Compensatory cell movements confer robustness to mechanical deformation during embryonic development

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Abstract

Embryonic development must proceed despite both internal molecular fluctuations and external perturbations. However, mechanisms that provide robustness to mechanical perturbation remain largely uncharacterized. Here, we use light-sheet microscopy, comprehensive single-cell tracking, and targeted cell ablation to study the response of *Caenorhabditis elegans* embryos to external compression. Compression changes the relative positions of many cells and causes severe distortions of the embryonic axes. A large-scale movement of cells then corrects this distortion. Only a small number of specific cells are required for these compensatory movements, and in particular one cell, ABarppap, appears to generate force, dramatically changeing as it moves to its correct local cellular environment. During these movements, we also observed 'egressions', cells moving from the interior of the embryo out onto the surface, and lineages that undergo both ingression and egression. In total, our work describes how the embryo responds to a major mechanical deformation that can occur during the early development of this organism *in situ* and puts forward a model to explain how the response is coordinated.

Introduction

Robustness is a universal feature of biological systems and refers to their ability to function despite external and internal perturbations (Wagner, 2007). Identifying the mechanisms and design principles that underlie robustness is a central goal of systems biology (Kitano, 2004). Mechanisms that allow organisms to be robust to genetic perturbations and stochastic molecular fluctuations have been investigated. They include functional redundancy (Nowak et al., 1997), negative feedback (Ji et al., 2013), and gene regulatory network topology with signaling across specifically positioned cells (Barkoulas et al., 2013). In animals, reduced robustness, for example due to an inherited mutation in a gene duplicate, can result in variable phenotypic outcomes because of variation in gene expression during development (Burga et al., 2011; Casanueva et al., 2012; Raj et al., 2010).

However, in addition to the requirement to function robustly despite stochastic or environmentally-triggered variation in the concentration of molecules, developing embryos - and biological systems more generally - also need to be robust to mechanical perturbation. Cells in developing embryos can experience substantial mechanical perturbations. The morphogenetic processes themselves generate tensions and shear stresses on surrounding tissues (Heisenberg and Bellaiche, 2013), but also external perturbations will occur, for example due to movements and physical constraints during growth inside the mother, or due to the wider external environment in embryos that develop outside of their parents.

Biological systems have evolved diverse mechanisms for detecting physical forces, for example gravity perception in plants and force sensitive ion-channels that play a role in mechanotransduction from bacteria to higher animals (Arnadottir and Chalfie, 2010). In addition, the growth and organization of multicellular systems is regulated by physical contact between cells. In multiple systems an intimate coupling between mechanics and signal transduction has been demonstrated during development, and ‘mechanochemical’ patterning has been proposed as a widespread mechanism for spatial pattern formation (Howard et al., 2011). Here, however, we address a different question: how do embryos develop normally despite being subjected to substantial mechanical deformation?

C. elegans embryos are a good model in which to study robustness to mechanical deformation

as compression occurs during the natural life cycle of the animal, specifically when old or starved adult worms retain developing embryos inside their bodies. Compression may also occur transiently during normal egg laying and, experimentally, compression is applied when imaging embryos to reduce the depth of field (Bao et al., 2006; Schnabel et al., 1997; Sulston et al., 1983). Previous studies, including the original description of the cell lineage by Sulston, have described large-scale rotations of cells observed in compressed embryos (Hench et al., 2009; Schnabel et al., 2006; Schnabel et al., 1997; Sulston et al., 1983). In addition, Hench and co-workers tracked the positions of cells during the development of non-compressed embryos for the first time and concluded that the movements of non-ingressing cells during gastrulation are much more extensive in compressed embryos (Hench et al., 2009), a conclusion confirmed by more comprehensive tracking (Giurumescu et al., 2012). However neither the purpose of these movements nor the mechanism that underlies them has been addressed.

Although still a controversial idea (Hench et al., 2009), evidence for coordinated cell movements to correct cellular mispositioning in the early *C. elegans* embryo comes from the experiments of Schnabel and co-workers who reported differences in how some cells move when comparing between their tracked embryo datasets (Schnabel et al., 2006). By combining blastomeres from different embryos and by analyzing cell fate mutants they also provided evidence that certain cells move according to their fate and local environment (Bischoff and Schnabel, 2006). Anecdotal observations of possible local 'error correction' in tracked embryos further supports this (Moore et al., 2013; Pohl et al., 2012). In addition, documented cellular displacements in the early embryo do not necessarily lead to large defects in larvae, e.g. a syndecan null mutant causes a large change in the spindle rotation of founder cell ABar, but does not cause large phenotypes in hatched larvae (Dejima et al., 2014; Rhiner et al., 2005).

Here we use comprehensive single cell tracking to compare the positions and movements of all nuclei in compressed and uncompressed embryos and to describe both how compression distorts the embryos and how this distortion is corrected. Based on this data and the results of additional experiments we propose a model for how embryos compensate for compression based on the directed movement of specific cells to their correct local environment. The model represents a simple design for how an embryo can be robust to a major mechanical deformation that it experiences. Specific mechanisms that accomplish similar corrections may be a general feature of other multicellular organisms.

Results

Nuclear tracking in compressed and uncompressed embryos

We compressed embryos on a microscope slide using 20 μm polystyrene beads to control compression with the cover slip. Embryos expressing green fluorescent protein-tagged histones (Bao et al., 2006) were imaged through time-lapse microscopy and a semi-automatic nuclear tracking algorithm (Dzyubachyk et al., 2009; Dzyubachyk et al., 2010; Kruger et al., 2015) was used to follow the positions, movements and divisions of all nuclei in the early embryo at one minute temporal resolution. We were able to track nuclei in uncompressed embryos using two approaches: (1) confocal microscopy where embryos were mounted using 45 micron polystyrene beads to resist compression by the cover slip, and (2) light sheet microscopy (Huisken et al., 2004) where embryos were immobilized in low melting point agarose. The light sheet microscopy allowed for a reduction of the amount of laser light used for imaging avoiding phototoxicity. At least 336 nuclei were tracked in every embryo, leading to a dataset with over 72,000 data points. All embryos used in this study successfully developed and hatched. The complete dataset of nuclear positions over time is available as Supplementary Data File 1.

