RESEARCH ARTICLE

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

Running title: H⁺/K⁺ ion pump and gastrulation

Kaichi Watanabe^{1,*}, Yuhei Yasui¹, Yuta Kurose², Masashi Fujii¹, Takashi Yamamoto¹,
Naoaki Sakamoto^{1,*}, Akinori Awazu^{1,3,*}

¹ Graduate School of Integrated Sciences for Life, Hiroshima University, Higashi-Hiroshima, 739-8526, Japan

² Department of Mathematical and Life Sciences, Graduate School of Science, Hiroshima University, Higashi-Hiroshima, 739-8526, Japan

³ Research Center for the Mathematics on Chromatin Live Dynamics, Hiroshima University, Higashi-Hiroshima, Hiroshima, 739-8526, Japan

*Corresponding authors (kaichi1849@hiroshima-u.ac.jp (K.W.); naosaka@hiroshima-u.ac.jp (N. S.); awa@hiroshima-u.ac.jp (A. A.))

KEY WORDS: Gastrulation, H⁺/K⁺ ion pump, sea urchin, cytoskeleton, pH regulation

Summary statement: The H⁺/K⁺ ion pump is critical for normal gastrulation in sea urchin through pH regulation of cell-position dependent intracellular actin distributions.

ABSTRACT

Gastrulation is a universal process in the morphogenesis of many animal embryos. In sea urchin embryos, it involves the invagination of single-layered vegetal plate into blastocoel. Although morphological and molecular events have been well studied for gastrulation, the mechanical driving forces and their regulatory mechanism underlying the gastrulation is not fully understood. In this study, structural features and cytoskeletal distributions were studied in sea urchin embryo using an “exogastrulation” model induced by inhibiting the H^+/K^+ ion pump with omeprazole. The vegetal pole sides of the exogastrulating embryos had reduced roundness indices, intracellular pH polarization, and intracellular F-actin polarization at the pre-early gastrulation compared with the normal embryo. Gastrulation stopped when F-actin polymerization or degradation was inhibited by *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 (Serrano Nájera and Weijer, 2020; Shindo, 2018; Stower and Bertocchini, 2017). Species-specific variations in this process provides the basis for particular animal morphologies and leads 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 (Aihara and Amemia, 2001; McCain and McClay, 1994; Takemoto et al., 2016; Warner and McClay, 2014), neural network formations (Burke et al., 2014; McClay et al., 2018; Yaguchi et al., 2010), and gastrulation (Dan and Okazaki, 1956; Gustafson and Kinnander, 1960; Hardin and Cheng, 1986; Kominami and Takata, 2004; Martik and McClay, 2017) due to its evolutionary position as an ancestral deuterostome. Furthermore, the gene regulatory network controlling endomesoderm specification in sea urchin embryo has been well studied (Davidson et al., 2002; Oliveri & Davidson, 2004). Sea urchin gastrulation progresses through the following four steps (Kominami and Takata, 2004): 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 (Dan and Okazaki, 1956; Gustafson and Kinnander, 1956).

The 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 (Dan and Okazaki, 1956; Hardin and Cheng, 1986; Khurram et al., 2004). 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 by Rab35 knockdown that was also suggested to disturb whole embryonic cytoskeleton distribution (Remsburg et al., 2021).

Moreover, bending of the vegetal plate in step 2 of normally cultured embryos of the sea urchin, *Lytechinus pictus* (LP), occurs even when it is surgically isolated (Ettensohn, 1984). It is suggested that the tip of the archenteron should be bottle-shaped (Nakajima and Burke, 1996), and the area around the vegetal plate should be wedge-shaped (Burke et al., 1991) to initiate primary invagination (Kominami and Takata, 2004). 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” (Davidson et al., 1995; Odell et al., 1981). 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 gastrulation of sea urchins 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 embryos from the sea urchin, *Hemicentrotus pulcherrimus* (HP). The H^+/K^+ ion pump regulates the pH in cells and pH influences the structure and force of the actomyosin system (Köhler et al., 2012). Therefore, the partial exogastrulation was likely due to anomalous cytoskeletal behaviors. Second, knockout experiments on cytoskeleton-related molecules were performed in HP embryos and analyzed by fluorescence imaging. Third, simulations of morphogenic process of normal and H^+/K^+ ion pump inhibited (exhibiting exogastrulation) HP embryo during the pre-early gastrulation were performed using a mathematical model utilizing the cytoskeletal force parameters determined by the above experiments. The modeled shapes of the H^+/K^+ ion pump-inhibited embryos of the sea urchin, *Lytechinus variegatus* (LV,) 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 observed at the primary-secondary invagination stages and 36 hours 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 are as follows. 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 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 comparatively little elongation of the archenteron into the blastocoel compared to the control embryos. At 36 hpf, “partial” exogastrulation, in which the tip of archenteron folded inward by the 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 (Dan and Okazaki, 1956; Hoshi, 1979; Khurram et al., 2004), therefore, partial exogastrulation in this report was expected to occur by 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 by fluorescence of actinin-GFP, and intracellular pH was estimated with pH indicator, whose fluorescent intensity increased as pH decreased. The polarity of intracellular F-actin and pH distribution was evaluated by estimating the fluorescence intensities ratio between the apical and basal sides of cells.

