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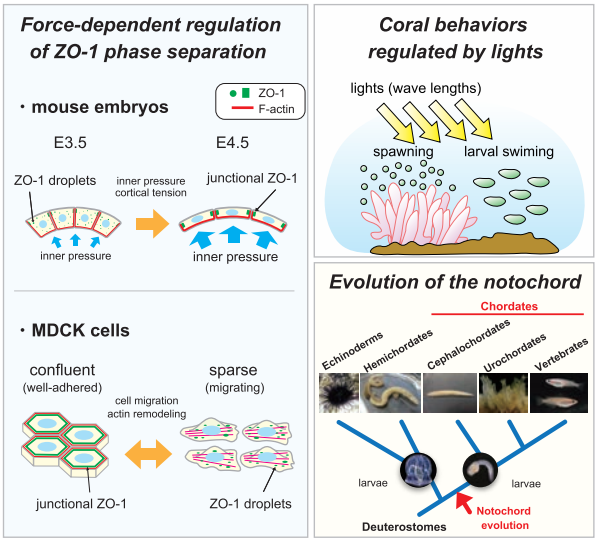
In addition to genes and proteins that are widely known to govern biological phenomena and have been extensively studied over the past several decades, a growing body of evidence now suggests that physical environments that include light, temperature, and internal and external forces influence both cellular and organismal behaviors. In particular, we are currently investigating the contribution of physical forces using mouse embryos and light-dependent behaviors using coral larvae to understand the impact of physical environments on organisms. As reported below, the inner pressure of the blastocyst cavity generates tensile force upon trophectodermal cells in mouse embryos, and enhances their cell junction. During this process, a physical phenomenon, liquid-liquid phase separation (LLPS) of the tight junction protein ZO-1, plays a pivotal role. The coral planula larvae change their swimming activities by sensing types of light of specific wavelengths to settle upon an appropriate place in the sea in which to live. We also investigate the evolution of the notochord using ancestral chordates.



A graphical overview of our laboratory's works.

I. ZO-1 condensates in mouse hatching embryos

Cells sense and respond to not only molecular factors but also mechanical forces, which in turn play various important roles in biological events, such as tissue homeostasis, differentiation, and cell migration. During early embryogenesis, various dynamic morphogenetic movements occur, including the convergent extension of the axial mesoderm and epiboly of the ectoderm. These movements generate physical forces at both the cellular and tissue levels. However, it is still not fully understood how these forces influence morphogenetic processes.

To investigate how embryonic cells respond to mechanical stimuli, we artificially applied mechanical forces to *Xenopus* embryos. We found that tensile force applied to ectodermal tissue induces a mesenchymal-epithelial transition (MET)-like phenotype, enhancing the junctional structure and increasing ectodermal tissue stiffness. Among these changes, we focused on the behavior of the tight junction protein, ZO-1. Before the force application, ZO-1 forms cytoplasmic granules, but the mechanical force induces its accumulation at the tight junction. Significantly, mechanical forces generated by gastrulation movements also induce similar changes in the developing ectodermal tissue.

To examine whether the behavior of ZO-1 protein is conserved across species, in mammals in particular, we first observed the localization of ZO-1 in the mouse embryo. We focused on E3.5 and E4.5 embryos since they hatch out of the zona pellucida (ZP) and expand their shape (Figure 1A). We immunostained E3.5 and E4.5 embryos with a ZO-1 antibody and found that the cells of E3.5 embryos showed a significantly higher number of ZO-1 puncta in the cytoplasm relative to E4.5 embryos. As development proceeded, the surface area of the trophectoderm (TE) cells expanded and became thinner. The number of cytoplasmic ZO-1 puncta was reduced, and ZO-1 signal intensity at the plasma membrane in E4.5 embryos became much higher