The effects of compression on early embryonic development

With these recordings we quantified the consequences of compressing the embryos. As has been previously demonstrated, cells in the very early (up to the eight-cell stage) embryo show highly constrained positions and movements in all embryos (Pohl and Bao, 2010). At the four-cell stage, all four nuclei are arranged in a plane, which in the case of compressed embryos is invariably orthogonal to the axis of compression (Bao et al., 2006). The first significant difference for compressed embryos involves a large early movement by the ABpl cell at the eight-cell stage that was described as important for establishing left-right asymmetry (Pohl and Bao, 2010). In the uncompressed embryo, however, the ABpl cell moves less and the movement of the ABpr cell is larger (Supplementary Data Figure 1). From this stage on, the compression causes a moderate misplacement of many cells (overall normalized misplacement, mean/1st quartile/3rd quartile = 4.1/2.6/5.3 μm). The compression does not have an influence on the timing of cell divisions or gastrulation in the early embryo nor does it cause large systematic differences in the embryo's highly directed division angles (Supplementary Data Figures 3, 4). The next major directed

movement in the *C. elegans* embryo is the start of gastrulation with the ingression of the two intestine precursor cells, Ea and Ep, and proceeds normally. However, at this stage a systematic misalignment of cells is apparent in compressed embryos: groups of cells that are positioned along the left-right and dorsal ventral axes in uncompressed embryos are not properly positioned relative to each other in compressed embryos (Figure 1A).

Compensatory cell movements realign the embryonic axes and misplaced cell groups

How does the embryo compensate for the cellular misalignments present during compression? Continued tracking of individual nuclei in compressed and uncompressed embryos reveals that the compressed embryo responds with concerted movements of cells, shortly after the 6th division of the AB lineage, about 90 minutes after the four-cell stage. When gastrulation proceeds with the ingression of the mesodermal lineage and some ABa derived lineages, the compressed embryos show a large-scale movement of cells on the dorsal/right surface of the compressed embryo not seen in the uncompressed embryos (Figure 1B and C). For example the eight descendants of ABplp cell move on average 8.2 microns in a compressed embryo, whereas they only move 4.6 in the uncompressed state. It is notable that the embryo undertakes these abrupt and large movements in the light of its very rapid development.

Compensatory movements during gastrulation involve many cells

During this large-scale rotation movement at least 34 cells (39% of cells at this stage of development) move differentially (Figure 1C, false discovery rate <0.1). The movements are not homogeneous throughout the embryo, and not all lineages move a larger distance in a compressed embryo during gastrulation. For example, several cells of the ABara lineage move a smaller distance in compressed than in uncompressed embryos (Figure 1C, median 59% of the distance covered in the uncompressed embryos). The overall effect of the movements is a re-alignment of the cells according to the main axes of the embryo (Figure 1A, Supplementary Data Movie 1), which can be quantified as a reduction of the mispositioning of the cells over the inferred axes (Figures 1D, see Materials and Methods for methodological details).

Ingressions during gastrulation do not drive the compensatory cell movements

What drives these coordinated movements that correct for the effects of compression? We first tested to what extent the movements could be explained or driven by the concurrent gastrulation movements. The forces associated with gastrulation are generated by apical constriction of the

ingressing cells, and have been well studied as a force generator (Nance et al., 2005). If gastrulation is the force generator, then the compensatory movements would be passive, that is, driven by the pressure generated by the apical contraction of the ingressing cells and coordinated by differential adhesion of the cells. However when we prevented the gastrulation at this stage by disrupting the MS cell with laser irradiation (Figure 2A), we found that the rotational movements continued in compressed embryos (Figure 2B-C).

Two cells, ABarppaa/p, show the largest differential movements in compressed embryos

We next examined the local, compression-dependent mis-positioning of the cells in relation to the rotational movements in greater detail. We defined a measure to quantify misplacement of cells by considering their context in the embryo and measuring how a cell's distances to other cells differ in the compressed embryos (Materials and Methods). This misplacement measure shows a moderate correlation to the differential movements, and highlights two cells, ABarppaa/p that show both strong misplacement and the largest differential movements (Figure 2D). The larger movements in the compressed embryo tend to reduce the misplacement, as reflected by a large reduction of the misplacement statistic (Figure 2E). This suggests that cells move according to the extent of the local misplacement from their normal location.

During gastrulation cells egress out of the embryo and traverse from one side of the embryo to the other

During our analysis of cell movements in the compressed and uncompressed embryos we noticed a series of unusual and, to the best of our knowledge, previously unreported, compression-independent movements by individual cell lineages. In particular, we observed that during gastrulation not only do some cell lineages ingress from the outside of the embryo into the interior (Supplementary Data Movie 2) - as is observed for the E lineage, the MS lineage and some ABa derived cells - but other cells actually move from the interior of the embryo out onto the surface ('egression' movements). For example, the ABarpap cell is born inside the embryo, but its two daughter cells subsequently move out onto the surface (Supplementary Data Movie 3). These egression movements occur at the same time as gastrulation (Figure 3B) and may be required to generate space for the ingressing cells. Further, some lineages first ingress from the surface of the embryo into the interior and then 'tunnel' across the embryo before moving back out onto the surface at a specific location (Supplementary Data Movie 4). The identities of the ingressing and egressing cells are listed in Tables 1 and 2, with lineages showing coupled

ingressions and egressions marked. The timing of their movements into and out of the embryo are presented in the tables and also shown in Figure 3A. The compression of the embryo does not change the cells that ingress or egress and the timing of the movements is not strongly influenced.

