H⁺/K⁺ Ion Pump Enhances Cytoskeletal Polarity to Drive Gastrulation in Sea Urchin Embryo

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RESEARCH ARTICLE

TITLE: H⁺/K⁺ ion pump enhances cytoskeletal polarity to drive gastrulation in sea urchin embryo

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ABSTRACT

Gastrulation is a universal process in the morphogenesis of many animal embryos. In sea urchin embryos, it involves the invagination of a single-layered vegetal plate into the blastocoel. Although morphological and molecular events in gastrulation have been well studied, the mechanical driving forces and the regulatory mechanism underlying gastrulation is not fully understood. In this study, structural features and cytoskeletal distributions were studied in sea urchin embryos using an “exogastrulation” model induced by inhibiting the H^+/K^+ ion pump with omeprazole. The vegetal poles of the exogastrulating embryos showed reduced roundness indices, intracellular pH polarization, and intracellular F-actin polarization at the pre-early gastrulation stage compared with normal embryos. Gastrulation stopped when F-actin polymerization or degradation was inhibited via *RhoA* or *YAP1* knockout, although pH distributions were independent of such a knockout. A mathematical model of sea urchin embryos at the early gastrulation reproduced the shapes of both normal and exogastrulating embryos using cell-dependent cytoskeletal features based on F-actin and pH distributions. Thus, gastrulation required appropriate cell position-dependent intracellular F-actin distributions regulated by the H^+/K^+ ion pump through pH control.

INTRODUCTION

Gastrulation is a universal and essential morphogenic process in various animals, wherein a ball of single-layered cells (blastula) differentiates into a multilayered gastrula or early embryo [1-3]. Species-specific variations in this process provide the basis for particular animal morphologies and lead to the generation of internal organs. The basic mechanism of whole embryonic transformations induced by organizational rearrangements and cell movements are expected to be universal.

Sea urchin embryos are commonly used for studying various morphogenetic processes universally observed in animals, such as the left-right symmetry breakage [4-7], neural network formations [8-10], and gastrulation [11-15] due to its evolutionary position as an ancestral deuterostome. Furthermore, the gene regulatory network controlling endomesoderm specification in sea urchin embryos have been well studied [16,17]. Sea urchin gastrulation progresses through the following four steps [14]: Step 1 (after hatching), the embryo becomes an epithelial monolayer with a thickened vegetal pole; step 2 (pre-gastrulation), the vegetal plate bends to invade the blastocoel; step 3 (early gastrulation), the tubular archenteron is formed, and secondary mesenchyme cells (SMCs) appear at the tip; step 4 (mid-late gastrulation), the archenteron elongates by pulling on the filopodia of the SMC. Steps 1 and 2 refer to the primary invagination stages, and steps 3 and 4 are those of the secondary invagination stages [11,18].

Embryos of some sea urchin species exhibit anomalous morphogenesis (exogastrulation) under various perturbations where the vegetal plate of the embryo prolapses outward instead of invaginating. For example, the archenteron of embryos

cultured with LiCl or sugar is completely evaginated due to the depolymerization of SMC microtubules in the blastocoel space connecting the primary intestine and ectoderm [11,13,19]. These studies suggest that lifting the primary intestine from the mid-gut by microtubules plays an important role in step 4 of gastrulation. Exogastrulation was also observed via Rab35 knockdown, which was also suggested to disturb whole embryonic cytoskeleton distribution [20].

Moreover, the vegetal plate bending in step 2 of normally cultured embryos of the sea urchin, *Lytechinus pictus* (LP), occurs even when it is surgically isolated [21]. It is suggested that the tip of the archenteron should be bottle-shaped [22], and the area around the vegetal plate should be wedge-shaped [23] to initiate primary invagination [14]. Additionally, mathematical models of vegetal poles suggest that primary invagination occurs under the appropriate force conditions for “apical constriction,” “cell tractor,” “apical contractile ring,” “apicobasal contraction,” and “gel swelling” [24,25]. However, it is unclear which of these effects are essential for development up to pre-early gastrulation (steps 2 and 3).

In this study, the roles of the dominant factors in steps 2 and 3 of sea urchin gastrulation were determined through experimental analyses and mathematical modeling. First, specific inhibition of H^+/K^+ ion pump activity by omeprazole caused “partial” exogastrulation without the loss of microtubules in the blastocoel space of *Hemicentrotus pulcherrimus* (HP) embryos. The H^+/K^+ ion pump regulates the pH in cells and the pH influences the structure and force of the actomyosin system [26]. Therefore, the partial exogastrulation was likely due to anomalous cytoskeletal

behaviors. Second, knockout experiments of cytoskeleton-related molecules were performed in HP embryos and analyzed using fluorescence imaging. Third, simulations of morphogenic processes of normal and H^+/K^+ ion pump-inhibited (exhibiting exogastrulation) HP embryos during the pre-early gastrulation were performed using a mathematical model utilizing the cytoskeletal force parameters determined using the above experiments. The modeled shapes of the H^+/K^+ ion pump-inhibited *Lytechinus variegatus* (LV) embryos, were compared to those of HP to reveal the contributions of the H^+/K^+ ion pump to the regulation of cytoskeletal behaviors.