Similar apical/basal ratio distributions of actinin-GFP intensities 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 intensities in step 2 were similar between control and inhibited embryos only in the animal half (Fig. 3E, Fig. S1C). The ratios of the control embryos from the equator to the vegetal pole was larger than that of inhibited embryos, while nearest the vegetal pole, control embryos showed smaller ratio than that of 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 in 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 on 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 in the control embryo, while this pattern disappeared in the inhibited embryo (Fig. 3F, Fig. S2). Thus, the polarity of the pH indicator fluorescent intensities was positively correlated with that of actinin-GFP 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), a F-actin scaffold in the apical lamina of the apical pole (Burke et al., 1998; Nakajima and Burke, 1996).

Gastrulation starts but does not progress in embryos with knocked out cytoskeleton regulators

CRISPR-Cas9-mediated knockout of the typical enhancer and repressor of F-actin formation (*RhoA* and *YAP1*) (Beane et al., 2006; Dupont et al., 2011) was performed by microinjection of sgRNAs designed with *Cas9* mRNA (Fig. S3) to elucidate the contribution of F-actin to the gastrulation process. Although the mutation frequencies of *RhoA* and *YAP1* knockout were 55.6% and 77.8%, respectively, and the frameshift rate was 22.3% in either knockout (Fig. S3), the gastrulation stopped at step 2 at the primary invagination stage in either *RhoA* or *YAP1* knockout embryos (Fig. 4A) and normal gastrulation and exogastrulation were not observed. Interestingly, the phenotypes between these gene knockout embryos were similar, although the gene functions are 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 was unchanged in *RhoA*-knockout embryos compared with 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 that of gastrulation in *Nematostella vectensis* (Tamulonis et al., 2011) that consisted of cells constructed by springs and beads circularly connected to form a 2-dimensional cross section of the embryo. The following assumptions were made based on experimentally known facts.

I) Each embryo contained three cell types: pigment cells (Takata and Kominami, 2004), wedge cells (Burke et al., 1991), and other cells (Fig. 5). The pigment cells near the entrance to the cavity were assumed to be bottle-shaped (Takata and Kominami, 2004) because of the site-specific force in these cells. This may explain the bending of the vegetal plate occurring in embryos with knocked out F-actin polymerization regulators (Fig. 4), or a surgically removed animal pole (Ettensohn, 1984). 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 to previously observed embryos (Kominami and Takata, 2004).

II) The width of the apical and basal sides changes 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 intensities at step 2 (Fig. 5A).

III) The cell divisions and 3-dimensional mutual cell invasions mainly contributing to late gastrulation were not included because these processes were rarely observed in early gastrulation (Mizoguchi, 1999). Therefore, the present model described embryo shape dynamics of the control and inhibited embryos as 2-dimensional motions and deformations of the apical and basal sides of 64 cells (Fig. 5A).

The simulations showed different shapes (Fig. 5B), although each shape was like 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 data (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, with this model named: “over-polarized

embryo” (Fig. 5A, B). The whole embryonic shape of this model resulted 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. The simulation results showed that such expected features in the vegetal half may progress to normal early gastrulation in the control embryos and partial exogastrulation in the inhibited embryos (Fig. 5). Therefore, appropriate embryonic intracellular apical F-actin distribution in the vegetal half is required for gastrulation.

One recent study suggested an outer intestinal embryo is formed by Rab35 knockdown that might disturb the whole embryonic intracellular F-actin polarity (Remsburg et al., 2021). 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 (Burke et al., 1991).