Cellular movements adjust to cellular environment

To further understand how cells move in the embryo, we compared the movements of these lineages with coupled ingressions and egression in the compressed and uncompressed embryos (Figure 3B and C). Since the relative positions of other cells and also their movements differ depending upon the compression, we were able to distinguish if the cell movements are directed towards a correct location or if only the initial direction of movement is specified. Analysis of the paths of the cells reveals that they exit the embryo at specific local cellular environments, with different courses depending upon the extent of the compression. For example, the ABalaap lineage moves into the embryo from the ventral-left surface and, after division, both cells move back out onto the surface about 10 μm from the ingression site. If we align the compressed and uncompressed embryos at the moment of ingression (based on the position of the MS lineage), the ingression sites coincide, but the paths of the lineage do not (Figure 3B). In an uncompressed embryo, the lineages tend to follow a shorter path across the embryo, which can include abrupt turns (Figure 3C). However, despite the altered paths, in both cases the egression occurs in the same local cellular context (Figure 3D, E and F). Thus, the movement of these cells must be directed by extracellular, location-dependent cues that facilitate the lineage finding its correct position within the embryo. This observation illustrates that cells in the early embryo can move to their correct local environments.

Cell irradiation experiments identify a lineage required for the compensatory movements

To identify the cells that contribute to the coordinated movements in compressed embryos, we systematically ablated cells with a high-powered laser and then observed the effects on the movements. We first systematically irradiated all cells in turn at the eight-cell stage, and scored the effect by observing the movements on the dorsal surface. Only the irradiation of one cell, ABar, prevented the corrective movements in compressed embryos (Figure 2C). We then proceeded to ablate the two daughter cells of ABar to determine their relevance for the phenomenon. Ablation of ABarp gave a phenotype, but ablation of ABara did not.. Similarly, by irradiating the daughters of ABarp, we found that ABarpp but not ABarpa contributed to the dorsal movements: disruption of this cell reduces movements to a level similar to the

uncompressed embryos (Figure 2B). Notably, this cell is the grandparent of the two cells highlighted as strongly displaced and moving the most by the cellular misplacement analysis (Figure 2D-E). Therefore the lineage that undergoes the largest differential movements and a strong reduction of misplacement in the compressed embryo is also necessary for the movement of the other cells. The irradiation of ABarpp does not cause large cell displacements or ruptures of the cell membranes (Supplementary Figure 5, which shows the dorsal surface of an embryo with ABarpp ablated).

Cytoskeletal activation in the moving cells

To observe which cells contribute actively to the movements, we directly observe the generation of forces. Non-muscle myosin was visualized in the rotating embryo using a fluorescent NMY-2 fusion protein. NMY-2 has previously been reported as apically enriched in all of the characterized ingression movements (Harrell and Goldstein, 2011). The images revealed a apical enrichment of NMY-2 in ABarppap and an ensuing contraction during the cellular movements in both compressed and uncompressed embryos (Figure 4A and B, Supplementary Movie 5).

To systematically evaluate if the NMY-2 enrichment is specific for the ABarppap cell or common to more dorsal cells we manually segmented cells in the first six μm of the dorsal surface of three embryos for 9-10 time-points, measuring the average NMY-2 fluorescence signal of cells for every plane. The final dataset contained nearly 3000 data points, with the plane with the highest average signal, typically the apical surface, used to quantify NMY-2 activity in each cell. The NMY-2 signal of ABarppap is stronger than all other cells on the dorsal surface (Figure 4B, Tukey posthoc test, $p<0.05$) with the exception of ABalapap. ABalapap is a cell on the anterior end of the dorsal surface and moves posterior/left and contacts ABarppaa, sister to ABarppap, but did not show (consistent) differential movement in the compressed embryo. Thus, during these time points the NMY-2 activity in ABarppap is highly specific to this and one other dorsal cell.

Transient internalization of ABarpp descendants has been previously noted (Pohl et al., 2012) and partial ingressions are visible in our nuclei tracked lineages. The NMY-2 accumulation and contraction coincide with the movements on the surface (Figure 4C). In addition, the ABarppap cell undergoes a large shape change, with the cell flattening and developing a basal protrusion

towards the left side of the embryo (Figure 4A). In these lower planes we also see additional build up of NMY-2 between ABarppap and ABplappa, and later ABplappp (Figure 4A). This suggests additional force generation in addition to the apical constriction. Consistent with this, we observe that for ABarppap the largest mispositioning is relative to ABplappp, followed by its sister ABplappa, and that the movements correct this mispositioning (Figure 4D).

Taken together, the requirement of the ABarppap lineage for the compensatory movement, the oriented shape change of ABarppap, the specific activation of its cytoskeleton, the large displacement of the cell and the large correction of this displacement all support a model where ABarppap plays a crucial role in the corrective movements.

Discussion

Due to the planar arrangement of cells both at the four-cell stage and of the E cells following their ingression, compression distorts the early *C. elegans* embryo in a predictable manner, with the four-cell stage plane and the E-lineage plane aligned perpendicular to the axis of compression. Compression occurs physiologically with embryos stacked one after the other in the uterus, with the longer AP axis aligned with the vulva. This causes forces orthogonal to the AP axis, especially when worms don't lay eggs, as in older or starved worms, and many embryos are constrained in a confined space. Compression induces severe but predictable distortions in the embryonic axes. It is therefore reasonable to assume that a specific mechanism has evolved to compensate for the reproducible effects of compression. Based on our comprehensive single cell tracking, imaging and perturbation experiments we propose that it is the ability of a small number of specific cells to detect that they are in the wrong local environment and initiate compensatory directed movements that underlies this correction.