RESULTS

H^+/K^+ ion pump inhibition in HP embryos caused partial exogastrulation

The development of HP embryos exposed to the specific H^+/K^+ ion pump inhibitor, omeprazole, immediately after fertilization exhibited anomalous morphogenesis with partial exogastrulation at the primary-secondary invagination stages and 36 h post fertilization (hpf) (Fig. 1). Detailed comparisons of the structural features around the vegetal pole between the normal (control) and inhibited embryos at each gastrulation step were performed. In step 1, control and inhibited embryos exhibited equivalent vegetal pole thickening (18-20 hpf). In step 2, primary invagination occurred in both control and inhibited embryos at the same time. However, intestinal invagination was slightly shallower in the inhibited embryos than that in the control embryos (20-22 hpf). In step 3, inhibited embryos did not show further elongation of the archenteron into the blastocoel, and the shape of inhibited embryos elongated along the animal-vegetal axis

(22-24 hpf). In step 4 (secondary invagination stage), the outward protrusion of the vegetal pole side was more pronounced in the inhibited embryo (24 hpf). The inhibited embryo had a comparatively little elongation of the archenteron into the blastocoel compared to the control embryos. At 36 hpf, “partial” exogastrulation, in which the tip of the archenteron folded inward due to normal primary invagination but the remaining part extended outward during the secondary invagination, was observed in the inhibited embryo.

The migration of SMCs was observed in the control embryos and in the inhibited embryos (Fig. 2) indicating that filopodia were formed. Previously reported exogastrulation was induced by the loss of pseudopodia [11,19,27]; therefore, partial exogastrulation in this report was expected to occur through a different mechanism.

H⁺/K⁺ ion pump inhibition perturbed whole embryonic pH and cytoskeletal distributions

The intracellular distribution of F-actin and pH was analyzed at the primary invagination stage (steps 1 and 2) to reveal the mechanism underlying the morphological changes in the inhibited embryos at the secondary invagination stage (steps 3 and 4), (Fig. 3a, b). F-actin was visualized via fluorescence imaging using actinin-GFP, and intracellular pH was estimated using a pH indicator; fluorescent intensity increased as pH decreased. The polarity of intracellular F-actin and pH distribution was evaluated by estimating the fluorescence intensity ratio between the apical and basal sides of cells.

Similar apical/basal ratio distributions of the actinin-GFP signal were obtained between the control and inhibited embryos, while the ratios gradually increased closer to the vegetal pole in both embryos at step 1 (Fig. 3c, d, Fig. S1a, b). The distribution of the apical/basal ratios of actinin-GFP signal intensities in step 2 was similar between control and inhibited embryos only in the animal half (Fig. 3e, Fig. S1c). The ratio of the control embryos at the equator of the vegetal pole was larger than that of inhibited embryos, whereas close to the vegetal pole, control embryos showed a smaller ratio than the inhibited embryos. Therefore, the formation of an anomalous embryonic shape of inhibited embryos correlated with the deviation of intracellular F-actin polarity in the vegetal half of the embryos during primary invagination.

The apical/basal ratio of the pH indicator was greater than one in both control and inhibited embryos in step 2 (Fig. 3f, Fig. S1d, S2) indicating that the pH of the apical side of each cell was always lower than that on the basal side. Additionally, this ratio was higher in the vegetal half than that in the animal half of the control embryo, while this pattern disappeared in the inhibited embryo (Fig. 3f, Fig. S2). Thus, the polarity of the pH indicator fluorescent intensity was positively correlated with that of actinin-GFP signal intensity in whole embryos (Fig. 3e, f).

Meanwhile, inhibition of the H^+/K^+ ion pump did not influence the whole embryonic distribution of fibropellin-1 (Fig. 3g, h), an F-actin scaffold in the apical lamina of the apical pole [22,28].

