. In panel C, indications for "correct" cell sorting can be found (see also Movie S2, Supplementary material). Three of the four ABala-derived regions orient by each other. The ABalaaaaa cells are in close neighborhood in an anterior position. One of the ABala-derived regions is trapped by the ABarp-derived regions in a posterior position. (D) ABala-derived cells are located at the anterior and ABarp-derived cells at the posterior pole. This structure resembles a normal embryo with an a–p axis, however, the ABarp-derived regions do not elongate. Three of the four ABalaaaaa cells are well sorted at the anterior pole of the embryonic fragment.

embryonic fragments, the density of instructive neighbors is relatively low. To test whether or not the number of cells is crucial for cell sorting, we increased the cell number by culturing two AB blastomeres together and, indeed, extensive cell sorting and far-ranging cell movements could be observed (Movie S3, Supplementary material). We were surprised to find that only "birostral" (two-headed) structures were formed (Fig. 4; $n = 6$): these have an area of ABarp-derived regions, positioned in between two poles consisting of ABala-derived cells. The pattern of early cell deaths in the ABala-derived

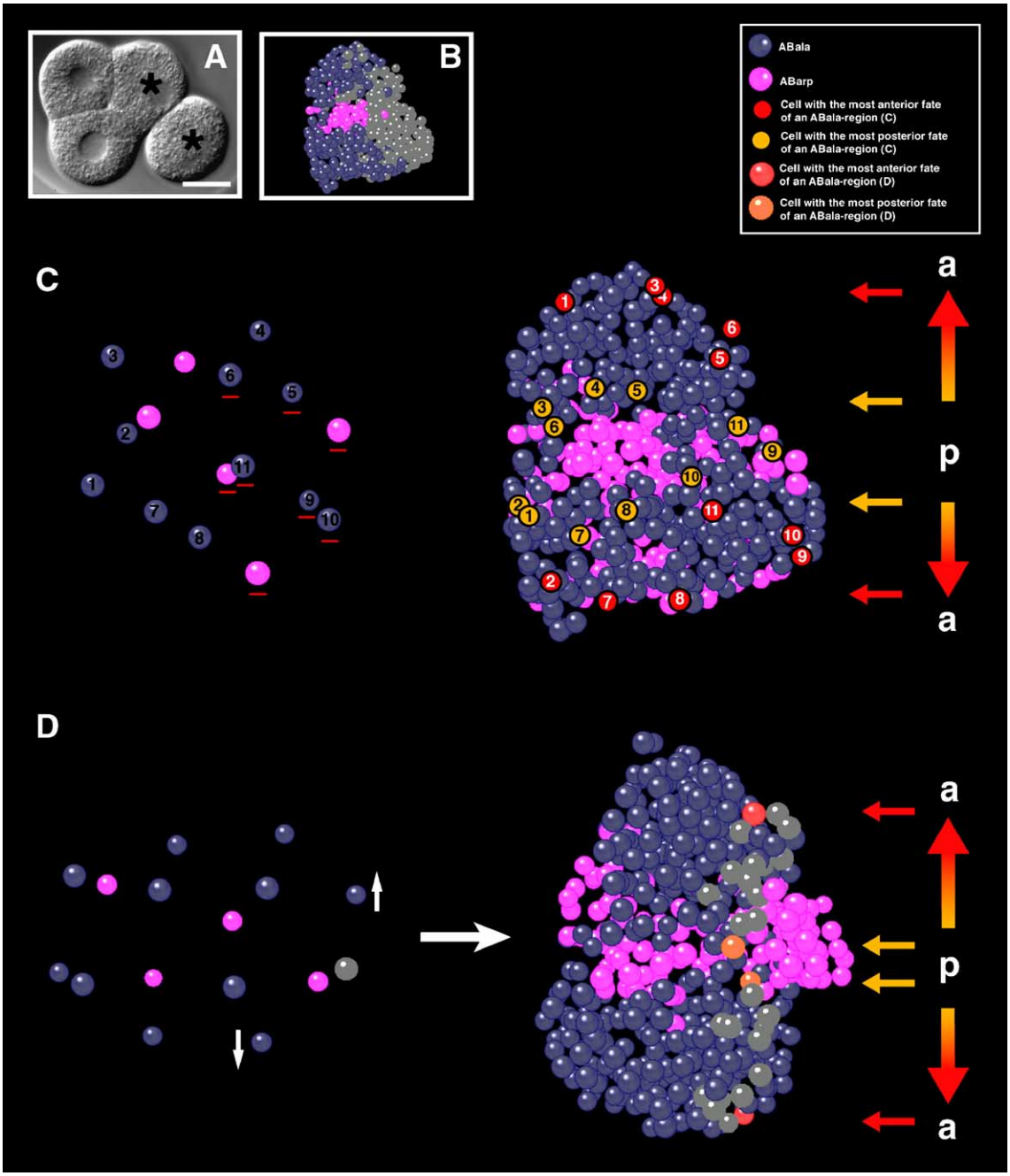


Fig. 4. In vitro combination of two AB blastomeres. DIC image and 3D representations. Colors refer to cell fates as shown in the legend. (A–C) Embryonic fragment #1. (A) DIC image shortly after combination. The daughters of the right AB blastomere are marked with asterisks. Scale bar: 10 μm. (B) 3D representation of the premorphogenetic stage. A "birostral" structure forms in which two anterior poles consisting of ABala-derived cells flank a posterior pole of ABarp-derived cells. The descendants of the right AB blastomere are colored in grey illustrating that both blastomeres contribute to both anterior poles. (C) 3D representations of the two times 8-AB cell and the premorphogenetic stage. The 8-AB-derived cells of the right AB blastomere are underlined with red bars. All ABala blastomeres are numbered in the two times 8-AB cell stage. According to these numbers, the most anterior and the most posterior descendants of each ABala blastomere are marked in the premorphogenetic stage in red and orange, respectively. This color code reveals that the polarities of the two anterior