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Review

Dynamic regulation of the microtubule and actin cytoskeleton in zebrafish epiboly ☆

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ABSTRACT

Gastrulation is a key developmental stage with striking changes in morphology. Coordinated cell movements occur to bring cells to their correct positions in a timely manner. Cell movements and morphological changes are accomplished by precisely controlling dynamic changes in cytoskeletal proteins, microtubules, and actin filaments. Among those cellular movements, epiboly produces the first distinct morphological changes in teleosts. In this review, I describe epiboly and its mechanics, and the dynamic changes in microtubule networks and actin structures, mainly in zebrafish embryos. The factors regulating those cytoskeletal changes will also be discussed.

Contents

1. Introduction

A single-cell fertilized egg first undergoes rapid embryonic cleavage to create enough cells for further development. The embryo then establishes three germ layers and body axes to build a body blueprint. The three germ layers, ectoderm, mesoderm, and endoderm, are formed during gastrulation. Gastrulation is a period when coordinated cell movements take place and bring cells to their proper layers and positions. To do so, several types of cellular

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movements occur during gastrulation, internalization of the epiplast to form mesoderm and endoderm (mesendoderm), convergence and extension to lay out dorsoventral and anterior–posterior axes, and epiboly to enclose the yolk cell with the blastoderm. Epiboly is the first morphologically distinct movement during gastrulation in teleosts. The zebrafish (*Danio rerio*) is one of the favorite teleost models for studying gastrulation due to its accessibility, ease of observation, and the feasibility of transient and permanent genetic manipulation. Global and cellular changes in initiation, progression, and closure of epibolic movements can be observed in zebrafish embryos under microscopy. Dramatic and dynamic changes in the cytoskeleton, including its actin filaments and microtubules, occur during epiboly. Pharmacological perturbations of either of these cytoskeletal structures notably inhibit or retard epibolic movement. Factors affecting cytoskeleton dynamics are often critical for epiboly. This review describes the morphological and cytoskeletal changes during epiboly and discusses factors affecting epiboly and the regulation of dynamic changes in the cytoskeleton of zebrafish embryos.

2. Epiboly mechanics

The zebrafish embryo is one of the best models, which fertilizes and develops *in vitro*, for studying the massive changes that happen during early embryogenesis. Upon fertilization, the chorion is elevated and cytoplasmic yolk streams vegetally to clear yolk from the animal pole and form the embryo disc. The standard culture temperature for zebrafish embryos is 28.5 °C, and the developmental timing and staging described here is according to Kimmel et al. [1]. About 40 min post-fertilization, the embryo proper begins rapid cleaving at 15 min intervals to become a cap of blastomeres, called the blastoderm, sitting on top of an uncleaved yolk cell. The blastoderm is composed inside of blastomeres called deep cells, which will give rise to future embryonic tissues, and an outside covering called the enveloping layer (EVL). At 3 h post-fertilization (hpf), the marginal blastomeres at the interface between the blastoderm and the yolk collapse to form a sycytium, the yolk syncitial layer (YSL), containing multiple nuclei. Epiboly begins at 4 hpf, which is indicated by the doming of the yolk cell into the blastoderm through an unclear mechanism. During doming, deep cells move radially outward [2]. Concurrently, the YSL also domes to form an internal YSL (I-YSL) and leaves the external YSL (E-YSL), which will move vegetally during epiboly. EVL, deep cells, and YSL all migrate toward the vegetal pole initially until they reached the equator of the yolk cell (50% epiboly stage). Cells temporarily pause and lateral cells converge on the future dorsal side to form the embryonic shield. At the shield, some epithelial cells involute anteriorly to become the hypoblast layer, which contains future mesendodermal cells, and non-involuting cells become the epiblast layer. The EVL, E-YSL, and epiblast continue to move vegetally and eventually the dorsal lip is closed, with the entire yolk cell enclosed at the 100% epiboly stage. For a detailed description of morphological changes during zebrafish embryonic development, readers are referred to a classical work by Kimmel et al. [1]. Here, I will focus on epiboly mechanics in more detail.

Before epiboly, the blastoderm is composed of three layers, a single-cell thick epithelium, the EVL, and deep cells. The EVL is tightly connected to the YSL appears around the midblastula transition at 3 hpf. YSL nuclei (YSN) divide several more times and become a wide E-YSL belt at the periphery of the blastoderm. By the sphere stage, at 4 hpf, the YSN are evenly distributed beneath the blastoderm to form the I-YSL. Within the YSL, the yolk is an active gene expression and signaling region that connects to the overlying blastoderm via gap junctions [3].

Trinkaus and colleagues pioneered epiboly research on the teleost *Fundulus heteroclitus*, which is similar to the zebrafish in many ways during development. Zebrafish epiboly can be grossly divided to two phases: initiation and progression [4,5].