Gastrulation starts but does not progress in cytoskeleton regulator-knockout

embryos

CRISPR-Cas9-mediated knockout of the typical enhancer and repression of F-actin formation (*RhoA* and *YAP1*) [29,30] were performed via microinjection of sgRNAs designed using *Cas9* mRNA (Fig. S3) to elucidate the contribution of F-actin to gastrulation. Although the mutation frequencies of *RhoA* and *YAP1* knockouts were 55.6 and 77.8%, respectively, and the frameshift rate was 22.3% in either knockout (Fig. S3), gastrulation stopped at step 2 at the primary invagination stage in *RhoA* or *YAP1* knockout embryos (Fig. 4a); normal gastrulation and exogastrulation were not observed. Interestingly, the phenotypes between these knockout embryos were similar, although the gene functions were different (Fig. S3). Pigmented cells were observed at 45 hpf, indicating that development had not ceased. This showed that primary invagination occurred even in embryos with mild perturbation of F-actin function, but subsequent gastrulation processes required appropriate F-actin-derived forces.

The whole embryonic distribution of average intensity and apical/basal ratios of the pH indicator were unchanged in *RhoA*-knockout embryos compared with those in the control, and therefore, perturbation of F-actin polymerization did not affect the pH gradient of the cell (Fig. 4b, c).

Mathematical model of embryonic shape formations in early gastrulation considering cell-dependent F-actin polarity

A mathematical model of cell motion at the cross-section including animal and vegetal poles during steps 1-3 of gastrulation was constructed to examine the influence of

intracellular F-actin distribution on the formation of embryonic shapes. The model was constructed based on the gastrulation model of *Nematostella vectensis* [31] that consisted of cells constructed using springs and beads circularly connected to form a two-dimensional cross-section of the embryo. The following assumptions were made based on experimentally known facts.

I) Each embryo contained three cell types as follows: Pigment cells [32], wedge cells [23], and other cells (Fig. 5). The pigment cells near the cavity entrance were assumed to be bottle-shaped [32] because of the site-specific force in these cells. This may explain the bending of the vegetal plate in F-actin polymerization regulator-knockout embryos (Fig. 4), or embryos with a surgically removed animal pole [21]. The apical length was sufficiently shorter than the basal length and the apical sides of wedge cells were wider than the basal side (Fig. 5a) to form a similar shape as previously observed embryos [14].

II) The width of the apical and basal sides changed in a cell-dependent manner in control and inhibited embryos due to cytoskeletal forces generated by F-actin. The apical side elongation was expected to correlate with F-actin concentration because higher F-actin concentration was expected to generate stronger cell cortical forces. Thus, the lengths of the apical and basal sides of cells, except pigment cells and wedge cells, were determined based on their apical/basal ratios of actinin-GFP signal intensities at step 2 (Fig. 5a).

III) Cell divisions and three-dimensional mutual cell invasions mainly contributing to late gastrulation were not included because these processes were rarely observed in

early gastrulation [33]. Therefore, the present model described embryo shape dynamics of the control and inhibited embryos introducing two-dimensional motions and deformations of the apical and basal sides in 64 cells (Fig. 5a).

Simulations showed different shapes (Fig. 5b), although each shape was similar to that experimentally observed at step 3 (Fig. 1). Similar temporal changes in roundness indices (Fig. 5c) of control embryos (0.85 ~ 0.9) and inhibited embryos (0.75 ~ 0.8) were observed in the simulated (Fig. 5d, Fig. S4a) and experimental data (Fig. 5e, Fig. S4b).

The simulation where the elongation ratio of the apical side of each cell was a little larger than that of control was also performed, named “over-polarized embryo” (Fig. 5a, b). The whole embryonic shape of this model showed in a wider and larger roundness index than the control embryos (Fig. S4a). This suggested that the apical/basal ratio polarity of F-actin distribution in each cell was sensitive to the whole embryonic shape.

DISCUSSION

Inhibition of the H^+/K^+ ion pump resulted in abnormal embryo shape with partial exogastrulation due to intracellular apical F-actin distribution at the vegetal pole (Fig. 3, 5). F-actin concentrations generating cell cortical forces were significantly larger at the apical side of each cell than that of the basal side in the vegetal half of the control embryos, except for cells at the vegetal pole. Therefore, the apical planes of wedge cells and other cells in the vegetal pole side were expected to be significantly wider than the basal planes. Conversely, the apical plane of cells in the vegetal half of the inhibited

embryo were not significantly wider compared to the basal sides due to lower apical F-actin levels. Simulation results showed that such expected features in the vegetal half may lead to normal early gastrulation in control embryos and partial exogastrulation in inhibited embryos (Fig. 5). Therefore, appropriate embryonic intracellular apical F-actin distribution in the vegetal half is required for gastrulation.

One recent study suggested that an outer intestinal embryo is formed upon Rab35 knockdown that may disturb the whole embryonic intracellular F-actin polarity [20]. The results of the present study support this suggestion, and further indicate that the intracellular F-actin polarity on the vegetal pole side of the embryo is particularly important for accurate gastrulation and the intracellular pH controlled by H^+/K^+ ion pump is involved in this process.