Taken together, our results suggest a model for how the effects of compression are detected and corrected for within embryos. Figure 5A shows a reconstruction of the dorsal surface of a compressed embryo before and after the compensatory movements and Figure 5B shows a slice through ABarppap and the embryo; cell lineages and movements are highlighted. Ablation of the ABarpp lineage reduced the rotational movements considerably. The role of this lineage could be active or it could be mainly passive, e.g. required for conducing tensions generated by other cells or for maintaining the integrity of the epidermis. The evidence for directed force generation and

shape change leads us to propose that ABarppap contributes actively to the corrective movements. The cell senses mis-positioning by interaction with ABplappa and/or ABplappp, and in response to misplacement and lack of contact, generates a force by directed cell shape changes, ingressing, and then finding and pulling on ABplappa and ABplappp. The generated forces are likely transmitted to specific neighboring cells by adhesion, such as its sister cell ABarppaa that moves in perfect concert with ABarppap but without any visible NMY-2 foci.

This model makes a number of specific experimental predictions that we have not yet been able to test for technical reasons. First, inactivation of myosin specifically in the ABarppap cells should prevent the corrective movements. Second, manipulation of the distance or contact between ABarppap and ABplappa/p should correspond to the size of the dorsal movements and inhibition of the contact should interfere with the movement. Third, the movements depend on some currently unknown cell recognition and guidance process and interference with this process should interfere with the movements. However, identifying these molecules may prove difficult because many of the candidate pathways such as Wnt and Notch signaling are essential for multiple earlier and concurrent development processes, necessitating the use of temporally and spatially restricted inactivation and the exclusion of indirect effects.

ABarppap movements are, however, unlikely to be the only actor, with other forces and movements contributing to the compensatory behavior of specific cells within compressed embryos. The egressing cells, for example, also play a role, as they fill the space left by the moving cells just ventrally of ABarppap, and allow the underlying right / ventral cells to remain mostly stationary, while maintaining the integrity of the epidermis. The anterior moving cells from the ABplp lineages relative to the posterior moving ABpla lineage cells likely provide another contribution: NMY-2 builds up on their interface (data not shown), likely pulling on ABplappp and connecting cells, contributing to the observed movements. Finally, the movements coincide with the tight packing and start of ingression of the MS cells and the active movements of the adjacent cells to cover the ingressing cells (Pohl et al., 2012). These movements provide space for the ABplp lineage cells to move to and the process generates tensions that could contribute. However, stopping the ingressions does not stop the rotational movement, demonstrating that these tensions are not essential for the correction.

An additional observation that we have reported here is that during gastrulation cells also

move out (egress) from the interior of the embryo onto the surface and other cell lineages first ingress into the interior and subsequently egress out onto the surface. One function of these movements may be to free up space in the interior of the embryo. Previously it has been suggested that volumetric constraints are the reason why gastrulation proceeds piecemeal in *C. elegans*, with the ingression of only a small number of cells into the interior at any one time accompanied by thinning of the ectoderm by co-planer cell divisions (Pohl et al., 2012). The egression of cells may be an addition solution to the volumetric constraints imposed by the eggshell. However, the egression movements may also play more active roles during development, for example facilitating compensatory movements such as those described above. Possible functions for the additional egressions and traversing cells with coupled ingressions and egressions will need to be systematically investigated in future work.

In total, our study adds to previous work describing examples of error correction in the *C. elegans* embryo involving the local movement of cells (Bischoff and Schnabel, 2006; Moore et al., 2013; Pohl et al., 2012; Schnabel et al., 2006). Our observations differ from the work by Bischoff and Schnabel (Bischoff and Schnabel, 2006; Schnabel et al., 2006) in that we observe that one lineage (ABarppap) contributes to the correction of mispositioning of other cells (amongst others, ABpla cells). Also the correction takes place predominantly orthogonal to the AP axis, which makes it unlikely that solely an AP positioning cue, as suggested by Schnabel et al., is sufficient to explain the positional awareness of the cells. The *C. elegans* embryo appears therefore to be rather regulative - both local 'mistakes' that occur during normal development and large-scale but reproducible distortions that are caused by an external perturbation can be corrected. Together with mechanisms that reduce the effects of molecular fluctuations (Barkoulas et al., 2013; Burga et al., 2011; Casanueva et al., 2012; Ji et al., 2013; Raj et al., 2010), these regulative cellular behaviors underlie the remarkable robustness of embryonic development to both internal and external perturbations.

Figure legends

Figure 1

A: Compression causes a mispositioning of cells that is corrected by concerted cellular movements. The left panels mark the positioning right after the sixth AB lineage division round at about 90 minutes after the four-cell stage, the right panels are taken just before the seventh division round at about 115 minutes. The first column shows the cell lineages with different colors, highlighting groups that will align along the principal axes of the late embryo. The MS cells and the ABarp cells are normally positioned along the dorsal ventral axis, and the following lineages are positioned symmetrically along the left-right axis: the MSa and MSp, ABpl and ABpr, and Ea/pl and Ea/pr descendants. The second column illustrates the mispositioning by showing an inferred dorsal-ventral axis - the green bar through the MS and ABarp cells and left-right axes - the lilac bars through the MSa-MSp descendants and the blue through ABpl-ABpr. During early development until the moment of the concerted cellular movements these groups are not aligned according to the axes in the compressed embryos, but are aligned in the uncompressed embryos. The panels for the later time point demonstrate that the movements correct the alignment of the axes and the relative positioning of the cells in the compressed embryos.

B: Visualization of the coordinated movements of the cells on the dorsal surface. For the later time-point traces are drawn to track the movements of the cells from the earlier time-point.

