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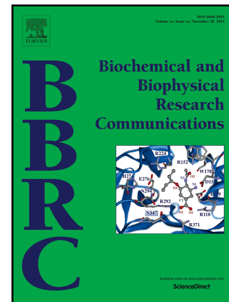

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Rac1 signalling coordinates epiboly movement by differential regulation of actin cytoskeleton in zebrafish

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Abstract

Dynamic cytoskeleton organization is essential for polarized cell behaviours in a wide variety of morphogenetic events. In zebrafish, epiboly involves coordinated cell shape changes and expansion of cell layers to close the blastopore, but many important regulatory aspects are still unclear. Especially, the spatio-temporal regulation and function of actin structures remain to be determined for a better understanding of the mechanisms that coordinate epiboly movement. Here we show that Rac1 signalling, likely functions downstream of phosphatiditylinositol-3 kinase, is required for F-actin organization during epiboly progression in zebtafish. Using a dominant negative mutant of Rac1 and specific inhibitors to block the activation of this pathway, we find that marginal contractile actin ring is sensitive to inhibition of Rac1 signalling. In particular, we identify a novel function for this actin structure in retaining the external yolk syncytial nuclei within the margin of enveloping layer for coordinated movement toward the vegetal pole. Furthermore, we find that F-actin bundles, progressively formed in the vegetal cortex of the yolk cell, act in concert with marginal actin ring and play an active role in pulling external yolk syncytial nuclei toward the vegetal pole direction. This study uncovers novel roles of different actin structures in orchestrating epiboly movement. It helps to provide insight into the mechanisms regulating cellular polarization during early development.

Key words: gastrulation; Rac1; phosphatiditylinositol-3 kinase; epiboly; actin cytoskeleton; zebrafish

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1. Introduction

During early development, cells in different regions of the embryo undergo extensive morphogenetic movements, which play a critical role in actively moving various cell populations to the correct positions within the embryo. In zebrafish, epiboly is the earliest morphogenetic movement that occurs before gastrulation. At the mid-blastula stage when zygotic transcription starts, there are about 4000 cells forming the blastoderm disc situated on a large syncytial yolk cell [1]. Epiboly movement begins at dome stage when the large yolk cell elevates into the blastoderm cells toward the animal pole, by a process called doming [2-4]. The blastoderm cells then spread toward the vegetal pole to completely cover the yolk cell at the end of gastrulation.

At the onset of epiboly, the zebrafish embryo is organized into three distinct layers: a single-cell superficial enveloping layer (EVL), the deep cell layer (DCL), and the multinucleated yolk syncytial layer (YSL) that is formed by a collapse of marginal blastomeres into the yolk cell and as a result of the absence of cytokinesis between daughter cells [5,6]. The YSL nuclei (YSN), deposited by these marginal blastomeres, are located at the periphery of the blastoderm margin. Soon after the initiation of epiboly, parts of the YSL cytoplasm and YSN spread underneath the blastoderm, resulting in the formation of the internal YSL (I-YSL), whereas the external YSL (E-YSL) with its cytoplasm and nuclei (E-YSN) remains at the blastoderm margin [4,7]. During epiboly progression, the E-YSL continuously advances across the yolk cell and pulls the EVL along behind it [1]. Thus, concurrent with other gastrulation movements, the YSL actively moves toward the vegetal pole in a coordinated manner to eventually cover the yolk cell.

The cellular and molecular mechanisms that coordinate the epiboly process are evolutionarily conserved and have been extensively studied, but many mechanistic aspects remain elusive. Dynamic regulation of actin cytoskeleton is central for epiboly. At the beginning of blastodisc flattening, cortical F-actin is organized in the EVL cells. Furthermore, a punctate marginal actin ring forms ahead of the leading edge of E-YSL later during epiboly, and F-actin is also present in the vegetal cortex of the yolk cell [8]. These F-actin networks have been thought to be required for the circumferential constriction of the margin during late epiboly stages, or for maintaining the

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structural integrity of the yolk cell [3,8,9]. However, how the actin dynamics at different location is regulated, and how different actin structures coordinate epibloy movements of various cell layers, remain largely unknown.