Gastrulation does not proceed when apical lamina is inhibited [23]. The whole embryonic distributions of apical lamina along cells were nearly identical between the control and inhibited embryos, although large differences were observed between the cell shapes in the vegetal half and whole embryos. This indicated that the generated force was independent of the relative amounts of the apical lamina in each cell. Embryos with a partial knockout of F-actin-regulating factors (*RhoA* or *YAPI*) showed inhibited gastrulation at the primary invagination step (Fig. 4a, b). This indicated that the secondary invagination was inhibited without appropriately regulated F-actin, even if the apical lamina existed. Therefore, we suggested that the apical lamina is the scaffold for apical F-actin to generate the force required for deformation processes driving the secondary invagination of gastrulation and whole embryonic deformation.

CRISPR-Cas9-mediated *RhoA* knockout showed an intermediate level (55.6%) of mutation frequency (Fig. S3) and the knockout embryos exhibited primary invagination. However, LV embryos expressing dominant-negative *RhoA* failed to initiate primary invagination [29]. Therefore, the contribution of F-actin is considerable for the primary invagination, but the extent of the dependency on F-actin function is small and a mechanism other than the F-actin-related system might be involved in this initial process. As mentioned above, F-actin function must be essential for secondary invagination.

F-actin network formation, generating cell cortical forces, was drastically enhanced with decreased pH *in vitro* [26]. The lower pH at the apical side compared with the basal side was maintained by H⁺/K⁺ ion pump activity. Whole embryonic distributions of apical/basal ratios of the pH indicator at step 2 correlated with those of intracellular actinin-GFP signal intensities (Fig. 3d, f), suggesting that F-actin concentration was high when the pH was low at the apical side of cells. Additionally, the apical/basal F-actin polarity in each cell significantly decreased in the vegetal half of the embryo upon inhibiting H⁺/K⁺ ion pump activity (Fig. 3e). Conversely, the whole embryonic distribution of intracellular pH and polarity was unchanged even when F-actin polymerization was directly perturbed by the knockout of F-actin-regulating factors (Fig. 4d). This suggested that the intracellular pH controlled by the H⁺/K⁺ ion pump is a one-sided regulator of F-actin polymerization.

Inhibition of the H⁺/K⁺ ion pump did not influence the whole embryonic distribution of the apical lamina (Fig. 3g, h). This suggested that the regulation of

F-actin intracellular distribution by pH is independent of the apical lamina. The embryo exhibited partial exogastrulation if the intracellular pH polarity at the primary invagination stage was weakened upon inhibiting H^+/K^+ ion pump activity. This indicated that H^+/K^+ ion pump activity in the primary invagination stage provided a dominant contribution to the progression of the normal secondary invagination to form appropriate intracellular F-actin polarity in the whole embryo.

The influence of pH on F-actin network formation and force generation is a universal biochemical feature confirmed via *in vitro* experiments [26]. Inhibition of H^+/K^+ ion pump activity significantly decreases intracellular pH in the LV embryo [34], while here, an increase in the intracellular pH was observed in the majority of cells in the HP embryo (Fig. S5). A decrease in the intracellular pH of LV may induce an increase in the apical/basal polarities of intracellular pH and F-actin concentration similar to the “over-polarized embryo” model. The over-polarized embryo model exhibited a wider embryonic shape with a larger roundness index than the control embryos (Fig. 5b, S4b), which is consistent with LV imaging [34]. This supported the suggestion that H^+/K^+ ion pump activity significantly contributed to early gastrulation through the regulation of intracellular F-actin apical/basal polarizations.

Omeprazole treatment of sea urchin embryos disrupts the left-right asymmetric formations of the adult rudiment although gastrulation is completed [35-37]. The left-right asymmetric *nodal* gene expression observed immediately after gastrulation is important for establishing this asymmetry [36]. Frogs, chickens, zebrafish, and ascidians show disrupted left-right asymmetry upon inhibition of H^+/K^+ ion pump

activity [38-40]. Disrupted left-right asymmetry in HP is observed at lower (~ 3/5 times) omeprazole concentrations [37], different to what was observed in the present study. Inhibition of H^+/K^+ pump activity using dilute omeprazole solutions induced weak perturbation of gut formation in the present study, which may disrupt cell-cell interactions and regulate whole embryonic *nodal* gene expression.