C: Comparison of cellular movements between compressed and uncompressed embryos. For the bars, the whiskers and center points indicate the max, min and median values over 5 observations for both compressed and uncompressed embryos. Dot, asterisk and double asterisk reflect 0.1, 0.05 and 0.01 significance levels (ANOVA; F-test) corrected for multiple testing (Benjamini-Hochberg).

D: Median differences of the cellular positions in the compressed embryo compared to the uncompressed embryo during early embryonic development (5 embryos per state). The embryos are aligned according to the inferred axes (anterior-posterior (AP), dorsal-ventral (DV), left-right (LR)) over time to compare the cellular positions directly and normalized for size differences over the axes (Methods). The dotted line indicates the start of the concerted movements, which cause a drop mostly in misplacement along the inferred dorsal-ventral axis as well as the overall misplacement.

Figure 2

A: Irradiation of the MS cell prevents gastrulation. Confocal microscopy image of the ventral surface of a compressed embryo expressing histone::GFP fusion protein to identify the nuclei. The MS cell was hit earlier with a high-powered infrared laser, which perturbed and delayed cell divisions, but did not damage surrounding cells. The image is taken just before the 7th division round of the AB cells, after the time the concerted cellular movements take place. The daughters of the MS cell did not gastrulate and remain on the surface and are indicated with an asterisk (based on three observations).

B: Size of rotational movements on the dorsal surface between the sixth and seventh division of the AB lineage. Compressed embryos are represented with C, uncompressed with U. C-MS and C-ABarpp represent compressed embryos with respectively MS and ABarpp irradiated. Ablation of the MS cell prevents gastrulation of the MS daughters, but does not stop the movements. Ablation of the ABarpp cell reduces movement to the level of the uncompressed embryo. One asterisk represents a significance level of 0.05, three asterisks a level of 0.005. The dorsal surface movements are quantified by tracking the ABplapaa cell.

C: Screen to identify the cells that contribute to the concerted movements in compressed embryos. Cells in the early embryo were systematically perturbed by laser irradiation, and then movements on the dorsal surface of the compressed embryo were followed. All squares indicate one experiment, at least three repeats per ablated cell. To quantify the movements, the dorsal cells that move more in the compressed embryo were followed. For reference the size of the movements in the compressed and uncompressed embryo are also shown. The screen highlighted the role of the ABar cell for the movements. The daughters of ABar were then irradiated, which highlighted the role of the posterior daughter but not the anterior daughter. Similarly for ABarp, the essay showed the posterior daughter has a much larger effect on the movements than the anterior daughter. The data underlying the figure are in Supplementary Table 1.

D. Differential movement of the cells correlates with misplacement. The differential movements are the differences in movements between compressed and uncompressed embryos. The relative misplacement is calculated from differences in the distances between cells shortly after the sixth AB division round. The measure reflects the relative positioning of cells to each other and does not require aligning embryos. The black line demonstrates the modest, but significant correlation (linear regression, $p<0.05$, dotted lines indicate 95% CI), which is stronger when only considering the moving exterior cells, and not the interior E and ingressing MS cells.

The ABarpp descendants are outliers with both large differential movements and considerable misplacement. Analysis based on 5 tracked embryos per state.

E. The larger movements in the compressed embryos tend to decrease misplacement. The difference in the relative misplacement is plotted against the differential movements. For simplicity only the cells are shown that exist for the whole time between the sixth and seventh AB divisions. The black line again demonstrates the correlation between the two measures ($p<0.001$, linear regression), with 95%CI. The large ABarppap/a movements reduce misplacement considerably. Analysis based on 5 tracked embryos per state.

Figure 3

A: Ingressions (red) and egressions (blue) over time. The vertical bars indicate the divisions of the AB lineage, the dotted line highlights the division preceding the rotational movements. Time represents minutes after the 4-cell stage, median time over 5 observations.

B: The coupled ingression and egression of cells. Shown is the path of ABalaap(p) from ingression at the bottom of the image to egression in 5 compressed and 5 uncompressed embryos (red uncompressed, blue compressed). The embryos are aligned at the time of ingression of ABalaap and the diverging paths of the cells are visible.

C: Further illustration of the coupled ingression and egression movement of ABalaap(p). Upper panels show the embryos before the sixth AB division round (indicated minutes after the four-cell stage), with ABalaap on the embryo's surface. The lower panels show the embryos just before the seventh AB division round. ABalaapp has tunneled through the embryo and egressed on the other side of the embryo. The cell moves in concert with sister cell ABalaapa, but this was omitted to simplify the figure. The movement of the cells differs between the compressed and uncompressed embryos. In the example shown there is a strong turn in the uncompressed embryo and a more straight movement in the compressed embryo.

D and E: Approximate average contact maps of the ABalaapp cell. The cellular environment of the ABalaapp cell is very similar in uncompressed (D) and compressed (E) embryos, with an unchanged egression site between the ABara and ABarp lineages. As a range for contacts a max cell radius of 4 μm was used, the radius of the larger E cells was estimated by means of the proportionally larger volume due to the fewer divisions. Few differences exist, including a brief contact with ABalpppp and the contact with ABpraaap at the end of the movement in the uncompressed embryo. The latter contact arises in the compressed embryos after ABalaapp

division, presumably because the egression movement completes faster in the uncompressed embryos. The egression movement tends to follow the division in the E lineage and contact with Eara. A cell is indicated as "absent" if not present in the embryo at that time point, i.e. the cell either already divided or has not been born. Analysis based on 5 tracked embryos per state.

F: Quantification of the similar cellular contexts between compressed and uncompressed embryos. Dice coefficients are calculated to quantify the similarity of the contact map of five compressed embryos to the average contact map for the uncompressed embryos. The coefficient is given by $\frac{2|A \cap B|}{|A|+|B|}$, where A and B represent the sets of neighbors. To cancel timing differences, for all time points the maximum score is given over the lifetime of ABalaapp in the compressed embryos.