In this work, we show that Rac1 is differentially required for F-actin organization during epiboly, likely downstream of phosphatiditylinositol-3 kinase (PI3K). The marginal actin ring is more sensitive to inhibition of Rac1 and PI3K, followed by vegetal cortex F-actin of the yolk cell. In particular, we find that marginal actin ring is necessary for maintaining the E-YSN within EVL margin for synchronous vegetal movement, whereas vegetal cortex F-actin plays an active role in pulling the E-YSN toward the vegetal pole. These findings provide novel insight into the actin dynamics in coordinating cell movements during epiboly progression.

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2. Materials and Methods

2.1. Zebrafish maintenance and microinjection

Zebrafish adult and embryos were maintained at 28.5°C, and staged as described [1]. Microinjections were performed using a PLI-100A Picoliter microinjector (Harvard Apparatus).

2.2. Inhibitor treatments

The Rac1 inhibitor, NSC23766, and the PI3K inhibitor, LY294002 (MedChem Express), were prepared as a stock concentration of 10 mM in DMSO. Embryos were incubated in different working concentrations of these inhibitors diluted in E3 buffer for appropriate periods. All control embryos were treated with 1% DMSO at 1-cell stage.

2.3. Plasmid construct

The plasmid corresponding to a dominant negative form of human Rac1 was linearised by EcoRI, and synthetic capped mRNA was in vitro transcribed using SP6 RNA polymerase as described [10]. The mRNA was injected into the yolk cell at 1-cell or 512-cell stage.

2.4. YSL labelling, F-actin staining, and confocal microscopy

Rhodamine-Lysine-Dextran (RLDx) was injected into the yolk cell at 512-cell stage. Embryos were fixed and samples for confocal microscopy were prepared as described [11]. Briefly, the embryos were first incubated with fluorescein-labelled (FITC) phalloidin and counterstained with DAPI (Sigma-Aldrich). They were analysed under a confocal microscope (Zeiss LSM700), and Z-stack projections were obtained by using the z-projection function.

2.5. Time-lapse imaging

Embryos at 65% epiboly stage were mounted in a cavity microscope slide in 1% low-melting agarose. Cell movements were recorded under an upright microscope (Leica, LM2500), using the function of differential interference contrast. The embryos were imaged every 15 seconds for a total period of 20 minutes. Time-lapse movies were generated using ImageJ software.

3. Results

3.1. Inhibition of Rac1 signalling disrupts F-actin organization during epiboly

Rac1 is a small G protein that regulates actin dynamics in a variety of cultured cells [12], but whether it modulates actin organization during epiboly remains unclear. We used the dominant negative mutant of Rac1, dnRac1 or N17-Rac1 [13], to inhibit Rac1 activation. When control embryos reached 75% epiboly stage, phalloidin and DAPI staining indicated that regular and strong cortical F-actin was present in EVL cells, and F-actin bundles aligned along the animal-vegetal direction could be also visualized in the vegetal cortex of the yolk cell (Fig. 1A). We designate this vegetal actin structure as vegetal cortex F-actin. In addition, a strong marginal punctate actin ring was formed ahead of the leading edge of E-YSL, and moved vegetally in close contact with the E-YSN (Fig. 1A-A”). Injection of synthetic *dnRac1* mRNA (100 pg) in the yolk cell at 1-cell stage resulted in a strongly reduced cortical F-actin in the EVL and a weak actin ring in a large majority of embryos. The organization of vegetal cortex F-actin was also strongly disrupted, and epiboly progression was severely delayed (Fig. 1B). In these embryos, all E-YSN were closely associated with the front of EVL (Fig. 1B’, B” and F). When *dnRac1* mRNA was injected at 512-cell stage, which leads to the accumulation of lower amounts of translated dnRac1 protein and its restriction in the yolk cell, we found that epiboly progression was moderately affected in 45% of embryos, along with an absence of marginal actin ring (Fig. 1C and F). However, the organization of vegetal cortex F-actin bundles remained relatively intact. Most intriguingly, we found that some E-YSN completely separated from marginal actin ring, forming an independent “E-YSN ring” that moved faster toward the vegetal pole (Fig. 1C’ and C”). These results suggest that Rac1 signalling is required for actin cytoskeleton organization during epiboly, and that marginal actin ring is particularly sensitive to modulation by Rac1 signalling.