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 apical lamina in each cell.

Embryos with partial knockout of F-actin regulating factors (*RhoA* or *YAPI*) stopped the

gastrulation at the primary invagination step (Fig. 4A, B). This indicated that the

secondary invagination is inhibited without appropriately regulated F-actin, even if the

apical lamina exists. Therefore, the apical lamina was suggested to be the scaffold for

apical F-actin to generate the force required for the deformation processes driving the

secondary invagination of gastrulation and whole embryonic deformation.

CRISPR-Cas9-mediated *RhoA* knockout showed intermediate level (55.6%) of

mutation frequency (Fig. S3) and the knockout embryos exhibited the primary

invagination. However, LV embryos expressing dominant negative RhoA failed to

initiate primary invagination (Beane et al., 2006). Therefore, contribution of F-actin

function is considerable for the primary invagination, but the extent of the dependency

on F-actin function is less 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 the secondary invagination.

The F-actin network formation generating cell cortical forces was drastically

enhanced with decreased pH *in vitro* (Köhler et al., 2012) (25). 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 pH indicator at step 2 correlated with those of intracellular actinin-GFP (Fig. 3D, F) suggesting that the 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 by 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 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 on 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 by 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 progress 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 by *in vitro* experiments (Köhler et al., 2012). Inhibition of H⁺/K⁺ ion pump activity significantly decreases intracellular pH in the LV

embryo (Schatzberg et al., 2015), while an increase in the intracellular pH was observed in the majority of cells in the HP embryo (Fig. S5). Decreases in the intracellular pH of LV may induce increases in the apical/basal polarities of intracellular pH and F-actin concentration like 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 (Schatzberg et al., 2015). 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 (Bessodes et al., 2012; Duboc et al., 2005; Hibino et al., 2006). The left-right asymmetric *nodal* gene expression observed immediately after gastrulation is important for establishing this asymmetry (Duboc et al., 2005). Frogs, chickens, zebrafish, and ascidians disrupt left-right asymmetry by inhibiting H^+/K^+ ion pump activity (Kawakami et al., 2005; Levin et al., 2002; Shimeld and Levin, 2006). Disrupted left-right asymmetry in HP is observed at lower (~ 3/5 times) omeprazole concentrations (Hibino et al., 2006) than those in this study. Inhibition of H^+/K^+ pump activity by dilute omeprazole solutions induced weak perturbation of gut formation in this 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 was 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 morphogenesis molecular mechanism. 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 Seto Inland Sea or Tateyama Bay. Eggs and sperm were obtained by coelomic injection of 0.55 M KCl. Fertilized eggs were cultured in filtered seawater at 16°C.

Inhibition of 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 to a final concentration of 100 μ M and cultured at 16°C.

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

Sea urchin embryos were fixed by immersion 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 1 x phosphate buffered saline (PBS), stored in 100% ethanol at -20°C, and used within 1 week. Fixed embryos were washed three times with 1 x 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 1 x PBS, mounted in SlowFade Gold antifade mountant with DAPI (Molecular Probes) and observed using a 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 µM and 5 µM, 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 m excitation/emission for SNARF-1, respectively.

Actinin and fibropellin-1 were visualized following 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 by 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 restriction sites EcoRI and XhoI. 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., (2019).

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*-stack image with the largest area by averaging seven *z*-axis images with a total thickness of 6 μm.

Quantification of experimental images

The fluorescent images of embryos were acquired according to the angle θ (0°~180°) 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 with the width of 3.126 μm from the apical and basal sides.

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

F-actin and apical lamina were stained by mRNA microinjections of actinin-GFP and fibropellin-1-GFP to 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 by 2 $\times$ [number of observed embryos].

Gene knockout by CRISPR-Cas9

Knock out of endogenous *RhoA* and *YAP1* in HP was performed using the method

described by Liu et al., (2019). The oligonucleotide sequences used in sgRNA

preparations were listed in Table S2.

Heteroduplex mobility assay (HMA) and DNA sequencing analysis

Analysis of knockout embryos by HMA and DNA sequencing was performed according

to Liu et al., (2019). The primer sequences used to amplify each target region were

listed in Table S3.