Figure 4

A: The ABarppap cell changes shape and exerts force during the compensatory movements. Confocal microscopy images are shown of the dorsal surface of compressed embryos, with in red the cell membranes (PH::mCherry fusion protein), and in green non-muscular myosin (NMY-2::GFP fusion protein). A focal high-density of non-muscular myosin is associated with force generation (Nance et al., 2005). The first row of images shows part of the dorsal side of the embryo close to the surface (1-3 μm), followed by images from deeper in the embryo (4-6 μm). The first image shows the ABarppap cell (indicated with the plus sign) shortly after the sixth AB lineage division. At t=960s NMY-2 built up is visible on its apical surface and apical contraction can be seen, followed by relaxation (t=1440s) prior to the next division round. The cell Caaa is indicated with an asterisk for reference of the posterior side of the embryo. The arrow highlights NMY-2 build up, and the stripe highlights the sister cells ABplappa and ABplappp (posterior). The second row shows contact is established between ABarppap and ABplappa/p with NMY-2 build up at the contact site. Finally, the third row illustrates the shape changes and the formation of an extending foot of the ABarppap cell as reconstructed from the microscopy images. Observations based on three embryos, Supplementary Figure 6 shows the shape changes for 2 more embryos. B: Quantification of NMY-2 signal between 6th and 7th AB division in cells on the dorsal surface. Cells were manually segmented for the first six μm of the dorsal surface of three embryos for 9-10 time-points, measuring the average NMY-2 fluorescence signal of cells for every plane. The plane with the highest average signal, typically the apical surface, was taken to represent NMY-2 activity in a cell. The lines shown for 21 cells are based on a natural spline

fit, with random effects for embryos and temporal pseudo-replication and normalization for bleaching effects. The image shows the 95% confidence interval fit over three embryos for ABarppap. The cell had higher NMY-2 intensity according to a Tukey post-hoc test ($p<0.05$) than all other dorsal cells except ABalapap. C: Dorsal movements coincide with ABarppap apical NMY-2 build up. Movement of ABplapap is measured for the three embryos analyzed as in B. The 95% confidence interval of the fit is shown. D. Cellular distances to ABarppap in uncompressed (black) and compressed (red) embryos. The left panel presents distances before the coordinated movements to all cells positioned on average within 14μm in the uncompressed embryos, which includes all known touching cells. The right panel shows the distances after the coordinated movements moments before the 7th division of the AB lineage. The star indicates significance at the 5% FDR level.

Figure 5

A. Reconstructions of the dorsal surface of a compressed embryo, based on imaging of the cell membrane (PH::mCherry fusion protein). The embryo is oriented with its posterior side up. Left: the state just after the 6th AB division round, just before the 7th. Relevant cells and lineages are colored. The white arrow indicates the movement of the ABarppap cell, the lilac arrows indicate egression movements, the orange arrows indicate the movements of the ABpla and ABplp lineages. ABarppap's sister ABarppaa, the pink cell immediately anterior to ABarppap (the white cell), moves and distorts together with ABarppap, but does not show NMY-2 foci.

B. Slice through the reconstructed embryos at the height of the ABarppap cell, illustrating the displacement and shape change of ABarppap, as well as the egression of ABarpapp. Part of the MS lineage ingression movements are also visible.

Tables

Table 1

Ingressing cells with time of ingression initiation. The cells were scored as ingressing based on an automated analysis and then confirmed as correct and complete by 2 manual annotators in both compressed and uncompressed embryos (RJ and BL). The mean times of ingression are

given as minutes after the 4-cell stage. The list is complete and confirmed in various embryos until 150 minutes. The following cells were previously reported to ingress (Harrell and Goldstein, 2011), but were not confirmed in the 5 compressed and 5 uncompressed embryos we considered: ABprpaap, ABalpaapp. ABplppap ingresses only in some embryos. Ingression of ABprppap was not reported earlier (Harrell and Goldstein, 2011).

Name	**Time**	**Name**	**Time**
Ep	48	ABaraappp	128
Ea	50	ABaraapap	129
ABalaap	80	Dap	130
ABalpppp	90	ABaraapaa	134.5
ABalpppa	90	ABaraaaap	138
ABarappp	96	Cappa	139
ABplppap	103	ABaraappa	139.5
MSpaaa	107	MSaapap	143
MSaaap	107	MSaapaa	143
ABprppap	107	ABarapaap	143
MSpppp	108	Cppap	147
MSapap	108	Capaa	147
Z2	109	MSpapap	148
MSapaa	109	Cappp	149.5
MSppap	111	Cppaa	150
MSappp	111	MSpapaa	151
MSpaap	111.5	Cpppp	151
Z3	112	Capap	152.5
MSppaa	114	Cpppa	153
MSappa	115	ABalpaaap	156.5

MSaaaa	117	ABprpapaa	158
MSpppa	117.5	MSaappp	162
Dpa	126	MSpappp	178
Daa	126	MSpappa	178
Dpp	128	MSaappa	181

Table 2

Egressing cells with moment of egression completion. As for the egressing cells, we identified cells based on an automated analysis and then 2 manual annotators confirmed as correct and complete the listing in both compressed and uncompressed embryos. The mean times of egression are given as minutes after the 4-cell stage and the list is complete and confirmed in various embryos until 150 minutes. These egressing cells were not previously annotated. Cells that egress after a previous ingression of the same or mother cell are marked with an asterisk (*). ABarpppppp and ABarpppppa appear to egress in only a subset of embryos.