This report revealed the close relationship between pH, F-actin, and H^+/K^+ ion pump activity inducing normal/abnormal gastrulation in sea urchins through intracellular F-actin regulation. Embryonic F-actin distribution on the apical side of each cell is important for normal gastrulation, and H^+/K^+ ion pump activity may be involved in F-actin distribution. This finding provides key insights into the evolutionary relationship between development and morphogenesis. Intracellular apical/basal polarity of pH showed different cellular features between vegetal and animal poles in step 2 of gastrulation. However, the formation of these position-dependent intracellular states was not explored. Therefore, upstream regulators of H^+/K^+ ion pump activity should be investigated to reveal the detailed molecular mechanism of morphogenesis. The universal effect of inhibiting H^+/K^+ ion pump activity on early embryo formation among different animals will be tested in the future.

MATERIALS AND METHODS

Animals and embryos

Adult Japanese sea urchins (HP) were collected from the Seto Inland Sea or Tateyama Bay. Eggs and sperms were obtained via coelomic injection of 0.55 M KCl. Fertilized

eggs were cultured in filtered seawater at 16°C.

Inhibition of the H⁺/K⁺ ion pump by omeprazole

A 100 mM stock solution of omeprazole (FUJIFILM Wako Pure Chemical Corporation, Japan) dissolved in dimethyl sulfoxide (DMSO) was immediately added to fertilized eggs at a final concentration of 100 µM and cultured at 16°C.

***In situ* nucleus and F-actin visualization in embryos**

Sea urchin embryos were fixed by immersing in fixative III (4% paraformaldehyde, 32.5% filtered seawater, 32.5 mM 3-(N-morpholino)propane sulfonic acid (MOPS), pH 7.0, 162.5 mM NaCl) for 16 h at 4°C. Embryos were washed three times using 0.5 mL 1x phosphate-buffered saline (PBS), stored in 100% ethanol at -20°C, and used within 1 week. Fixed embryos were washed three times with 1x PBS, permeabilized with 0.5% Triton X-100 for 20 min at room temperature, washed three times with 1 x PBS, and stained with 100 nM phalloidin (in 1 x PBS) for 45 min at room temperature. Stained embryos were washed three times with 1x PBS, mounted using SlowFade Gold antifade mountant with DAPI (Molecular Probes), and observed using confocal laser scanning microscopy (Carl Zeiss LSM 700, Germany) with laser illumination at 405/435 nm excitation/emission for DAPI and 555/573 nm excitation/emission for phalloidin, respectively.

Live imaging of pH, actinin, and apical lamina

Intracellular pH of sea urchin embryos was visualized using pHrodo Red AM intracellular pH indicator or 5-(and-6)-carboxy SNARF-1 (C-1270) (ThermoFisher Scientific, USA) at final concentrations of 10 and 5 μ M, respectively, and stained at 16°C for 30 min. The embryos were washed in filtered seawater and observed using confocal microscopy with laser illumination at 555/585 nm excitation/emission for pHrodo and 555/573 nm excitation/emission for SNARF-1, respectively.

Actinin and fibropellin-1 were visualized following the fusion of the proteins with GFP. RNA was extracted from mesenchyme blastula stage HP embryos using ISOGEN (Nippongene, Japan) according to the manufacturer's instructions. Actinin- and fibropellin-1-coding sequences were amplified via RT-PCR using SuperScript™ III Reverse Transcriptase (Thermo Fisher Scientific, USA) with the primers stated in Table S1 and cloned into pGreenLantern2-derived plasmid at EcoRI and XhoI restriction sites. mRNA (actinin-GFP, fibropellin-1-GFP, in that order) was synthesized *in vitro* using an mMESSAGE mMACHINE T7 ultra transcription kit (ThermoFisher Scientific, USA) and purified using an RNeasy mini kit (Qiagen, Nederland). mRNA was microinjected into fertilized eggs as described by Liu et al. [41].

Fluorescent images were acquired by confocal microscopy using an excitation of 488 nm, and emission of 515 nm. The images of the animal and vegetal poles of the embryo were analyzed based on the z-stacked image with the largest area by averaging seven z-axis images at a total thickness of 6 μ m.

Quantification of fluorescent signals

Fluorescent images of embryos were acquired according to the angle θ ($0^\circ \sim 180^\circ$) between the vegetal and animal poles along the circumference (Fig. S6) and transformed into a band-like image using the "Polar Transformer" function (<https://imagej.nih.gov/ij/plugins/polar-transformer.html>) of ImageJ 2.1.0. Each band-like image was filtered using a median filter (radius = 1.0). The filtered images were binarized using "Mean" (pHrodo indicator), "IsoData" (actinin), and "Triangle" (fibropellin-1 and SNARF-1) functions, respectively, to obtain the cellular regions of the embryo. The apical/basal ratios of the pH indicator, actin-GFP, and fibropellin-1-GFP for θ were determined using average fluorescent intensities over the regions at a width of 3.126 μm from the apical and basal sides (Fig. S6).