To more conveniently analyse the temporal requirement of Rac1 signalling during epiboly, we employed the specific Rac1 inhibitor, NSC23766 [14], which blocks Rac1 activation both in cultured cells and in zebrafish embryo [15,16]. We found that treating embryos at 512-cell stage with NSC23766 (150 μM) resulted in strong epiboly defects. Phalloidin and DAPI staining revealed

that nearly half of the embryos showed severely reduced marginal actin ring and vegetal cortex F-actin (Fig. 1D-D” and F). In this case, the leading edges of EVL and E-YSN moved alongside during epiboly (Fig. 1D’ and D”). When treated at 50% epiboly stage, most embryos showed a strongly reduced actin ring, but with relatively normal vegetal cortex F-actin. In this case, an “E-YSN ring” was formed (Fig. 1E-E” and F). This suggests that vegetal cortex F-actin is less sensitive to inhibition of Rac1 signalling at more late stages.

3.2. Inhibition of PI3K similarly disrupts actin cytoskeleton organization

PI3K is an important component of various signalling events leading to cell polarization. Especially, phosphorylated lipids produced by PI3K recruit and activate key modulators of Rac function, such as guanine exchange factor proteins and GTPase activating proteins, leading to activation of Rac signalling [12]. To examine whether PI3K functions as Rac1 in epiboly, we inhibited its activity using a specific inhibitor, LY294002 [17]. Embryos were treated at different stages with LY294002 (7.5 μM), and phalloidin and DAPI staining was performed at 65% epiboly. In DMSO-treated embryos (77/79), a gap was present between the leading edges of EVL and DCL, and E-YSN moved vegetally along with marginal actin ring (Fig. 2A-A”). In contrast, all embryos treated at 1-cell stage (40/40) displayed a complete absence of marginal actin ring, and strongly reduced actin structures in the EVL and in the vegetal cortex. The gap between the leading edges of EVL and DCL disappeared (Fig. 2B-B”). In most embryos treated at 512-cell stage (69/78), marginal actin ring was also absent, and cortical F-actin in the EVL cells was reduced and the organization of vegetal cortex F-actin was severely disrupted (Fig. 2C-C”). When treated at 50% epiboly stage, a weak marginal actin ring was present, but no gap was formed between the leading edges of EVL and DCL. In these embryos, cortical F-actin staining in the EVL was weak, whereas vegetal cortex F-actin was present as bundles and aligned in the animal-vegetal direction (Fig. 2D). Accordingly, a regular “E-YSN ring” was formed and moved away from the leading edge of the EVL, (Fig. 2D’ and D”). Importantly, they seemed to attach to the vegetal cortex F-actin bundles (Fig. 2D”). These results suggest that PI3K may function in same pathway as Rac1 in actin organization during epiboly.

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3.3. Attraction of E-YSN by the vegetal cortex F-actin

The above observation implies a possible attraction of E-YSN by vegetal cortex F-actin toward the vegetal pole. We further tested this possibility by time-lapse imaging. Because PI3K inhibitor exerts the same effects as Rac1 inhibitor on actin organisation, and was found to be more efficient, it was used in the following experiments. Under differential interference contrast microscopy, E-YSN and the gap between the leading edges of EVL and DCL were clearly apparent at 65% epibloy stage. In control embryos, E-YSN always associated with the leading edge of EVL or in its vicinity during epibloy progression (Fig. 3A-E; Supplementary movie S1). In embryos treated with LY294002 at 512-cell stage, which disrupts both marginal actin ring and vegetal cortex F-actin, no gap was formed between the leading edges of EVL and DCL, and E-YSN were associated with the common front of EVL and DCL (Fig. 3F-J; Supplementary movie S2). However, when embryos were treated at 50% epiboly stage, which reduces marginal actin ring, but keeps vegetal cortex F-actin relatively intact (see Fig. 2D-D"), E-YSN moved away from the leading edge of EVL. Interestingly, some E-YSN were stretched into a very elongated shape when they were still associated with marginal actin ring (Fig. 3K and L; Supplementary movie S3). Once detached, they moved extremely faster toward the vegetal pole to rapidly join the existing "E-YSN ring", and resumed a rounded shape (Fig. 3M-O; Supplementary movie S3). This result was further confirmed using Rac1 inhibitor following phalloidin and DAPI staining, which revealed the presence of elongated E-YSN that were "squeezing" through the actin ring (Fig. 3P-R). These observations suggest that vegetal cortex F-actin likely possesses a contractile activity and may actively pull E-YSN toward the vegetal pole during epiboly progression.