Name	Time	Name	Time
ABarpapa	104.5	ABalpppap*	136
ABarpapp	106	ABarpppppp	137
ABalaapp*	110	ABarpppppa	137
ABalappa	112	ABalpppaa*	140.5
ABalaapa*	117	ABalppppp*	141
ABarapppp*	128.5	ABalapppa*	146
ABarapppa*	129.5	ABalappap	146
ABpraappp	131	ABalppppa*	148
ABpraappa	134	ABalapppp*	149.5

Supplementary data file 1. A zipped file containing the tracked nuclear positions and names of all nuclei at all time points for the embryos analyzed in this manuscript in Starrynite format (Murray et al., 2006). Dataset compressed-1 is from (Bao et al., 2006)

Materials and methods

C. elegans maintenance and strains

All strains were maintained under standard conditions at 20°C (Brenner, 1974). Wild-type is the Bristol N2 strain. Animals expressing histone-GFP fusion proteins (Murray et al., 2006) were fed on OP50-seeded nematode growth media (NGM) plates and maintained by daily transferring the worms to maintain a healthy, not-starved population with high-quality eggs. To track the nuclei during embryogenesis we used RW10029 [zuIs178 [his-72::HIS-72::SRPVAT::GFP + unc-119(+)], provided by the Caenorhabditis Genetics Center (University of Minnesota, Minneapolis). Non-muscular myosin was observed using a cross between OD70, ltIs44pAA173; [pie-1p-mCherry::PH(PLC1delta1) + unc-119(+)], and JJ1473, zuIs45 [nmy-2::NMY-2::GFP + unc-119(+)], kindly shared by Zhirong Bao of the Sloan Kettering Institute.

Time-lapse fluorescence microscopy

Eggs isolated from young adults on day three and four after hatching were used for imaging. For embryo extraction, young adult worms were picked and placed into a drop of Boyd buffer/methyl cellulose in a clean, pre-chilled watch glass, as described (Murray et al., 2006). Early stage embryos were selected.

Embryos compressed for imaging were transferred onto a slide with 20μm polystyrene beads diluted 1:30 in Boyd's buffer. This reduced the diameter of the eggs, thus obtaining a more intense fluorescence signal at the deeper planes whilst avoiding damage to the eggs (Murray et al., 2006). Imaging was performed using an upright Leica TCS SP5 confocal microscope with a 63x 1.4NA Plan Apo oil immersion objective using the 488-nm excitation line of a 65 mW argon laser. To enhance the detected signal while minimizing the required excitation, a relatively large pinhole size (2 Airy units), high detector gain (1,000–1,100), and an amplifier gain of 1.25 were used. To minimize signal loss, we used the RSP 500 filter in the light path. To balance between noise and imaging speed, we used a scan speed of 400Hz and bidirectional scanning. Due to spherical aberration, the GFP fluorescence signal decreases rapidly towards the far side of the embryo. To compensate this, we used the "auto Z" feature to increase laser power by 50% with scanning depth through the embryo. Images were arranged so that the anterior–posterior axis was aligned in parallel to the x-axis of the image. Each 2D image consists of 712 × 512 pixels (pixel size 130 nm) and each z-stack consists of 26-36 (typically 28) slices with a spacing of 1.0 μm. Stacks were taken every 1 minute resulting in a ≈ 12 -gigabyte series for a 10h time-lapse. The

temperature for the samples was kept between 23-25 degrees Celsius.

Uncompressed embryos were imaged using a bespoke Selective Plane Illumination Microscope (SPIM) (Huisken et al., 2004; Swoger et al., 2011). For SPIM imaging of the uncompressed HIS-72::GFP embryos, an argon ion laser with a 488 nm line was used. A fixed Keplerian telescope couples the output from the slit into a cylindrical lens, which focuses the light in one dimension into the back focal plane of the 10x 0.3NA PL FLUOTAR illumination objective lens. The output of this objective forms the selective plane of illumination at the position of the sample. The detection arm of the SPIM consists of an a 63x 0.9NA water dipping objective lens, a filter wheel to block scattered excitation wavelengths, a tube lens, and a cooled CCD camera (C4742-80- 12AG, Hamamatsu Photonics K.K.). The objective and tube lenses form a telecentric optical system that images the plane of interest in the sample onto the sensitive area of the camera. The microscope system was computer controlled through a program written in the Labview programming language (National Instruments).

For imaging the embryos in the SPIM, the sample was mounted in low-gelling-temperature agarose on a 5mm diameter cover slip. The cover slip was glued to a glass rod and lowered into the sample chamber from above. This rod was attached to a manipulation stage that allows translation of the sample with sub-micron precision along the 3 principle axes of the instrument. The sample was scanned with a z-spacing of 1.0 μm and a pixel size of 100nm.

Cell Irradiation

For the irradiation of selected cells we used a strong IR laser (Mai Tai BB Deep See (Spectra Physics) tunable (710-990nm) pulsed laser used at 910nm, average power > 1.35 W), normally used for multi-photon excitation of fluorophores and imaging. The system was connected to the Leica TCS SP5 Upright CFS microscope run under LAS-AF software, which was also used for normal imaging of the compressed embryos as described above. First an embryo was followed with normal confocal microscopy through the early phases of development to identify the correct cell to target. By using multi-positioning and placing two embryos in a field of view, up to 6 embryos could be irradiated in a single time-lapse. A targeted nucleus was irradiated 3 times at 10 distributed points (bleach points in the LEICA software) where the laser beam was parked for 50ms each. After the irradiation the embryos were imaged, as described above, at least until completion of the 7th division of the AB lineage.