The fluorescent intensity of the pHrodo indicator was defined as the average fluorescent intensity over the intracellular region at a width of 6.232 μm from the apical side (Fig. S6). Fluorescent signal intensity values were estimated by calculating the ratio between the observed fluorescence intensity and the average background fluorescence intensity around the entire embryo.

F-actin and apical lamina were stained via mRNA microinjections of actinin-GFP and fibropellin-1-GFP into fertilized eggs. Nonnegligible variations was inevitable in the concentration of injected mRNA among fertilized egg samples, which would be amplified during development. Therefore, only the apical/basal ratios were used to evaluate the intracellular features of F-actin and apical lamina distributions.

Two data points were obtained for each θ from each fluorescence image of the embryo since the left and right sides of the embryo were considered axis symmetric

against the animal-vegetal axis. Both data were used to estimate the sample average and 95% confidence interval of each value, where the number of samples (n) was given as 2 × [number of observed embryos].

Gene knockout using CRISPR-Cas9

Knock out of endogenous *RhoA* and *YAP1* in HP was performed using the method described by Liu et al. [41]. The oligonucleotide sequences used for sgRNA preparations are listed in Table S2.

Heteroduplex mobility assay (HMA) and DNA sequencing

Analysis of knockout embryos using HMA and DNA sequencing was performed as previously published by Liu et al. [41]. The primer sequences used to amplify each target region are listed in Table S3.

Mathematical model of sea urchin embryos

A two-dimensional particle model describing cellular motion at the cross-section of animal poles and vegetal poles of sea urchin embryos during steps 1 to 3 of gastrulation was constructed. The determined number of cells was based on that at the equatorial plane of the embryo at the blastula stage [33]. The following assumptions were made: 1) each cell was represented by 16 particles with radii (r) of 1.125 μm, and each embryo was represented by 64 cells connected in a ring (Fig. S7); 2) each cell perimeter was 36 μm and the height and width of the embryo at step 1 were 110 and 100 μm, respectively,

which was consistent with sea urchin embryo observations (results not shown); 3) the number of cells was constant because cell divisions and cell invasions from other cross-sections were rarely observed during the primary invagination stage [33]; 4) the motion of each particle obeyed the overdamped limit of the equation (1) of motion,

$$\frac{\partial \mathbf{X}_{ij}}{\partial t} = -\frac{1}{\gamma} \frac{\partial V}{\partial \mathbf{X}_{ij}} \quad (1)$$

where $\mathbf{X}_{ij} = (x_{i,j}(t), y_{i,j}(t))$ is the position of the j -th particle constructing the i -th cell ($i = 0, 1, 2, \dots, 63$ and $j = 0, 1, 2, \dots, 15$) on the x-y plane at time t (Fig. 5, Fig. S7), γ is the coefficient of the drag force acting on each particle, and V is the potential of the entire system; 5) the y-axis was parallel to the animal-vegetal axis of the embryo model.

We rewrote $\frac{V}{\gamma} = U$ and U was calculated as follows:

$$U = U_{bond} + U_{collision} + U_{actin} + U_{adhesion} + U_{ca} + U_{ea} \quad (2)$$

where U_{bond} is the elastic force potential between each neighboring pair of particles in each cell to maintain each cell perimeter, denoted as follows:

$$U_{bond} = \sum_{i=0}^{63} \sum_{j=0}^{15} \frac{k_b}{2} (d_{(i,j),(i,j+1)} - 2r)^2 \quad (3)$$

where k_b , $d_{(i,j),(i,j+1)}$, and r are the coefficient of elasticity, the distance between the j -th and $j+1$ -th particle ($j+1 = 0$ for $j = 15$) in the i -th cell at time t , and the particle radius, respectively.

$U_{collision}$ is the potential of excluded volume effects among all particles denoted as follows:

$$U_{collision} = \sum_{i < k} \sum_{j < l} \theta(2r - d_{(i,j),(k,l)}) \frac{k_c}{2} (2r - d_{(i,j),(k,l)})^2 \quad (4)$$

412 where k_c is the coefficient of repulsion between two particles, and $\theta(x)$ is the
 413 Heaviside step function.
 414 U_{actin} is the elastic force potential to form and sustain cell shape with the expansion
 415 and contraction of the apical and basal sides by the cytoskeleton, denoted as follows:
 416
$$U_{actin} = \sum_i \frac{k_a}{2} ((d_{(i,10),(i,14)} - l_i^a)^2 + (d_{(i,2),(i,6)} - l_i^b)^2) \quad (5)$$