3.4. Marginal actin ring maintains E-YSN within the EVL margin

The appearance of "E-YSN ring" in embryos with disrupted marginal actin ring, but with intact vegetal cortex F-actin, suggests that E-YSN may be normally retained in the EVL margin. To test this possibility, we labelled the YSL region by injecting RLDx at 512-cell stage, and treated embryos with LY294002 at 50% epiboly and shield stages to disrupt marginal actin ring, but not vegetal cortex F-actin. Confocal microscopic analyses following phalloidin and DAPI staining at 65%

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epiboly stage showed that RLDx-labelled YSN and YSL region were located between the leading edges of EVL and DCL (Fig. 4A-F). However, when embryos were treated at 50% epiboly stage, marginal actin ring was severely, but not completely disrupted (Fig. 4G), some E-YSN were separated from the EVL margin to form an “E-YSN ring” (Fig. 4H-L). When treated at shield stage, marginal actin ring was only weakly affected (Fig. 4M), and the organization of vegetal cortex F-actin was not affected, there were still E-YSN “escaped” from the EVL margin, forming an incomplete “E-YSN ring” (Fig. 4N-R). These observations suggest that marginal actin ring may function as a mechanical “filet” that prevents E-YSN from pulling outside the EVL margin, and synchronizes the vegetal movements of E-YSL and EVL during epiboly.

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4. Discussion

Actin dynamics plays essential roles in the progression of epiboly movement in zebrafish, but the regulatory mechanism remains largely elusive. We show in this work that Rac1 signalling differentially regulates the organization of various actin structures during epiboly. More importantly, our findings demonstrate that vegetal cortex F-actin bundles exert a pulling force to attract E-YSN vegetally, and that marginal actin ring plays an important role in maintaining the E-YSL and EVL ensemble. All together, our results suggest that marginal actin ring and vegetal cortex F-actin bundles act in concert to synchronize and drive the movements of E-YSL and EVL during epiboly progression.

Both PI3K and Rac1 are required for epiboly progression by regulating F-actin organization in the embryo. This is consistent with previous findings showing that PI3K is required for polarized cell behaviours during gastrulation [18], and that a Rac GTPase activating protein, α2-chimerin, is implicated in epiboly progression [19]. Furthermore, it was shown that heterotrimeric G proteins $G\alpha_{12}$ and $G\alpha_{13}$ regulate actin cytoskeleton organization in epiboly upstream of RhoGEF/Rho signalling pathway [20], and that Rho mediates cell migration in epiboly through a ROK/ROCK-dependent pathway [21]. These observations support a role for small G proteins in regulating actin dynamics during epiboly. Our results further reveal a differential regulation of actin structures by Rac1 signalling.

Marginal actin ring is formed by the accumulation of actin and non-muscle myosin II at the interface between EVL and YSL after 50% epiboly [8,22]. It has been thought that this contractile actomyosin ring is implicated in the circumferential contraction of the margin and in the generation of a pulling force that drives EVL epiboly [22,23]. The E-YSL, with its cytoplasm and E-YSN, also plays an important role in epibloy by connecting EVL at its margin [22, 24]. Conversely, the EVL may also actively contact the YSL by filopodia formation [25]. Thus, the recruitment of actin and myosin II within the YSL functions to coordinate EVL cell shape changes during epiboly progression. Indeed, a correctly formed marginal actin ring correlates with the coordinated vegetal movement of EVL, YSL and DCL. However, in embryos with disrupted marginal actin ring, but with

intact vegetal cortex F-actin, E-YSN detach from the EVL margin and move extremely faster toward the vegetal pole, forming a regular "E-YSN ring" that largely outpaces the leading edge of EVL. Thus, our observations suggest that marginal actin ring, once formed, not only functions as a force-generating structure, but also acts as a mechanical "filet" that retains E-YSN with EVL for synchronized movement.