poles are opposed (red/orange arrows). The most anterior cells are placed at the tips of the two poles, and the most posterior cells lie close to the ABarp-derived cells. Only the orientation of one ABala-derived region (#11) deviates from this pattern. Cells from both AB blastomeres contribute to both anterior poles. (D) Embryonic fragment #2. Reversion of the polarity of a group of cells placed in an environment where the neighboring cells show opposite polarity. For simplicity, the behavior of only one ABala-derived region—marked in grey—is shown. The polarity of the future regions of the two neighboring ABala-derived blastomeres is indicated by white arrows in the two times 8-AB cell stage. In the premorphogenetic stage, the grey ABala region has divided in two subregions, each joining one anterior pole. Color coding of the most anterior and the most posterior cells of the two subregions in red and orange, respectively, shows that the two subregions have opposite polarities (red/orange arrows). Furthermore, all ABala-derived regions of each anterior pole are oriented away from the ABarp-derived cells placed in the middle like in the embryonic fragment shown in panels A–C (arrows). Analysis of a time-lapse movie of this embryonic fragment (Movie S5, Supplementary material) reveals that this is due to a reorientation of the posterior part of the ABala-derived region by cell sorting.

regions shows that the most anterior cell of each ABala-derived region is always positioned at the tip of the corresponding anterior pole, indicating that the poles have opposite polarity (Fig. 4C). In addition, descendants of both AB blastomeres contribute to both anterior poles (Fig. 4B). The most parsimonious explanation for these findings is that no general cellular polarity is used for a directional sorting of AB blastomeres, but that the polar arrangement of cells is established locally according to the environment—as already observed in the P_1 removal experiment shown in Fig. 3A. If cells used a general polarity for sorting, they should not contribute to two opposingly polarized anterior poles. Only local cell sorting is consistent with the formation of "local minima" like the two anterior poles. These results also suggest that a larger number of cells improve cell sorting. The same results are obtained when three AB blastomeres are cultured together (data not shown).