 417 where the apical and basal sides of the i -th cell consisted of particles with $j = 10 - 14$
 418 and $j = 2 - 6$, respectively. The wideness of the apical and basal sides at time t were
 419 given by $d_{(i,10),(i,14)}$ and $d_{(i,2),(i,6)}$, respectively. The basic wideness of the apical and
 420 basal sides were given by l_i^a and l_i^b , respectively. k_a was assumed by the coefficient
 421 of elasticity to sustain the wideness of the apical and basal sides of the cell.
 422 $U_{adhesion}$ is the potential force for cell adhesion by proteins, such as cadherin, denoted
 423 as follows:
 424
$$U_{adhesion} = \sum_i \sum_j \frac{k_h}{2} (d_{(i,j),(i+1,k(j))} - 2r)^2 \quad (6),$$

 425 where k_h is the coefficient for the adhesive forces between the particles of the i -th and
 426 $i + 1$ -th cells ($i + 1 = 0$ for $i = 63$), and $k(j) \equiv 6, 7, 8, 9, 10$ for $j \equiv 2, 1, 0, 15, 14$,
 427 respectively (Fig. S7).
 428 U_{ca} is the potential of the forces to conserve each cell area, denoted as follows:
 429
$$U_{ca} = \sum_i \frac{k_{ca}}{2} (s_i - s_i^{ini})^2 \quad (7),$$

 430 where k_{ca} is the coefficient of elasticity required to maintain each cell area. The area
 431 of the i -th cell was estimated by $s_i = \sum_{j=0}^{15} |\mathbf{X}_{t,j} - \mathbf{X}_i^{cc}|$ with $\mathbf{X}_i^{cc} = \frac{1}{16} \sum_{j=0}^{15} \mathbf{X}_{t,j}$, and

432 s_i^{ini} referred to s_i given by $\mathbf{X}_{i,j}$ at the initial state.

433 U_{ea} is the potential of the forces to maintain the area (volume) of the sea urchin

434 embryo, denoted as follows:

435
$$U_{ea} = \frac{k_{ea}}{2}(S - S^{ini})^2 \quad (8),$$

436 where k_{ea} is the coefficient of elasticity required to maintain embryo area. The area

437 was estimated by $S = \sum_{i=0}^{63} |\mathbf{X}_i^{cc} - \mathbf{X}^{ec}|$ with $\mathbf{X}^{ec} = \frac{1}{64} \sum_{i=0}^{63} \mathbf{X}_i^{cc}$, and S^{ini} referred to

438 S given by $\mathbf{X}_{i,j}$ at the initial state.

439 U_{ca} and U_{ea} were considered alternatives for the volume-conserving forces in real

440 3-D cells and embryos as internal pressures in each cell and embryo were considered to

441 be isotropic.

442

443 **Simulation method for the mathematical model**

444 The simulations of the present mathematical model were performed through the

445 integrals of the equation of motion (1) using the Euler method at time intervals of

446 0.000064 h with conserved $\mathbf{X}^{ec} = (0, 0)$. In all models, the parameters

447 k_{bond} , $k_{collision}$, k_{actin} , $k_{adhesion}$, k_{ca} , and k_{ea} were given empirically as 9375, 6250,

448 8125, 8125, 625, and 0.00625 hour⁻¹, respectively, because there were no experiments to

449 measure or estimate them. The qualitative features of the results were independent of

450 the details of these values if the order was maintained. The model formed the embryo

451 shape at step 1 of gastrulation if the appropriate cell type-dependent values of l_i^a and

452 l_i^b , $= l_i^{a,ini}$ and $l_i^{b,ini}$ were given for the models of pigment cells, wedge cells, and

other cells (Fig. 5). This configuration gave the particle positions $\mathbf{X}_{i,j}$ at time $t = 0$ (initial configuration) in all simulations.

The early gastrulation processes were simulated by the change in l_i^a and l_i^b respectively, from $l_i^{a,ini}$ and $l_i^{b,ini}$ to $l_i^{a,fin}$ and $l_i^{b,fin}$ at time $t = 0$. Here, $l_i^{a,fin}$ and $l_i^{b,fin}$ were assumed to obey $l_i^{a,fin} + l_i^{b,fin} = l_i^{a,ini} + l_i^{b,ini} = \text{constant}$ among cells except pigment cells based on the expectation that F-actin was constant. The model showed similar structural behaviors to the early gastrulation as the relaxation process of $d_{(i,10),(i,14)} \rightarrow l_i^{a,fin}$ and $d_{(i,2),(i,6)} \rightarrow l_i^{b,fin}$.

Statistical analysis

All experiments were performed independently twice or more with four replicates or more per experiment. Statistical test was performed using SciPy library (<https://www.scipy.org/>). The roundness index of the vegetal side of each embryo was evaluated using the following: [embryo width half-way between the bottom and middle of the embryo]/[embryo length] (Fig. 5c).