2.1. Epiboly initiation

Zebrafish epiboly initiates during the transition from the sphere stage (4 hpf) to the dome stage (4.3 hpf), with the yolk cell "doming" into the blastoderm and the blastoderm becoming an inverted cap by radial intercalation to the top of the yolk cell dome [2]. At the sphere stage, the blastoderm is piled high atop the rounded yolk cell. The blastoderm is then compressed downwards to flatten the blastoderm–yolk interface and force the embryo to assume a spherical shape. In *Fundulus*, the EVL is stretched by tight junctions attached to the YSL [6]. Similarly, tension exists within the EVL that may act to maintain the orientation of mitotic spindles in the plane of the cell sheet [7]. During doming, the E-YSL is flattened and the YSN are packed along the dorsoventral axis [8]. In the meantime, the yolk cell expands upward into the overlying blastoderm, causing a thinning of the blastoderm that makes it appear like an inverted cup on top of the yolk cell [2]. The mechanism of doming is still unclear. At the midblastula transition, deep cells become motile with active blebs extending in random directions until cells begin to move radially outward [2]. It is generally believed that deep cells are pushed outward by the doming yolk cell due to the fact that most cells disperse into the center of the embryo [9]. However, whether deep cells move actively or passively during epiboly initiation needs further investigation. We also know very little about the factors affecting doming.

The transcriptional inhibitor α-amanitin inhibits doming if it is administrated to embryos during the cleavage stage [10], suggesting that active transcription is required for doming. However, no zygotic mutants with defective doming have been identified [10]. There is only one maternal mutant halt (*srh*) recovered that fails to dome [11,12]; however, *srh* mutant embryos exhibit early developmental arrest. Thus, its doming defect may very likely be secondary to aborted zygotic gene expression.

2.2. Epiboly progression

Once epiboly is initiated, the blastoderm thins and spreads toward the vegetal pole, resulting in a large increase in the blastoderm's surface (4.3–10 hpf). Deep cells, the EVL, and YSL margins move dynamically throughout epiboly progression, and the tightly attached YSL and EVL margins are always running in front of the blastoderm margin. E-YSL nuclei are gradually covered by the blastoderm at 30–40% of the epiboly stage. At the shield stage (soon after 50% epiboly), deep cell migration temporarily ceases while cell internalization is initiated and the blastoderm engulfs the widest part of the yolk. At this time, EVL and YSL expand past the blastoderm margin to cover 65% and 70% of the yolk surface, respectively, just when the deep cells have attained 60% epiboly [8]. The internalization of deep cells splits them into outer ectodermal epiblasts and inner mesendodermal hypoblasts. In contrast to the initiation phase, and while epiboly occurs without other gastrulation movements, concurrent ingression and convergence take place during most of the progression phase. After the blastoderm has crossed over the equator of the yolk, the actomyosin ring at the blastoderm margin starts to constrict circumferentially to close the blastopore [13].

2.3. Driving force for epibolic movement

It is commonly accepted that the yolk cell may function as an epiboly motor to pull the EVL and deep cells along with it, as evidenced by the fact that the YSL moves faster during epiboly in

the absence of the blastoderm [4,6]. The YSL is connected to the EVL via tight junctions and thus exerts its pulling force on the blastoderm. To serve as an epiboly motor, the yolk surface exhibits dynamic changes in the cytoskeleton that coincide with epiboly progression. From the shield stage to the end of epiboly, an estimated 87% of the external membrane is removed in a zebrafish embryo [13]. In addition, the lost external yolk cell membrane can be recovered by active endocytosis in a region vegetal to the running front of the blastoderm in both *Fundulus* and zebrafish [8,14]. It appears that endocytosis may be important for epiboly, but this remains to be tested.

3. Dynamic changes and regulation of the microtubule network

3.1. Dynamic changes of the microtubule network during epiboly

A microtubule network exists in the zebrafish embryo before doming. Two distinct types of microtubule arrays are present in zebrafish embryos and actively involved in epibolic movements. Short parallel intercrossing microtubule arrays tether YSN to the E-YSL. The perpendicular arrays along the animal–vegetal axis are in the yolk cytoplasmic layer (YCL) [8]. YCL microtubules arise from microtubule organizing regions in the marginal YSN and extend through the yolk cell to the vegetal pole. Strähle and Jesuthasan first observed the delay in zebrafish epiboly by disrupting microtubules with ultraviolet light or the microtubule depolymerizing agent nocodazole [5]. Later work by Solnica-Krezel and Driver revealed that nocodazole only affects epiboly progression, not the initiation of epiboly, and microtubule-dependent YSL contraction is not required for initiation [8]. These results demonstrate the necessity of YCL microtubule arrays for regulating the speed of epiboly. Furthermore, the epiboly of EVL is least affected by nocodazole [8] because it does not inhibit endocytosis in the blastoderm's marginal rim, which is in part driving the epibolic movement of the EVL [4].