Reversion of local polarity according to the environment

We explained the observation that two AB blastomeres form only "birostral" fragments by an exclusively local sorting of cells independent of a general polarity. If cells rearranged along the local a–p axes in "birostral" fragments, depending on the polarity of the environment, this would provide strong evidence for local cell sorting. It would also demonstrate that cell sorting is independent of the direction of cell cleavages and the underlying cellular polarity. We could, indeed, document such cases of cell rearrangements. In the "birostral" fragment shown in Figs. 4A–C and Movie S4 (Supplementary material), a mitosis places the daughter with the posterior fate anterior to the cell with the anterior fate. During development, the descendants of these cells rearrange so that in the terminal stage the cells with the anterior fate are actually positioned anterior to the cells of the other ABala-derived regions of this pole. In another "birostral" fragment, we observed that five of the 12 ABala-derived regions each divide into two subregions. These subregions then join different anterior poles with opposing polarity (Fig. 4D). During this process, one of the two subregions—the one which moves to the anterior pole with a "wrong" polarity—re-orients in a–p direction to adapt to the polarity of its surrounding (Movie S5, Supplementary material). This leads to a situation in which both subregions display the polarity of their corresponding anterior pole. This indicates that isolated AB-derived cells do not use a general polarity—if it exists—or at a minimum that this polarity can be overcome by the local cell sorting.

Table 1
Elongation of embryonic fragments

No. of AB blastomeres	Elongation of embryonic fragment (mean ± SD)	
	Without P_2	With P_2
1	1.17 ± 0.28 (n = 15) **	2.15 ± 0.72 (n = 3) **
2	0.98 ± 0.18 (n = 6) *	2.27 ± 0.45 (n = 3) *

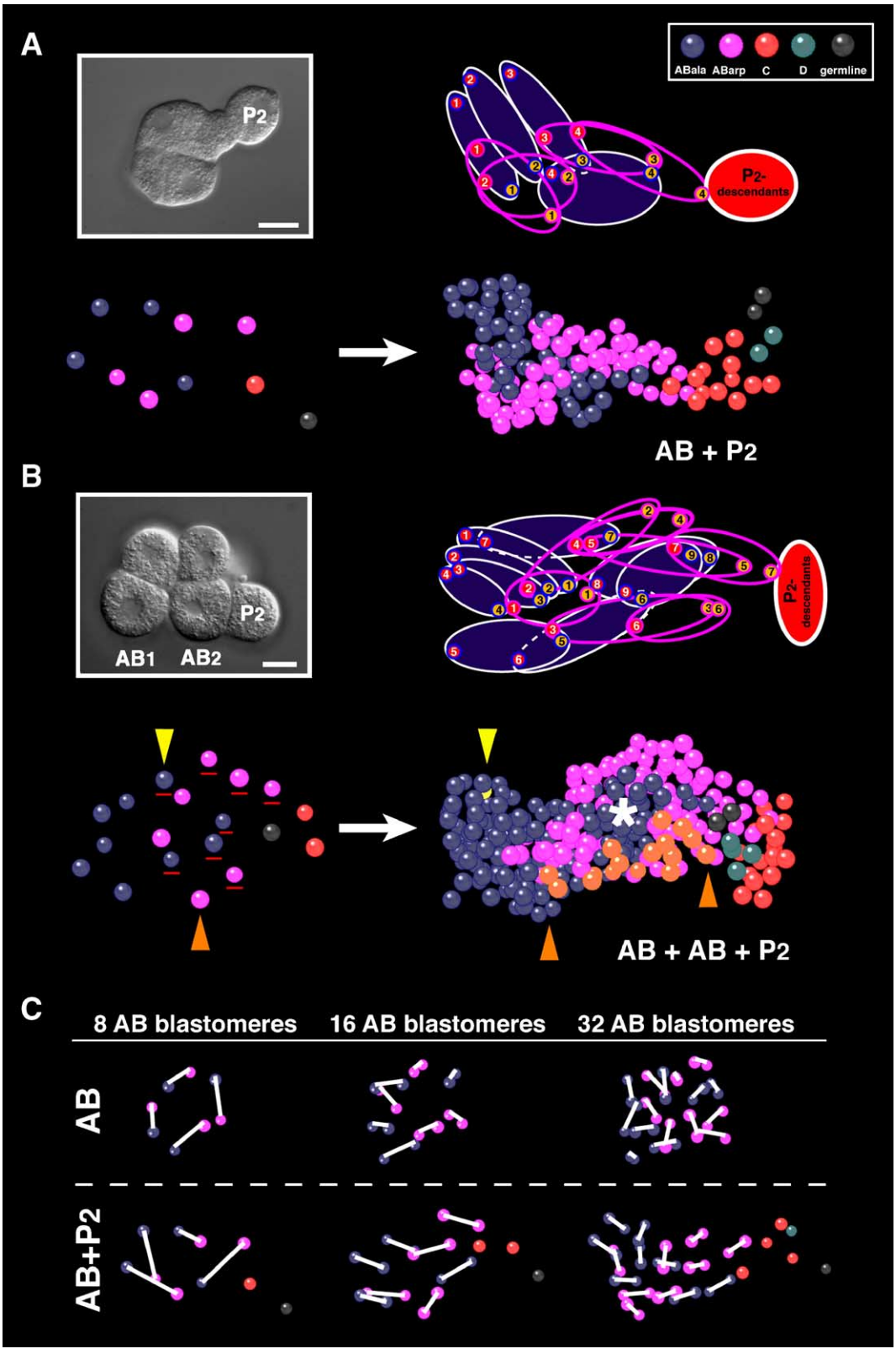
The elongation of embryonic fragments consisting of one and two AB blastomeres with and without P_2 is compared dividing the length of the fragment by its width. In both experiments, the addition of P_2 leads to a significant elongation of the embryonic fragment.
* Paired t test: $P = 0.004$.
** Paired t test: $P < 0.001$.