Mathematical model of sea urchin embryos

A 2-dimensional particles 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 (Mizoguchi, 1999). The following assumptions

were made: 1) each cell was represented by 16 particles with radii (r) of $1.125\ \mu\text{m}$, and

each embryo was represented by 64 cells connected in a ring (Fig. S7). 2) Each cell

perimeter was $36\ \mu\text{m}$ and the height and width of the embryo at step 1 was $110\ \mu\text{m}$ and

$100\ \mu\text{m}$, 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 (Mizoguchi, 1999). 4) The motion of each particle obeyed the

following overdamped limit of the equation 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 by:

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

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

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

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:

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

where k_c is the coefficient of repulsion between two particles, and $\theta(x)$ is the Heaviside step function.

U_{actin} is the elastic force potential to form and sustain cell shape with the expansion and contraction of the apical and basal sides by the cytoskeleton denoted as follows:

$$U_{actin} = \sum_i \frac{k_a}{2} ((d_{(i,10),(i,14)} - l_i^a)^2 + (d_{(i,2),(i,6)} - l_i^b)^2) \quad (5),$$

where the apical and basal sides of the i -th cell consisted of particles with $j = 10 - 14$ and $j = 2 - 6$, respectively. The wideness of the apical and basal sides at time t were given by $d_{(i,10),(i,14)}$ and $d_{(i,2),(i,6)}$, respectively. The basic wideness of the apical and basal sides were given by l_i^a and l_i^b , respectively. k_a was assumed by the coefficient of elasticity to sustain the wideness of the apical and basal sides of the cell.

$U_{adhesion}$ is the potential force for cell adhesion by proteins such as cadherin, and is denoted as follows:

$$U_{adhesion} = \sum_i \sum_j \frac{k_h}{2} (d_{(i,j),(i+1,k(j))} - 2r)^2 \quad (6),$$

where k_h is the coefficient for the adhesive forces between the particles of the i -th and $i + 1$ -th cells ($i + 1 = 0$ for $i = 63$), and $k(j) \equiv 6, 7, 8, 9, 10$ for $j \equiv 2, 1, 0, 15, 14$, respectively (Fig. S7).

U_{ca} is the potential of the forces to conserve each cell area denoted as follows:

$$U_{ca} = \sum_i \frac{k_{ca}}{2} (s_i - s_i^{ini})^2 \quad (7),$$

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

U_{ea} is the potential of the forces to maintain the area (volume) of the sea urchin embryo and denoted as follows:

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

where k_{ea} is the coefficient of elasticity required to maintain embryo area. The area 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 S given by $\mathbf{X}_{i,j}$ at the initial state. U_{ca} and U_{ea} were considered the alternatives of the volume-conserving forces in real 3-D cells and embryos as internal pressures in each cell and embryo are considered isotropic.

Simulation method of mathematical model

The simulations of the present mathematical model were performed through the integrals of equation of motion (1) using Euler method with time intervals of 0.000064 h with conserved $\mathbf{X}^{ec} = (0, 0)$. In all models, the parameters k_{bond} , $k_{collision}$, k_{actin} , $k_{adhesion}$, k_{ca} , and k_{ea} were given empirically as 9375, 6250, 8125, 8125, 625, and 0.00625 hour⁻¹, respectively, because there were no experiments to measure or estimate them. The qualitative features of the results were independent of the details of these values if the order was maintained. The model formed the embryo shape at step 1 of gastrulation if the appropriate cell type-dependent values of l_i^a and 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 2 times or more with 4 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 by: [embryo width half-way between the bottom and middle of the embryo]/[embryo length] (Fig. 5C).

Acknowledgements

We thank Prof. Masato Kiyomoto (Tateyama Marine Laboratory, Ochanomizu University) for supplying live sea urchins. We would like to thank Editage (www.editage.com) for English language editing.

Competing interests

No competing interests declared.

Author Contributions

K.W., N.S., and A.A. conceived and designed the study; K.W., Y.K., and N.S. conducted the experiments; K.W., Y.Y., M.F., and A.A. analyzed the data; K.W. and A.A. conducted the mathematical model construction and simulations; K.W., N.S., and A.A. wrote the manuscript with support from all authors; T.Y. supervised the work.

Funding information

This work was supported by JSPS KAKENHI [grant, award number: 17K05614, 21K06124 to A.A.]; and JSPS KAKENHI [grant, award number: 17K07241, 20K06602 to N.S.]; JSPS KAKENHI [grant, award number: 19K20382 to M.F.]; SASAGAWA SCIENTIFIC RESERCH GRANT, [award number: 2021-4023, to K.W.]; Hiroshima University Graduate School Research Fellowship, 2021-2024 (InformationAI Field, to K.W.);.