The stability of the microtubule appears to be critical for epiboly. Taxol-treated zebrafish embryos have stable microtubules and reveal epiboly defects [8]. Global or YSL-specific morpholino (MO) knockdown of cytochrome P450, subfamily XIA, polypeptide 1 (Cyp11a1), an enzyme catalyzing cholesterol into pregnenolone (P5), results in epiboly delay and blockage of blastopore closure [15]. The role of Cyp11a1 in epiboly is further strengthened by its restricted expression in the YSL during epiboly [16]. *Cyp11a1* MO-treated embryos (hereafter called morphants) have disruptive microtubule arrays and reduced polymerized microtubules, implying that P5 mediates epiboly via stabilizing microtubules. However, how P5 exerts its microtubule stabilizing activity was puzzling until the discovery of one of its binding proteins, cytoplasmic linker protein 1 (CLIP-170), which is a microtubule plus end-tracking protein, through the use of a P5 photoaffinity probe conjugated to diaminobenzophenone. P5 interacts with CLIP-170 at its coiled-coil domain and changes CLIP-170 to an open state that enhances its interaction with microtubules, the dynactin subunit $p150^{Glued}$, and the dynein-binding protein LIS1. It also promotes CLIP-170-dependent microtubule polymerization. More importantly, *clip-170* morphants show epiboly defects similar to *cyp11a1* morphants. Collectively, the P5-CLIP-170 signal appears to play an important role in regulating microtubule dynamics during epiboly [17]. However, questions still remain unresolved about the dynamic production and distribution of P5, its synthesizing and degrading enzymes, Cyp11a1 and Hasd3b, respectively, and their correlation to changes in microtubule dynamics during epiboly.

3.2. Transcriptional regulation of epiboly via microtubules

Several transcription factors have been shown to regulate epiboly by modulating microtubules. Among them, eomesodermin (EOMES), a T-box transcription factor involved in patterning and morphogenesis, is essential for gastrulation in *Xenopus* [18] and zebrafish [2,19,20]. A maternal/zygotic zebrafish mutant of *eomesa* shows distinct delay in epiboly initiation but not progression in a microtubule-dependent manner [21]. *Eomesa* mutant embryos can eventually complete epiboly with the concomitant recovery of the YCL microtubule array, possibly via other complementary genes like *pou5f1*. Pou5f1 also known as Oct4, is a POU domain transcription factor that is critical for maintenance of stemness in mice [22]. Zebrafish *pou5f1* maternal/zygotic mutant embryos (*spiel ohne grenzen*) are severely defective in gastrulation, dorsalization, and endoderm malformation due to alterations in microtubules and actin filaments in all three embryonic lineages [23]. In addition, monosaccharide O-linked beta-N-acetylglucosamine (O-GlcNAc) can modify Pou5f1/Oct4 in human embryonic stem cells. Up- or down-regulation of monosaccharide O-linked beta-N-acetylglucosamine (O-GlcNAc), which can modify Pou5f1/Oct4 in human embryonic stem cells, results in severe disturbance of microtubules and actin filaments and defective epibolic movement [24]. Collectively, transcriptional regulation of microtubule dynamics appears to be important in zebrafish epiboly, and other transcription factors that may be involved in microtubule dynamics are still waiting to be discovered.

3.3. Other factors mediate microtubule dynamics during epiboly

Non-canonical Wnt/planar cell polarity (Wnt/PCP) signaling mediates convergent and extension movements from ascidians through mammals by controlling the polarity of cell morphology and behavior [25]. By examining the interaction of Wnt/PCP and the centrosome or microtubule organizing center (MTOC), Sepich et al. (2011) demonstrated that a reciprocal requirement for Wnt/PCP polarity and positioning of MTOC establishes embryonic polarity. Once planar polarity is established, intact microtubules are no longer needed [26].

Microtubule-associated protein 9 (Map9/Asap) has recently been shown to be essential for early embryogenesis in zebrafish [27]. Map9 is associated with the mitotic spindle. The overexpression of *map9* results in an aberrant spindle and the loss of Map9 causes mitotic defects and cell death [28]. Injection of *map9* MO at the one-cell stage results in early epiboly arrest and disruption of YSL microtubule arrays that can be partially rescued by *map9* mRNA. To examine whether the Map9 effect is specific to the regulation of YSL microtubules, *map9* MO was injected into the YSL. Although similar epiboly arrest was observed in those embryos, the defect could not be rescued by the co-injection of *map9* mRNA. Thus, whether the effect of Map9 is specific to the regulation of YSL microtubules or due to its effect on mitosis and subsequent cell apoptosis is still uncertain.