P_2 polarizes an AB-derived embryonic fragment

Since isolated AB-derived cells do not show the normal preference for a–p directed cleavages, even though P_1 is removed rather late, the possibility arose that a general polarity that also organizes the spindles is induced later in development, but not by P_1. To test this possibility, we added a P_2 blastomere to the two daughters of an AB blastomere to see whether the addition of P_2 alters the behavior of the cells. To avoid the induction of ABp-derived fates and, thus, to ensure comparability of these experiments to those above, we used blastomeres from a *glp-1 (e2144)* mutant background (Hutter and Schnabel, 1994; Priess et al., 1987). The addition of P_2 causes the embryonic fragment to elongate by a factor of two (Table 1). The cleavages of the AB-derived cells no longer show the rotation of the cleavage directions by approximately 90° but align mainly with the P_2 blastomere (Fig. 5C). Thus, the elongation is due to an alteration of the direction of mitosis and not to an improved cell sorting—the cells do not move more

Fig. 5. Development of AB blastomeres touching P_2. (A–C) DIC images, schematic representations (for details refer to legend of Fig. 1A) and 3D representations. All cells are derived from *glp-1 (e2144)* mutant embryos. Colors refer to cell fates as shown in the legend. Scale bars: 10 μm. (A) P_2 added to AB. The embryonic fragment is elongated in a–p direction compared to isolated AB blastomeres (Fig. 3). Cells do not move very much, and the terminal pattern resembles the initial topology. However, the schematic representation illustrates that three of the four ABala-derived regions orient by each other and their ABalaaaaa cells lie in close neighborhood in the anterior pole of the embryonic fragment. White box: DIC image of the blastomeres shortly after combination. (B) P_2 added to two AB blastomeres, touching only one of them. The 8-AB-derived blastomeres of the posterior AB blastomere are underlined with red bars. In the two times 128-AB cell stage embryo, the embryonic fragment is elongated in a–p direction compared to isolated AB blastomeres (Fig. 3) as well as compared to two combined AB blastomeres (Fig. 4). Cell sorting occurs also by far-ranging cell movements. For example, the descendants of ABala #7 move relative to the descendants of the other more anteriorly positioned ABala blastomeres (#1–#5), and its most anterior descendant (ABalaaaaa) catches up with the most anterior descendants of the other ABala blastomeres (see the schematic representation; the positions of ABala #7 and its most anterior descendant are also indicated in the 3D representation by yellow arrowheads and a yellow sphere, respectively). Thus, at the terminal stage six out of nine ABalaaaaa cells are positioned "correctly" in an anterior position. However, the descendants of two ABala-derived blastomeres are "trapped" by ABarp-derived blastomeres located in an anterior position and are not able to overcome this "local minimum" (white asterisk). The elongation of an ABarp-derived region derived from the anterior AB blastomere (orange) in posterior direction, which also is mediated by cell movements, is shown by orange arrowheads. White box: DIC image of the blastomeres shortly after combination. The two AB blastomeres execute the second division. The descendants of the right AB blastomere are touched by P_2. (C) Cell cleavages of isolated AB-derived blastomeres with (bottom row) and without (top row) an added P_2 blastomere. Same embryos as in Fig. 3C and panel A. The directions of cell cleavages of the different stages are indicated by white bars connecting the two daughter cells directly after mitosis. In the isolated AB blastomere, the main direction of cell cleavages rotates by approximately 90° after each division. Adding a P_2 blastomere orients the direction of most cell cleavages towards the P_2 blastomere in all cleavages.