To identify an adequate strength for the irradiation we systematically irradiated cells in embryos and followed the effects over time. The effects were scored on the defects on the mitosis of the radiated cell, with delays and aberrant effects such as lagging chromosomes, but also a lack

of phenotypes in the surrounding cells during several division rounds. To transfer settings from one session to another, we needed to take into account that the effective strength of the laser can vary due to subtle changes in the alignment of the system. Therefore we calibrated the beam's strength prior to every experiment by imaging a fluorescent slide and fixing the read out intensity to a predetermined value. The required intensity was initially determined.

Image processing and cell tracking

After cropping the time-lapse images in ImageJ (http://rsbweb.nih.gov/ij/) we reduced the noise corruption of our images through fast interscale wavelet denoising as implemented in an ImageJ plugin (Luisier et al., 2010). To retrieve the nuclei positions over time we employed a segmentation and tracking algorithm based on a model-evolution approach (Meijering et al., 2009) the details of which were described before (Dzyubachyk et al., 2010). Briefly, the key idea of the approach is that each cell is represented by a surface, which is iteratively optimized (evolved) to fit a nucleus region in the image stack at one time point, and then used as the initial state for the fitting procedure in the next time point. The general-purpose cell tracking and segmentation algorithm described in (Dzyubachyk et al., 2010) has been customized for tracking *C. elegans* embryogenesis by, in particular, compensating for inter-scan motion for each nucleus, targeted detection and handling of cell divisions and recovery of false negatives. Such a model-evolution approach results in simultaneous tracking and segmentation of each nucleus at each time point. To prevent the propagation of errors, the algorithm allows the user to manually curate the segmentation and tracking and then restart the automated tracking at any time point. The algorithm was developed in the MATLAB (TheMathWorks, USA) environment, where the most computationally demanding operations were implemented in C/C++. Tracking results are exported in the StarryNite format (Bao et al., 2006).

Manual lineage correction and analysis

Lineage outputs from the tracking algorithm were analyzed with a custom-made lineager program. The program allows visual evaluation of the output lineages against the recorded images and correction of errors. After editing the lineage, cells were named automatically according to the canonical naming scheme (Sulston et al., 1983). The naming algorithm aligns the observed division angles of the nuclei to a set of manually named wild-type lineages. For every division the naming of the daughters is decided based on a majority vote. From the corrected lineages we determined cell cycle timings, division angles, cell movements, and cell positions relative to wild-type embryos. By both statistical analysis and the visual inspection of the plotted statistics across time and cell types we detected relevant deviations from wild-type.

Further analysis was carried out manually, visualizing the embryos in a 3D simulator in our lineaging software. In this program all cells can be visualized, highlighting different lineages. Different embryos can be overlayed, to facilitate comparisons.

Statistics and analysis

To evaluate observed differences in movements and timings between divisions, we employed hypothesis testing using ANOVA and an F-test in R. For figures with tests for multiple cells (e.g. Figure 1C), p-values were corrected for multiple testing through the Benjamini-Hochberg procedure.

For the median normalized misplacement of Figure 1D, embryos were aligned according to inferred cellular axes. First, the anterior-posterior axis was inferred to coincide with the first principal component of a principal component analysis over all nuclear positions in the first 100 minutes of development. The dorsal-ventral axis is orthogonal to anterior-posterior axis and proceeding through the center of the cells of the MS lineage that mark the ventral surface. The left-right axis is defined as orthogonal to the other two axis. The directionality of the axes is defined by the location of specific cells whose identity is readily derived from the invariant lineage, for AP, DV, LR respectively P2, MS (or descendants), and ABpr (or descendants). After aligning the embryos, size differences along the axes are normalized by scaling to the range.

For figures 2D and E we performed a linear regression in R, with a statistical analysis assuming normally distributed residuals. The relative misplacement measure used for this figure represents differences between uncompressed and compressed embryos in the distances between a selected cell and all the other cells at a given time. The measure is based on the mean cell-to-cell distance matrix between all cells. This matrix is calculated for both compressed and uncompressed embryos after which the difference between the two matrices is taken. As a single measure of misplacement for every cell, the standard deviation per row of the difference matrix is calculated. This measure captures the frequency and size of distance differences to the other cells between the two embryo states. This method does not depend on estimating the correct axes in the embryo and should be a robust overall measure of misplacement.

For figure 4B we generated a dataset of NMY-2 intensities based on manual segmentation of cells based on PH marked cell membranes, over 10 time points and five planes (spanning the full depth of most cells). The resulting segmentations for all planes were mapped to the appropriate cells. The data were analyzed in R, where we normalized for differences between embryos and overall signal decrease over time due to bleaching. The *nlme* package was then used to fit a mixed effects model to the data, taking into account a random effect for embryos and modeling

temporal pseudo-replication (corARMA(q=2)). For the lines in the figure a fixed effect over time was fit using a natural cubic spline (3 or 4 degrees of freedom, excluding intercept). To test significant differences between cells, a model was fit with the same random effects as before, but now using strain ID as a predictor. Significant differences where subsequently tested using a Tukey post-hoc test.

Identification of egressing and traversing cells

To identify ingressing and egressing cells the cell positions of the nuclei tracked embryos was used. A voronoi decomposition was used to identify cells that have contact with the exterior. Then we looked for cells that acquired or lost contact with the exterior for extended periods of time. These cells were then checked for ingression or egression manually by two observers (RJ; BL).

Reconstruction of cell shapes

Based on confocal images highlighting the cell membranes with the PH::mCherry fusion protein, we manually reconstructed the cells in the embryo, e.g. as shown in Figure 5. The TrakEM2 tool (Cardona et al., 2012) which is part of FIJI (Schindelin et al., 2012) was used for the manual tracking.

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Author contributions

RJ performed the experiments and analyses. RJ and BL conceived the study, interpreted the results, and wrote the manuscript. AK contributed early experiments. JS built the SPIM microscope and performed the SPIM measurements. TZ conceived the ablation methodology. AK, JS, TZ are listed in alphabetical order.

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