The yolk cell has been proposed to function as an epiboly motor because it provides much of the motive force [3,4,9]. Although no functional role has been attributed to vegetal actin microfilaments in epiboly at present, pioneer works in the teleost *Fundulus heteroclitus* have suggested that YCL displays extensive contractility [26,27]. By differentially disrupting actin structures during epiboly progression and by time-lapse analysis, we find that vegetal cortex F-actin bundles likely play an important role in driving E-YSN epiboly. There are several arguments supporting this conclusion. First, the regular "E-YSN ring" seems to be closely associated with vegetal cortex F-actin bundles, implying that vegetal actin structure may play a role in their movement. Second, time-lapse analysis indicates that, in embryos with partially disrupted marginal actin ring, some E-YSN were stretched into an elongated shape prior to be separated from the EVL margin. Once detached, they moved extremely faster and were able to join the existing "E-YSN ring" formed in more vegetal direction, where they resume a rounded shape. Finally, E-YSN never separate from the YSL region when marginal actin ring and vegetal cortex F-actin are simultaneously disrupted, instead, they become closely associated with the leading edges of EVL and DCL in this situation. These strongly correlate with an active role of vegetal cortex F-actin that underlies the contraction of YCL and exerts a pulling force on E-YSN during epiboly progression.

It is still unclear how vegetal cortex F-actin bundles contact E-YSN and drive their vegetal movement in normal epiboly. One possibility may be that they pass through marginal punctate actin ring in the EVL margin to directly link the E-YSN. This possibility may be supported by our time-lapse analysis showing that, when marginal actin ring is partially disrupted, there are E-YSN stretched into an elongated shape, probably by the pulling force exerted by vegetal cortex F-actin. Alternatively, vegetal cortex F-actin bundles may indirectly contact E-YSN, through interaction or

direct connection with marginal actin ring. Further study using appropriate approaches should help to clarify this situation.

In conclusion, we demonstrate that Rac1 and PI3K signaling functions to regulate F-actin dynamics during epiboly. We uncover a novel function for marginal actin ring in keeping the E-YSN for coordinated movement, and for vegetal cortex F-actin in pulling E-YSN toward the vegetal pole. All together, our results suggest that marginal actin ring and vegetal cortex F-actin coordinately drive E-YSN epiboly. These findings shed light on the regulation and function of dynamic changes in the actin cytoskeleton during morphogenetic movements.

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Conflict of interest

The authors declare no conflict of interest.

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Fig. 1. Inhibition of Rac1 disrupts F-actin organization. Embryos were either injected with *dnRac1* mRNA or treated with NSC23766 at indicated stages, followed by phalloidin and DAPI staining. (A-A") A control embryo at 75% epiboly stage, with a dense marginal actin ring (arrows) and associated E-YSN (arrowheads). (B-B") A representative embryo injected with *dnRac1* mRNA at 1-cell stage shows epiboly defects, and disruption of EVL cortical F-actin, marginal actin ring and vegetal cortex F-actin. No "E-YSN ring" is formed. (C-C") An embryo injected with *dnRac1* mRNA at 512-cell stage shows absence of marginal actin ring, but with normal vegetal cortex F-actin. An "E-YSN ring" (arrowheads) is formed. (D-D") A representative embryo treated with NSC23766 at 512-cell stage shows epiboly defects and disrupted EVL cortical F-actin, marginal actin ring and vegetal cortex F-actin. No "E-YSN ring" is formed. (E-E") An embryo treated with NSC23766 at 50% epiboly stage shows absence of marginal actin ring, but with normal vegetal cortex F-actin. An "E-YSN ring" (arrowheads) is formed. (F) Statistical analyses of different phenotypes. Numbers at the top of each stacked column indicate total embryos scored from three independent experiments using different batches of embryos. Scale bar: 200 μm.

Fig. 2. Inhibition of PI3K disrupts F-actin organization. Embryos were treated with LY294002 at indicated stages, and phalloidin and DAPI staining was performed at 65% epiboly. (A-A") A DMSO-treated control embryo shows intense vegetal F-actin staining and localization of E-YSN in the vicinity of marginal actin ring (arrowheads). A gap is apparent between the leading edges of EVL and DCL (arrows). (B-B") A representative embryo treated at 1-cell stage shows strongly disrupted actin structures in the EVL and in the yolk cell, with a complete absence of marginal actin ring. Notice also the tight association of E-YSN with the merged front of EVL and DCL. (C-C") A representative embryo treated at 512-cell stage shows moderately disrupted EVL cortical F-actin, and strongly disrupted marginal actin ring and vegetal cortex F-actin. (D-D") A representative embryo treated at 50% epiboly stage shows moderately disrupted EVL cortical F-actin and relatively intact vegetal cortex F-actin bundles. Weak marginal actin ring is present, and a regular "E-YSN ring" (arrowheads) is formed, through close contact with vegetal cortex F-actin bundles (inset). Scale bar: 200 μm.