than in embryonic fragments derived only from AB. The P_2 cell defines the posterior pole of the fragment (Fig. 5A; $n = 3$). This finding indicates that P_2 polarizes the whole embryonic fragment and not only the touching cell, as shown for the interaction between P_2 and EMS as well as for the interaction between a 4-AB cell stage cell and P_2 (Goldstein, 1995; Park and Priess, 2003; Bischoff and Schnabel, submitted for publication).



Cell sorting in polarized embryonic fragments

Since, as we reported above, the movements appear to be impaired if fragments are too small, we asked if P_2 influences cell sorting by adding P_2 to two AB blastomeres. The addition of P_2 again leads to a twofold elongation of the embryonic field (Table 1; $n = 3$). General cell sorting is improved as well, however, "local minima" still occur. The descendants of two of the nine ABala-derived blastomeres are trapped by ABarp-derived cells located in an anterior position (Fig. 5B). These cells are not able to overcome this obstacle and remain in a posterior position. Therefore, cells are not able to recognize cells with a posterior fate which are positioned more anteriorly as right neighbors on the wrong side. When three AB blastomeres are added to P_2 the embryonic fragment also elongates but cells optimize their neighborhoods by sorting perpendicularly to the a–p axis induced by P_2 (data not shown). This shows—together with the incapability to resolve "local minima"—that the polarity induced by P_2 aligns the mitotic spindles in an a–p direction but is not itself used for cell sorting.

Discussion

In this study, we test the "cell focusing" hypothesis designed to explain the robust cell sorting in the embryo of *C. elegans* (Schnabel et al., 2006). The "cell focusing" hypothesis proposes that cells produce autonomously—depending on their fate—positional values and use local cell–cell interactions to compare them to find their "correct" position. We use in vitro cell culture experiments combined with 4D microscopy to follow cell behavior in detail (Schnabel et al., 1997, 2006).

Cell sorting in altered environments

One prediction of the "cell focusing" hypothesis is that cell sorting should occur only locally, since cells should deduce their position exclusively from their neighbors. The analysis of the embryonic patterns formed after extensive manipulations of blastomere positions and fates showed that, independently of the kind of manipulation, cells sort "correctly" on a local scale. The most extensive cell sorting, including far-ranging cell movements, occurs in embryonic fragments derived from two AB blastomeres (Fig. 4; Movie S3, Supplementary material). However, in many experiments, the patterns formed by cell sorting do not resemble the normal global topology. Cells appear to be trapped in "local minima", defined as a locally correct neighborhood within a globally wrong pattern (Fig. 1D). This can, for example, be observed in "birostral" fragments which form two anterior poles (Fig. 4). The inability of resolving such "local minima" on the one hand and the generation of a locally "correct" pattern on the other hand suggest that cell sorting is, indeed, based only on cell–cell interactions between neighboring cells, as predicted by the "cell focusing" hypothesis.

The second prediction of the "cell focusing" hypothesis —cells of all kinds of fates should be able to sort out—is also supported by our results. We observed that cells of all fates sort even in different environments (Schnabel et al., 2006; this study). For example, all five ABala-derived regions presented in Fig. 2D move to find a locally "correct" position, although their neighborhoods differ significantly. Apparently, cells can obtain instructive information from any other cell, suggesting that cells use a universal signaling system to compare their positional values and, thus, to sort.