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475

476 **Competing interests**

477 No competing interests declared.

478

479 **Author contributions**

480 K.W., N.S., and A.A. conceived and designed the study; K.W., Y.K., and N.S. conducted

481 the experiments; K.W., Y.Y., M.F., and A.A. analyzed the data; K.W. and A.A.

482 conducted the mathematical model construction and simulations; K.W., N.S., and A.A.

483 wrote the manuscript with support from all authors; T.Y. supervised the work.

484

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492

493 **Data availability**

494 The datasets generated during and/or analysed during the current study are available

495 from the corresponding author on reasonable request.

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653 **Figures**

Fig. 1. Developmental stages of control and H^+/K^+ ion pump-inhibited HP embryos.

Typical bright-field images of the gastrulation process (steps 1-4) and 36 hpf embryos (scale bars: 30 μ m). Arrows indicate vegetal pole positions. Gastrulation did not progress after step 2 in the inhibited embryos, and the outward protrusion of the vegetal pole side was more pronounced at step 4. At 36 hpf, “partial” exogastrulation was observed in the inhibited embryo.

Fig. 2. *In situ* SMC visualization of HP embryo development with inhibited H^+/K^+ ion pumps. Confocal microscopy images at 24 hpf (a), 27 hpf (b), and 29 hpf (c) (scale bars: 25 μ m). The nucleus and F-actin in the embryos were stained with DAPI (magenta) and phalloidin (cyan), respectively. SMC migration was confirmed at all developmental stages as indicated by red triangles.

Fig. 3. Whole embryonic distributions of pH, actinin-GFP, and fibropellin-1-GFP.

(A-B) Confocal fluorescence microscopy images of co-stained intracellular pH (pH indicator: red) and actinin (actinin-GFP: green) at gastrulation step 1 (a) and step 2 (b) on the cross-section of animal poles and vegetal poles of control and inhibited embryos (scale bars: 30 μ m). (c) Definition of angle θ (0°~180°) from the vegetal pole (0°) to the animal pole (180°) along the circumference of the embryo cross-section. (d-f) Average apical/basal ratios and 95% confidence intervals (error bars) at angle θ of actinin-GFP

intensities at step 1 **(d)** and step 2 **(e)**, pH indicator intensities at step 2 **(f)**, and fibropellin-1-GFP intensities at step 2 **(g)**. Blue and orange curves and bars represent the control and inhibited embryo values, respectively. Gray bars indicate significantly different average values between the control and inhibited embryos according to Welch's t-test ($p < 0.05$) (see also Fig. S1.). The correlation coefficients of apical/basal ratios between actinin-GFP intensities **(e)** and pH indicator intensities **(f)** in control and inhibited embryos were 0.56 and 0.64, respectively. **(h)** Fluorescence images of fibropellin-1-GFP from control and inhibited embryos at gastrulation step 2 (scale bars: 30 μm).

Fig. 4. Effect of F-actin regulator knockout on gastrulation.

(a) Bright-field images of gastrulation (steps 2-3) in control, *RhoA*-knockout, and *YAP1*-knockout embryos at selected time-points. PMCs and pigment cells were observed in all embryos suggesting that development did not stop. The knockout embryos did not form the structure like prism larva observed at 45 hpf (step 3) in the control embryo (scale bars: 30 μ m). (b-c) Average fluorescence intensities (arbitrary

fluorescence units) and 95% confidence intervals (error bars) of intracellular pH indicator **(b)** and apical/basal ratio of pH indicator **(c)** of *RhoA*-knockout embryos and control embryos as a function of angle θ . The indications of colors and θ are stated in Fig. 3 and Fig. S1.

Fig. 5. Coarse-grained model and simulation of control, inhibited, and

over-polarized embryos. (a) Modeled cell lengths of apical and basal sides of control, inhibited, and over-polarized embryos; $l_i^{a,fin}$ and $l_i^{b,fin}$ refer to the final length of the apical and basal sides of the i -th cell, respectively (see Materials and methods). Distributions of the final length of the former two models were determined based on the distributions of apical/basal ratios of actinin-GFP intensities from control and inhibited embryos in step 2. The top center panel was a modification of Fig. 3d. Red, green, and blue circles in the top panel and panel **(b)** represent pigment cells, wedge cells, and other cells, respectively. **(b)** Snapshots of the initial embryo shape at 18 and 26 hpf of the three models. **(c)** Definition of the roundness of the vegetal side of embryos from simulation results and imaging. **(d-e)** Roundness indices of vegetal sides of modeled control and inhibited embryos **(d)** and experimentally determined values (E). Blue and orange # refer to the number of control and inhibited embryos at each time point, error bars indicated 95% confidence intervals, and * indicated that the roundness of control embryos was significantly larger than that of inhibited embryos according to Welch's t-test ($p < 0.05$, see Fig. S4b).

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