Fig. 3. Analyses of E-YSN behaviours during epiboly. Embryos were treated by LY294002 or with NSC23766 at indicated stages, and time-lapse imaging or phalloidin and DAPI staining was performed at 65% epiboly stage. Green and red horizontal broken lines demarcate the leading edges of DCL and EVL, respectively. Yellow, blue and black broken circles outline selected E-YSN. (A-E) E-YSN behaviours in a DMSO-treated embryo. (F-J) In an embryo treated with LY294002 at 512-cell stage, no gap is formed between the leading edges of EVL and DCL. E-YSN are tightly associated with the joined EVL and DCL front margins. (K-O) In an embryo treated with LY294002 at 50% epiboly stage, a gap between the leading edges of EVL and DCL is present. An "E-YSN ring" is formed, which detaches from the EVL margin, and moves faster vegetally to join existing "E-YSN ring" (see also Supplementary movies S1, S2 and S3). (P-R) Confocal microscopy images of an embryo treated with NSC23766 at 50% epiboly stage show some elongated E-YSN (arrows) detaching from marginal actin ring, and others resuming a rounded shape (arrowheads). Scale bars: (A-O) 50 μm; (P-R) 100 μm.

Fig. 4. Marginal actin ring retains E-YSN with the EVL margin. The YSL region (outlined by dotted lines) was labelled with RLDx, and embryos were treated with LY294002 at 50% epiboly or shield stage. Phalloidin and DAPI staining was performed at 65% epiboly. (A-F) A control embryo shows the association of E-YSN with marginal actin ring. (G-L) An embryo treated at 50% epiboly stage shows absence of marginal actin ring. E-YSN (arrowheads) detach from the EVL margin and contact vegetal cortex F-actin in a more vegetal direction to form an "E-YSN ring". (M-R) An embryo treated at shield stage shows reduction of marginal actin ring, and some E-YSN (arrowheads) separate from the EVL margin and are located near the leading edge of EVL. Scale bar: 100 μm.

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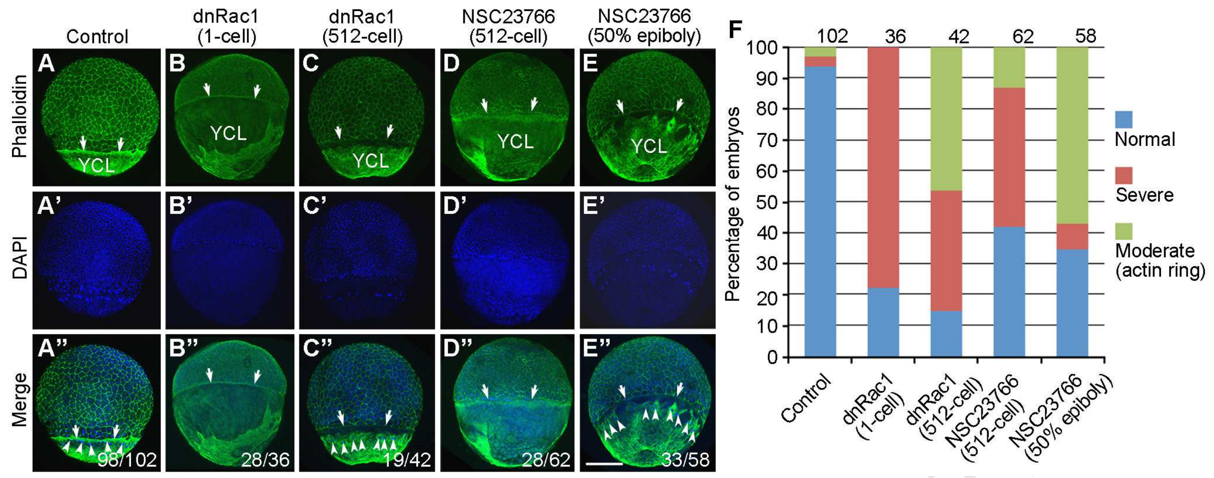