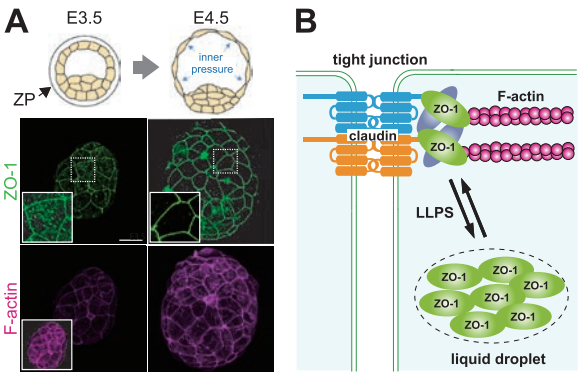


Figure 1. Change of ZO-1 localization in mouse hatching embryos.
A. At E3.5, the embryo is covered with the zona pellucida (ZP). During hatching, the embryo is enlarged and emerges from the ZP. Embryos were stained with an anti-ZO-1 antibody and Alexa Fluor 546 Phalloidin. Scale bar, 20 μm. The inset in the E3.5 phalloidin image was acquired with higher laser power, demonstrating that the structure of cortical F-actin is formed at E3.5 even though the signal intensity was weaker than that in the E4.5 embryo. B. Schematic diagram of ZO-1 behavior shuttling between the tight junction and cytoplasmic droplets.

than those of E3.5 embryos at the expense of their cytoplasmic pool. Importantly, this change coincides well with the accumulation of F-actin in the cell cortex in E4.5 embryos. This result suggests that the shuttling of ZO-1 protein from the cytoplasmic puncta to cell junctions occurred as development progressed.

To confirm that the tensile force induces this ZO-1 behavior in TE cells, we inhibited the expansion of said cells using two methods: one was the Na^+/K^+ ATPase inhibitor, ouabain treatment, and the other was piercing the embryo with a glass needle. Both methods reduced the inner pressure of the cavity and inhibited ZO-1 accumulation in the cell junction. These results suggested that tensile force being applied to the TE cells. Furthermore, we assumed that the ZO-1 puncta in E3.5 embryos were formed by phase separation and treated embryos with 1,6-hexanediol, which is known to dissolve LLPS assembly. As expected, 5% 1,6-hexanediol treatment reduced the number of particles within a few minutes, thus suggesting that these puncta are liquid droplets generated by phase separation, and that mechanical force regulates ZO-1 phase separation (Figure 1B).

II. Interaction with F-actin regulates ZO-1 phase separation

To analyze the nature of ZO-1 liquid droplets in various cell environments, we first expressed GFP-ZO1 in A6 cells. GFP-ZO-1 is mainly localized in the cell periphery and colocalized with F-actin bundles. We thus treated cells with latrunculin B, which disrupted F-actin (Figure 2B). We found that the formation and extinction of cytoplasmic ZO-1 granules depended on the destruction and development of the F-actin network. Furthermore, we confirmed that latrunculin B-induced ZO-1 granules rapidly recovered in the FRAP assay. These observations indicate that the efficiency and growth of ZO-1 condensation are negatively regulated by the interaction with F-actin in A6 cells.

To inhibit the interaction between ZO-1 and F-actin in a different way, we constructed GFP-ZO-1ΔABD, which lacks the actin-binding domain (ABD) (Figure 2A), and expressed it in A6 cells. GFP-ZO-1ΔABD subsequently formed droplets with a smooth surface (Figure 2C). This suggests that cytoplasmic droplet formation is restricted by the binding of ZO-1 to F-actin and that GFP-ZO-1ΔABD lost its capacity for the interaction. It is known that when proteins form liquid droplets, intrinsically disordered regions (IDRs) play an important role in phase separation. In human ZO-1, four IDRs are predicted (Figure 2A). Among these, we found that the C-terminal IDR4, is essential for phase separation. Since IDR4 includes ABD (refer Figure 2A), binding to F-actin may affect the LLPS-inducing activity of the IDR4.

We observed ZO-1 behavior in the wound healing assay using canine MDCK cells. Initially, most of the cells had few condensates in a confluent cell sheet. However, after scratching, cells started active migration toward the wound site, forming a significant number of droplets (Figure 2D). In these cells, F-actin distribution and intensity also changed (Figure 2E). Initially, cortical F-actin was well-developed in the non-migrating polygonal cells with junctional ZO-1. In contrast, migrating cells around the wound site, ones that were forming ZO-1 droplets, reduced cortical actin and developed more stress fibers. This correlation between ZO-1 and F-actin is consistent with our previous finding that the mouse E3.5 embryo with ZO-1 droplets had less F-actin than the E4.5 embryo with junctional ZO-1. Thus, ZO-1's phase separation is regulated by cell-cell interaction and F-actin remodeling.