The number of cells and the early topology of the embryo influence cell sorting

The isolation of AB results in a globally aberrant pattern, even though the cells are positioned "correctly" on a local scale. The enhanced cell sorting after doubling of the number of cells suggests a trivial explanation for this observation—the low number of cells produced by an isolated AB blastomere limits cell sorting. It appears that cells also maintain their ability to sort in AB-derived embryonic fragments, and also that the low cell number produced by a single AB blastomere interferes with the efficiency of cell sorting.

Another finding in isolated AB blastomeres is that, depending on the starting conformation, different terminal patterns were generated (Fig. 3). Thus, the starting configuration formed in early development appears to be essential for the outcome of cell sorting.

Both observations—the influence of cell number as well as of the starting configuration on cell sorting—provide further evidence for a cell sorting mechanism based on local cell–cell interactions as proposed by the "cell focusing" hypothesis.

Polarization of the embryonic fragment by P_2

The combination of AB and P_2 suggests that the P_2 blastomere has a polarizing capacity (Fig. 5). This is in accordance with observations of Park and Priess (2003) who showed that an in vitro addition of P_2 to a single blastomere derived from a 4-AB cell embryo results in an alignment of the mitotic spindle towards P_2. We found that in embryonic fragments consisting of P_2 and AB, most cell divisions—and not only those of the cells touched directly as described before (Goldstein, 1995; Park and Priess, 2003)—were influenced by the added P_2 blastomere (Fig. 5). These experiments suggest that isolated AB blastomeres lack a polarity which can be induced by P_2 (Bischoff and Schnabel, submitted for publication).

An isolated AB blastomere can still produce ABala- and ABarp-derived lineages in absence of a P_1 blastomere and its descendants. This observation is not in accordance with a study by Park and Priess (2003). These authors dispute the existence of the polarizing induction for AB by P_1 which was shown to specify the posterior ABarp fate out of the anterior ABala fate in the 12-cell embryo (Hutter and Schnabel, 1995a). They explain this cell fate specification by later interactions of P_1 descendants with AB-derived cells. We, however, found here that the removal of P_1 during the 2-cell stage results in an embryonic fragment consisting of ABala and ABarp fates (Fig. 3), and this

supports the "polarizing induction model" proposed by Hutter and Schnabel (1995a).

Cell sorting in polarized fragments

The in vitro culture of two AB blastomeres suggested that cells do not use a cellular polarity for sorting. The addition of P_2 to AB, however, raised the question whether polarized cells behave differently and actually use their polarization to sort in a directed manner. We addressed this question by analyzing embryonic fragments consisting of two or three AB blastomeres combined with a P_2 blastomere. Since also in these embryonic fragments "local minima" occurred and neighborhood conflicts along the a–p axis are not resolved (Fig. 5B), we conclude that an induced cellular polarity is not used by the cells for sorting. However, there is an indirect impact of cell polarity on cell sorting because the alignment of mitotic spindles alters the early topology of the embryo, and this is required for proper sorting on a global scale.

The "cell focusing" hypothesis

In summary, our experiments confirm two predictions of the "cell focusing" hypothesis. Firstly, cells appear to be able to obtain positional information from all other cells of the embryo—for example, cells derived from ABalp and ABprp sort together although they normally never touch (Fig. 2D)—and, secondly, cells appear to sort only locally. All the observations speak against any global cell sorting mechanism. They rather support the local "cell focusing" hypothesis; cells are guided by their neighbors. Moreover, we show that a general cellular polarity is not required for cell sorting. Our findings on how cells sort are compatible with the general outline of the embryogenesis of the worm. The stereotyped cleavages generate during early development a starting configuration (Sulston et al., 1983) which enables a correct global patterning on the basis of local cell sorting.

The behavior of cells in the in vitro experiments reveals properties of the cell sorting mechanism which are veiled in normal development. For example, an analysis of normal development would not have detected the potential of non-polarized cells to sort correctly. This important finding helps to understand the mechanism of cell sorting.

This work and the accompanying paper define a new mechanism for pattern formation in *C. elegans*. Our observations now allow us to predict phenotypes which should lead to the identification of genes involved in this cell sorting process by forward genetics. Since our work points to local cell–cell interactions, it is the molecular elements of either differential cell adhesion or local cell-to-cell signaling that should underlie cell sorting.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2006.03.005.

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