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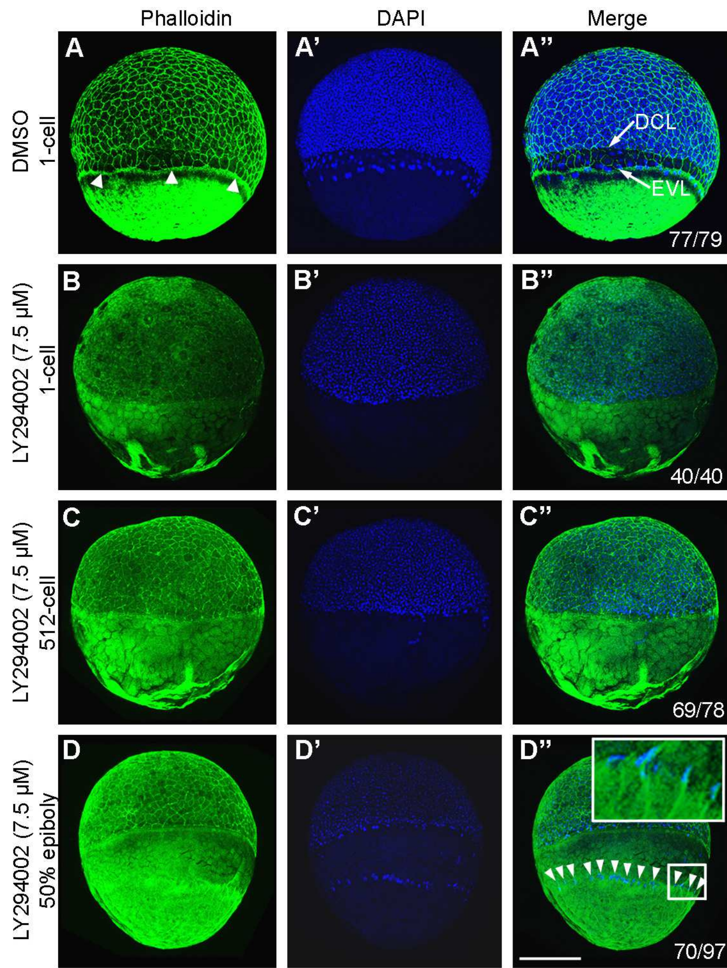

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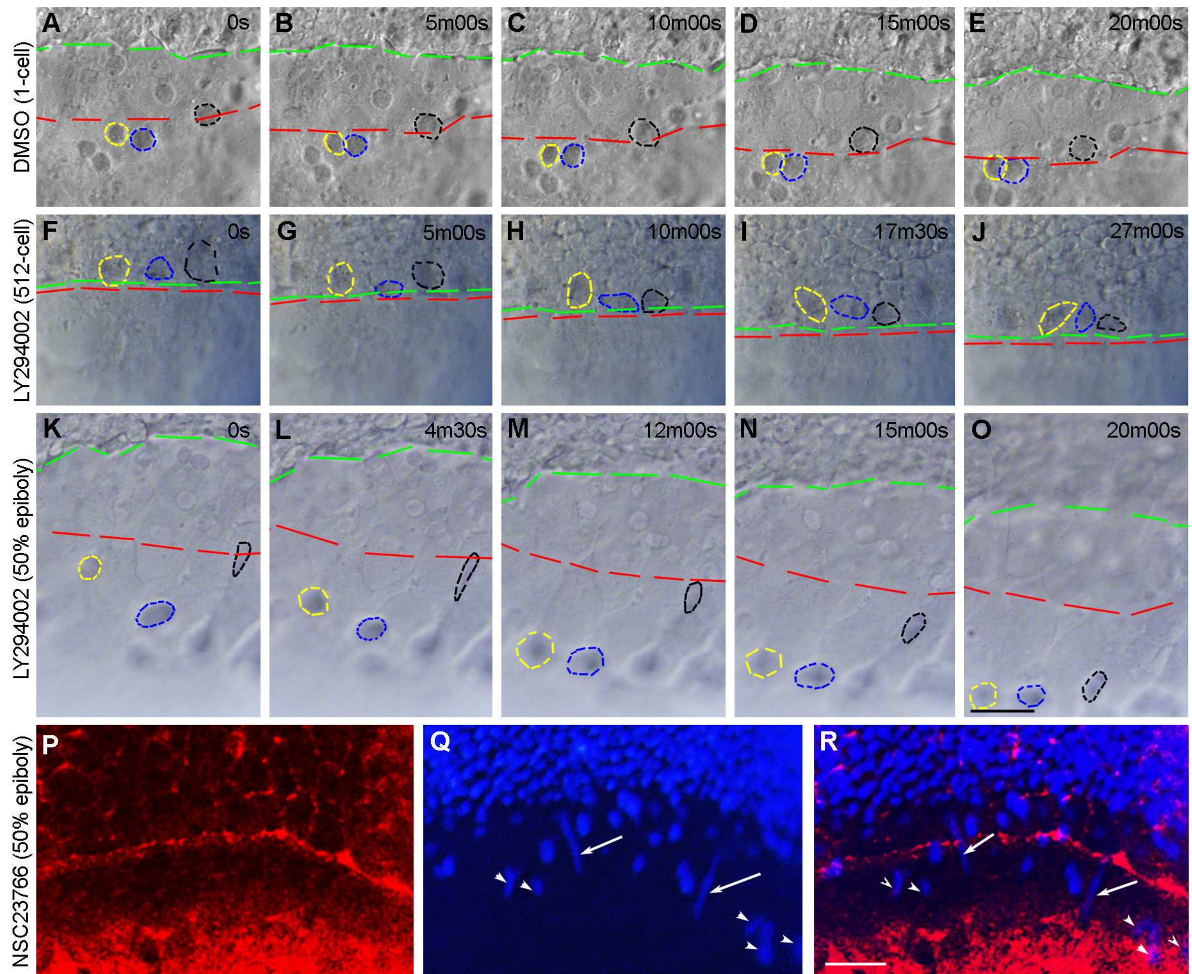