References

- Aihara M. and Amemiya, S.** (2001). Left-right positioning of the adult rudiment in sea urchin larvae is directed by the right side. *Development* **128**(24), 4935-4948.
doi:10.1242/dev.128.24.4935
- Beane, W. S., Gross, J. M. and McClay, D. R.** (2006). RhoA regulates initiation of invagination, but not convergent extension, during sea urchin gastrulation. *Dev. Biol.* **292**(1), 213-225. doi:10.1016/j.ydbio.2005.12.031
- Bessodes, N., Haillot, E., Duboc, V., Röttinger, E., Lahaye, F. and Lepage, T.**

(2012). Reciprocal signaling between the ectoderm and a mesendodermal left-

right organizer directs left-right determination in the sea urchin embryo. *PLoS*

Genet. **8**(12), e1003121. doi:10.1371/journal.pgen.1003121

Burke, R. D., Lail, M. and Nakajima, Y. (1998). The apical lamina and its role in cell

adhesion in sea urchin embryos. *Cell Adhesion Comm.* **5**(2), 97-108.

doi:10.3109/15419069809040284

Burke, R. D., Moller, D. J., Krupke, O. A. and Taylor, V. J. (2014). Sea urchin

neural development and the metazoan paradigm of neurogenesis. *Genesis* **52**(3),

208-221. doi:10.1002/dvg.22750

Burke, R. D., Myers, R. L., Sexton, T. L. and Jackson, C. (1991). Cell movements

during the initial phase of gastrulation in the sea urchin embryo. *Dev. Biol.*

146(2), 542-557. doi:10.1016/0012-1606(91)90255-2

Dan, K. and Okazaki, K. (1956). Cytoembryological studies of sea urchins: III. Role

of the secondary mesenchyme cells in the formation of the primitive gut in sea

urchin larvae. *Biol. Bull.* **110**, 29-42.

Davidson, E. H., Rast, J. P., Oliveri, P., Ransick, A., Calestani, C., Yuh, C. H.,

Minokawa, T., Amore, G., Hinman, V., Arenas-Mena, C., Otim, O., Brown,

C. T., Livi, C. B., Lee, P. Y., Revilla, R., Schilstra, M. J., Clarke, P. J., Rust,

A. G., Pan, Z., Arnone, M. I., Rowen, L., Cameron, R. A., McClay, D. R.,

Hood, L. and Bolouri, H. (2002). A provisional regulatory gene network for

specification of endomesoderm in the sea urchin embryo. *Dev. Biol.*, **246**(1), 162-

190. doi: 10.1006/dbio.2002.0635

- 540 **Davidson, L. A., Koehl, M. A., Keller, R. and Oster, G. F. (1995).** How do sea
541 urchins invaginate? Using biomechanics to distinguish between mechanisms of
542 primary invagination. *Development* **121**(7), 2005-2018.
543 doi:10.1242/dev.121.7.2005
- 544 **Duboc, V., Röttinger, E., Lapraz, F., Besnardeau, L. and Lepage, T. (2005).** Left-
545 right asymmetry in the sea urchin embryo is regulated by nodal signaling on the
546 right side. *Dev. Cell.* **9**(1), 147-158. doi:10.1016/j.devcel.2005.05.008
- 547 **Dupont, S., Morsut, L., Aragona, M., Enzo, E., Giulitti, S., Cordenonsi, M.,**
548 **Zanconato, F., Le Digabel, J., Forcato, M., Biciato, S. et al. (2011).** Role of
549 YAP/TAZ in mechanotransduction. *Nature* **474**(7350), 179-183.
550 doi:10.1038/nature10137
- 551 **Ettensohn, C. A. (1984).** Primary invagination of the vegetal plate during sea urchin
552 gastrulation. *Amer. Zool.* **24**(3), 571-588. doi:10.1093/icb/24.3.571
- 553 **Gustafson, T. and Kinnander, H. (1956).** Microaquaria for time-lapse
554 cinematographic studies of morphogenesis in swimming larvae and observations
555 on sea urchin gastrulation. *Exp. Cell Res.* **11**(1), 36-51. doi:10.1016/0014-
556 4827(56)90188-4
- 557 **Gustafson, T. and Kinnander, H. (1960).** Cellular mechanisms in morphogenesis of
558 the sea urchin gastrula: The oral contact. *Exp. Cell Res.* **21**(2), 361-373.
559 doi:10.1016/0014-4827(60)90268-8
- 560 **Hardin, J. D. and Cheng, L. Y. (1986).** The mechanisms and mechanics of
561 archenteron elongation during sea urchin gastrulation. *Dev. Biol.* **115**(2), 490-