We also analyzed LLPS using bacterially-produced ZO-1 *in vitro*. We found that ZO-1 IDR4 was sufficient to form liquid droplets *in vitro* (Figure 2F), and the full-length ZO-1 can bind to F-actin (Figure 2G). This system will be useful in analyzing detailed molecular mechanisms of ZO-1 phase separation.

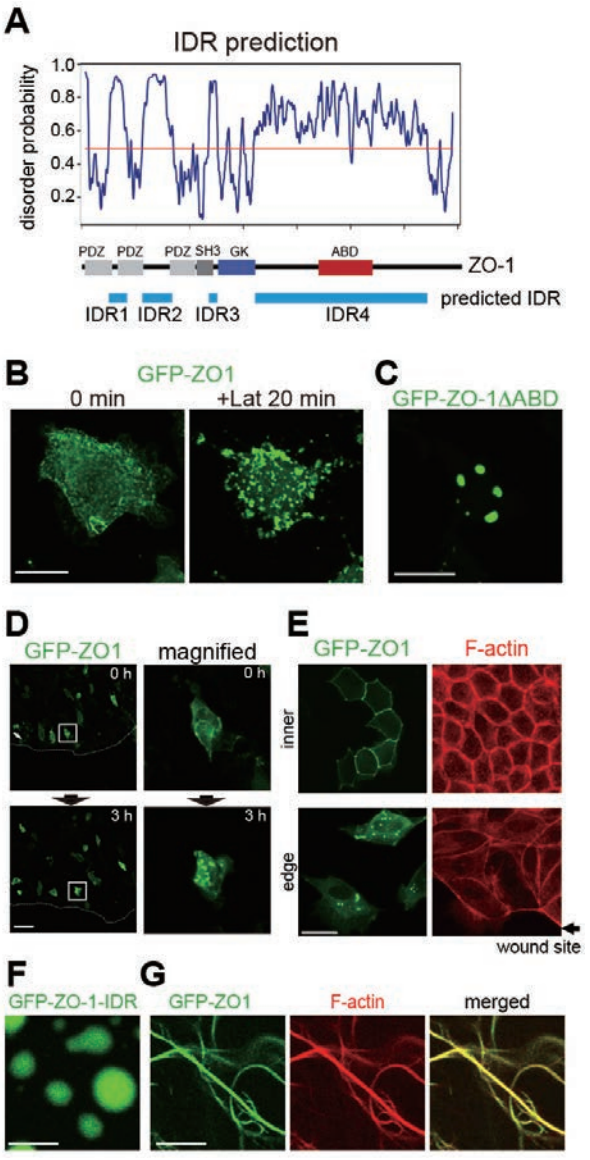


Figure 2. Interaction with F-actin regulates ZO-1 phase separation.
A. The domain structure and the intrinsically disordered regions (IDRs) of ZO-1. B. A6 cells were treated with latrunculin B (Lat) to disrupt F-actin. Bar = 20 µm. C. GFP-ZO-1 lacking the actin binding domain (ABD) was expressed in A6 cells. Bar = 20 µm. D. A wound healing assay using MDCK cells. Bar = 50 µm. E. 10 hours after the wound healing assay, cells close to the wound site ("edge") and in the inner region ("inner") were stained with phalloidin. Bar = 20 µm. F. Purified GFP-ZO1-IDR4 formed liquid droplets in vitro. Bar = 5 µm. G. Purified GFP-ZO-1 interacts with F-actin. Bar = 10 µm.

III. Cephalochordate *Brachyury* enhancers involved in the evolution of the chordate notochord and somites

The notochord and somite are the most central organs in chordates, and Brachyury (Bra) plays a pivotal role in their formation. The question of how Bra gained its enhancer activity in the notochord-specific expression is critical to our understanding of chordate evolution. In cephalochordates, Bra is duplicated into two genes (Figure 3). We examined the enhancer activity of *Branchiostoma floridae* Bra (BfBra1 and BfBra2) by lacZ reporter assay using a Ciona-embryo host system. In the 5', 3' regions and introns, the signal intensity was higher and broader in BfBra2 than BfBra1 (Figure 3). In some cases, lacZ expression is expanded to posterior muscle cells in the former. The intron enhancers displayed another difference; primary expression of BfBra1 occurred in the notochord, whereas both muscle and the notochord indicated BfBra2 expression. These results, along with other data, predicted an EvoDevo scenario in which BfBra2 is more ancestral and mother-like, while BfBra1 is daughter-like and duplicated by BfBra1. Originally, ancestral BfBra2 was likely to gain enhancer machinery for gene expression in muscle and notochord, as has been deduced from its spatial expression profile: the 5' region for somite expression, the 3' region for notochord expression, and introns for somite/notochord expression. In contrast, sister BfBra1 became more specialized for notochord expression using the intron enhancers.