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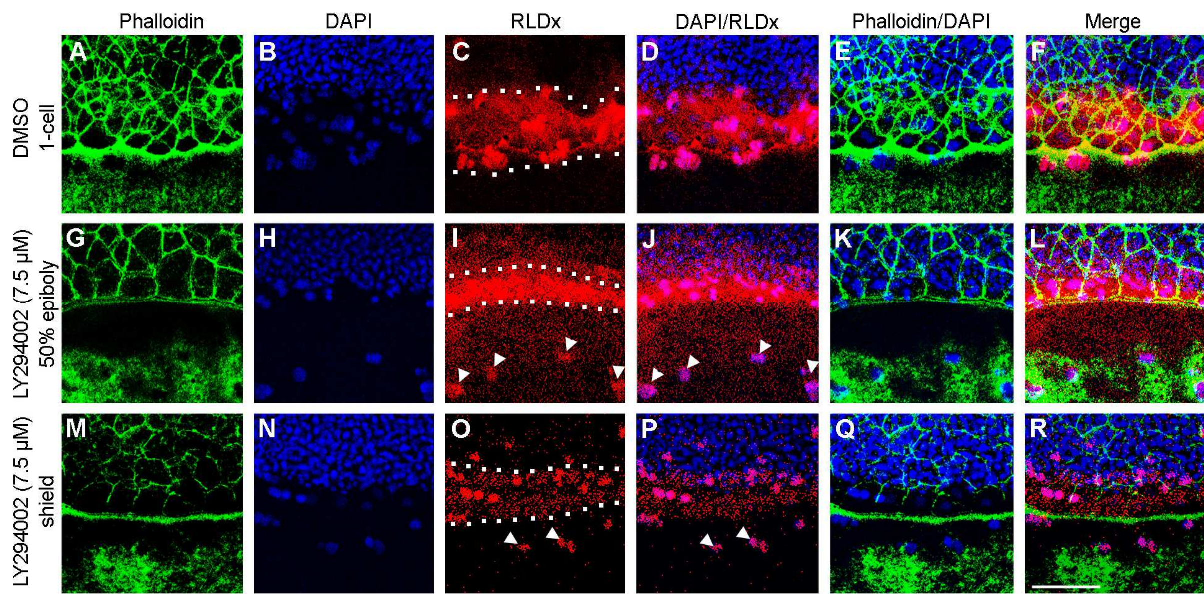

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Highlights:

Rac1 and PI3K signaling regulates actin dynamics during zebrafish epiboly

The contractile actin ring synchronizes epiboly of different cell layers

The vegetal cortex F-actin bundles exert a pulling force on yolk syncytial nuclei

The contractile actin ring and the vegetal cortex F-actin coordinate epiboly movement

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