562 501. doi:10.1016/0012-1606(86)90269-1

563 **Hibino, T., Ishii, Y., Levin, M. and Nishino, A.** (2006). Ion flow regulates left-right
564 asymmetry in sea urchin development. *Dev. Genes Evol.* **216**(5), 265-276.
565 doi:10.1007/s00427-005-0051-6

566 **Hoshi, M.** (1979). Exogastrulation induced by heavy water in sea urchin larvae. *Cell*
567 *Differ.* **8**(6), 431-5. doi:10.1016/0045-6039(79)90039-3

568 **Kawakami, Y., Raya, Á., Raya, R. M., Rodríguez-Esteban, C. and Izpisúa**
569 **Belmonte, J. C.** (2005). Retinoic acid signalling links left-right asymmetric
570 patterning and bilaterally symmetric somitogenesis in the zebrafish embryo.
571 *Nature* **435**(7039), 165-171. doi:10.1038/nature03512

572 **Khurram, M., Hernandez, A., Eskalaei, M., Badali, O., Coyle-Thompson, C. and**
573 **Oppenheimer, S. B.** (2004). Carbohydrate involvement in cellular interactions in
574 sea urchin gastrulation. *Acta Histochem.* **106**(2), 97-106. doi:
575 10.1016/j.acthis.2004.01.001

576 **Kimberly, E. L. and Hardin, J.** (1998). Bottle cells are required for the initiation of
577 primary invagination in the sea urchin embryo. *Dev. Biol.* **204**(1), 235-50.
578 doi:10.1006/dbio.1998.9075

579 **Köhler, S., Schmoller, K. M., Crevenna, A. H., Bausch, A. R.** (2012). Regulating
580 contractility of the actomyosin cytoskeleton by pH. *Cell Rep.* **2**(3), 433-439.
581 doi:10.1016/j.celrep.2012.08.014

582 **Kominami, T. and Takata, H.** (2004). Gastrulation in the sea urchin embryo: A model
583 system for analyzing the morphogenesis of a monolayered epithelium. *Dev.*

584 *Growth Differ.* **46**(4), 309-326. doi:10.1111/j.1440-169x.2004.00755.x

585 **Levin, M., Thorlin, T., Robinson, K. R., Nogi, T. and Mercola, M.** (2002).

586 Asymmetries in H⁺/K⁺-ATPase and cell membrane potentials comprise a very

587 early step in left-right patterning. *Cell* **111**(1), 77-89. doi:10.1016/S0092-

588 8674(02)00939-X

589 **Liu, D., Awazu, A., Sakuma, T., Yamamoto, T. and Sakamoto, N.** (2019).

590 Establishment of knockout adult sea urchins by using a CRISPR-Cas9 system.

591 *Dev. Growth Differ.* **61**(6), 378-388. doi:10.1111/dgd.12624

592 **Martik, M. L. and McClay, D. R.** (2017). New insights from a high-resolution look at

593 gastrulation in the sea urchin, *Lytechinus variegatus*. *Mech. Dev.* **148**, 3-10.

594 doi:10.1016/j.mod.2017.06.005

595 **McCain, E.R. and McClay, D.R.** (1994). The establishment of bilateral asymmetry in

596 sea urchin embryos. *Development* **120**(2), 395-404. doi:10.1242/dev.120.2.395

597 **McClay, D. R., Miranda, E. and Feinberg, S. L.** (2018). Neurogenesis in the sea

598 urchin embryo is initiated uniquely in three domains. *Development* **145**(21),

599 dev167742. doi: 10.1242/dev.167742

600 **Mizoguchi, H.** (1999). Cell numbers in the gut of the embryo of the sea urchin

601 *Hemicentrotus pulcherrimus*. *Zool. Sci.* **16**(2), 341-344. doi:10.2108/zsj.16.341

602 **Nakajima, Y. and Burke, R. D.** (1996). The initial phase of gastrulation in sea urchins

603 is accompanied by the formation of bottle cells. *Dev. Biol.* **179**(2), 436-446.