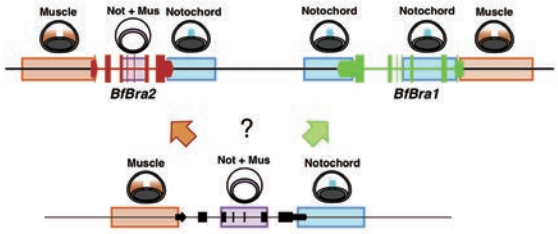


Figure 3. A summary of results of the present reporter assay and their possible interpretation.
Enhancer activity of 5' upstream sequences in muscle is shown in brown and that of the 3' downstream in notochord is in blue. The activity of *BfBra2* introns in muscle and notochord is shown in purple.

IV. A step-down photophobic response in larvae of the common reef coral, *Acropora tenuis*

Many reef-building corals form a symbiotic relationship with dinoflagellate algae of the genus Symbiodinium. Corals mostly depend on photosynthetic products from these symbionts as their energy source, and thus light conditions in habitats can influence post-settlement survival. Previous studies reported that light environments play an essential role in larval habitat selection. However, due to a lack of basic photobiological studies in corals, how coral larvae perceive and respond to the light in their environment remains largely unknown. To answer these questions, we analyzed the swimming behavior of larvae of the common reef coral Acropora tenuis (Figure 4) under various light conditions. In addition, we developed a mathematical model to test whether the observed light response resulted in aggregation or dispersal under specific light fields.

First, we precisely observed the larval swimming activity under fluctuating light conditions and found that larvae temporarily stopped swimming ~30 s after rapid light intensity reduction, thus exhibiting a step-down photophobic response. This behavior was also observed when we rapidly changed the spectral composition of light. Further experiments using the Okazaki Large Spectrograph revealed that the loss of short wavelengths of light (blue/green light) induced this type of behavior.

The analysis of mathematical simulations of this step-down photophobic response indicates that larvae will aggregate in the lighter areas of two-dimensional large rectangular fields. These results suggest that the step-down photophobic response of coral larvae may play an important role in determining where larvae settle on the reef.

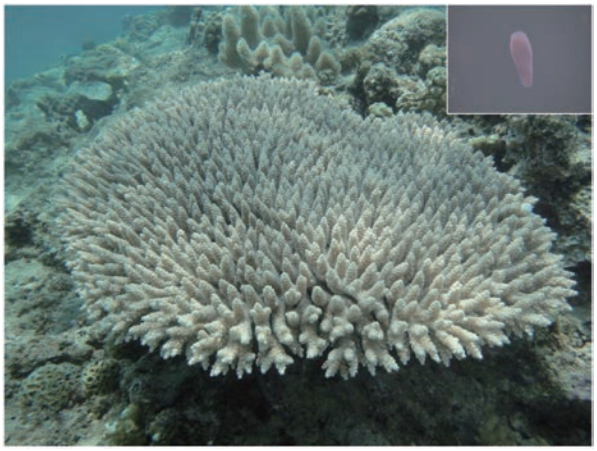


Figure 4. A wild colony of *Acropora tenuis* (photo taken by Masayuki Hatta at Ochanomizu University). The inset shows a planula larvae of A. *tenuis*.

Publication List:

〔Original paper〕

- Sakai, Y., Kato, K., Koyama, H., Kuba, A., Takahashi, H., Fujimori, T., Hatta, M., Negri, A.P., Baird, A.H., and Ueno, N. (2020). A step-down photophobic response in coral larvae: implications for the light-dependent distribution of the common reef coral, Acropora tenuis. Sci. Rep. *10*. DOI: 10.1038/s41598-020-74649-x