604 doi:10.1006/dbio.1996.0273

605 **Odell, G. M., Oster, G., Alberch, P. and Burnside, B.** (1981). The mechanical basis

606 of morphogenesis. I. Epithelial folding and invagination. *Dev. Biol.* **85**(2), 46-62.
607 doi:10.1016/0012-1606(81)90276-1

608 **Oliveri, P. and Davidson, E. H.** (2004). Gene regulatory network controlling
609 embryonic specification in the sea urchin. *Curr. Opin. Genet. Dev.*, **14**(4), 351–
610 360. doi: 10.1016/j.gde.2004.06.004

611 **Rensburg, C., Testa, M., Song, J. L.** (2021). Rab35 regulates skeletogenesis and
612 gastrulation by facilitating actin remodeling and vesicular trafficking. *Cells &*
613 *Dev.*, **165**(3), 203660. doi: 10.1016/j.cdev.2021.203660

614 **Schatzberg, D., Lawton, M., Hadyniak, S. E., Ross, E. J., Carney, T., Beane, W. S.,**
615 **Levin, M. and Bradham, C. A.** (2015). H⁺/K⁺ ATPase activity is required for
616 biomineralization in sea urchin embryos. *Dev. Biol.* **406**(2), 259-270.
617 doi:10.1016/j.ydbio.2015.08.014

618 **Serrano Nájera, G. and Weijer, C. J.** (2020). Cellular processes driving gastrulation
619 in the avian embryo. *Mech. Dev.* **163**, 103624. doi:10.1016/j.mod.2020.103624

620 **Shimeld, S. M. and Levin, M.** (2006). Evidence for the regulation of left-right
621 asymmetry in *Ciona intestinalis* by ion flux. *Dev. Dyn.* **235**(6), 1543-1553.
622 doi:10.1002/dvdy.20792

623 **Shindo, A.** (2018). Models of convergent extension during morphogenesis. (2018).
624 *WIREs Dev. Biol.* **7**(1), e293. doi:10.1002/wdev.293

625 **Stower, M. J. and Bertocchini, F.** (2017). The evolution of amniote gastrulation: the
626 blastopore-primitive streak transition. *Wiley Interdiscip. Rev. Dev. Biol.* **6**(2),
627 e262. doi: 10.1002/wdev.262

- Takata, H. and Kominami, T.** (2004). Pigment cells trigger the onset of gastrulation in tropical sea urchin *Echinometra mathaei*. *Dev. Growth Differ.* **46**(1), 23-35. doi:10.1111/j.1440-169X.2004.00726.x
- Takemoto, A., Miyamoto, T., Simono, F., Kurogi, N., Shirae-Kurabayashi, M., Awazu, A., Suzuki, K.-i. T. and Sakamoto, N.** (2016). Cilia play a role in breaking left-right symmetry of the sea urchin embryo. *Genes Cells* **21**(6), 568-578. doi:10.1111/gtc.12362
- Tamulonis, C., Postma, M., Marlow, H. Q., Magie, C. R., de Jong, J. and Kaandorp, J.** (2011). A cell-based model of *Nematostella vectensis* gastrulation including bottle cell formation, invagination and zippering. *Dev. Biol.* **351**(1), 217-228. doi:10.1016/j.ydbio.2010.10.017
- Voiculescu, O., Bodenstein, L., Lau, I. J. and Stern, C. D.** (2014). Local cell interactions and self-amplifying individual cell ingression drive amniote gastrulation. *eLife* **3**, e01817. doi: 10.7554/eLife.01817
- Warner, J. F. and McClay, D. R.** (2014). Left-right asymmetry in the sea urchin. *Genesis* **52**(6), 481-487. doi:10.1002/dvg.22752
- Yaguchi, S., Yaguchi, J., Angerer, R. C., Angerer, L. M. and Burke, R. D.** (2010). TGF β signaling positions the ciliary band and patterns neurons in the sea urchin embryo. *Dev. Biol.* **347**(1), 71-81. doi:10.1016/j.ydbio.2010.08.009

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 ($0^\circ \sim 180^\circ$) 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 μ m).

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 meanings of the colors and θ are stated in Fig. 3 and Fig. S1.

(B) Simulated initial embryo shape at 18 hpf

Simulated embryo shapes at 26 hpf

Control embryo Inhibited embryo Over-polarized embryo

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 was 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 hpf and at 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).