Calcium channel β4 subunit, a membrane-associated guanylate cyclase, regulates doming in a calcium-independent manner as demonstrated in MO knockdown assays. At a lower frequency, YSL-specific knockdown also blocked epiboly initiation, suggesting β4.1 and β4.2 genes have autonomous functions in the yolk. A similar phenotype was found in embryos treated with nocodazole, but yolk microtubules were not examined in the morphants and no mechanism is provided for the calcium channel-independent function [29].

4. Dynamic changes and regulation of actin filaments

4.1. Dynamic changes in actin filaments during epiboly

A classical paper by Andrew Miller's group described in detail three types of filamentous actin (F-actin)-based structures

appearing only after the shield stage [13]. There are two F-actin rings in the blastoderm margins of the deep cell and the EVL and a punctate actin band forms in the E-YSL. In addition, a vegetal actin cap appears between 30% and 85% epiboly stage. Disruption of these actin–based structures by cytochalasin B or a calcium chelator leads to the delay or arrest of epiboly, followed by the failure of yolk cell occlusion and lysis of the embryo. It suggests a pivotal role for these structures in the occlusion of the yolk cell during epiboly. Miller and colleagues proposed a purse-string mode that these actin rings at the EVL and deep cells leading margins may act as tighteners to promote active cell rearrangement and shape the change in EVL marginal cells needed to enclose the vegetal pole. This coordination may help drive epiboly by constricting the E-YSL and exerting tension on the attached margin of the EVL. Meanwhile, the yolk cell membrane is removed ahead of the advancing blastoderm as epiboly progresses [4,14]. Furthermore, the punctuate belt of F-actin is co-localized with a zone of active endocytosis that implies a role of dynamic actin remodeling in endocytosis during epiboly. However, it is bewildering that the endocytosis appears not to be affected by cytochalasin B [13].

4.2. Epiboly regulation via endocytosis

The dynamic turnover of E-cadherin is central to the effective and synchronized migration of mesendodermal cells during zebrafish gastrulation. Cooper and D'Amico (1996) first observed a cluster of endocytic cells, which are composed of EVL cells and one to two layers of deep cells [30]. This cluster of cells does not involute during epiboly and may be involved in the turnover of E-cadherin. Rab5, a small guanosine triphosphatase (GTPase), is well known for its role in the formation of early endosomes, which function to redistribute the endocytic membrane [31]. Wnt11 controls morphogenic cell movement during gastrulation by regulating Rab5-dependent adhesion of mesendodermal progenitor cells. The knockdown of Rab5c in wild-type embryos causes a posteriorly displaced and elongated prechordal plate at the end of gastrulation that resembles phenotypes of the *slb/wnt11* mutant, and the slb/wnt11 mutant can be rescued by overexpressing Rab5c. Wnt11 and Rab5c are both essential for the endocytosis of E-cadherin and E-cadherin-mediated cell cohesion that mediate mesendodermal involution [32].

Zebrafish Pou5f1/Oct4 maternal and zygotic *spg* mutant embryos are severely delayed in epiboly with an E-cadherin endosomal trafficking defect that results in E-cadherin accumulation at the plasma membrane. A mechanistic analysis revealed that Pou5f1-dependent EGF expression regulates endosomal E-cadherin trafficking via modulating p120 activity. This indicates that E-cadherin endosomal trafficking is required for the effective generation of new adhesion sites during epiboly cell movements [33].

Cells maintain a polarized orientation in a plane of tissue in a way called planar cell polarity (PCP). To do so, changes in cell adhesion and within the extracellular matrix (ECM) need to be tightly coordinated. Membrane type-1 matrix metalloproteinase (MT1-MMP or MMP14) is essential for PCP and convergent extension. A PCP protein, Vang-like 2 (VANGL2), regulates focal adhesion kinase-dependent endocytosis and the cell-surface availability of MMP14. Zebrafish trilobite/vangl2 mutant embryos have enhanced Mmp14 activity and reduced ECM. The knockdown of MMP14 can partially rescue the Vangl2-dependent convergence and extension defect. This suggests a link between VANGL2 and MMP14 trafficking and also that the establishment of PCP in migrating gastrula cells requires controlled proteolytic degradation or remodeling of the ECM [34].

Actomyosin contractility drives apical constriction in *Xenopus laevis* bottle cells [35] via endocytosis-driven membrane remodeling. Endosomes are located exclusively in bottle cells in the early gastrula, and the disruption of endocytosis with dominant-negative dynamin, a large GTPase involved in vesicle scission [36] or rab5 interferes with apical constriction. This suggests that endocytosis occurs downstream of actomyosin contractility to eliminate excess membrane tissue. The removal of the yolk cell membrane by endocytosis has long been proposed to be essential during epiboly. Using a combination of drugs and dominant-negative constructs to inhibit dynamin, dynamin-based endocytosis within the blastoderm but not in the yolk cell was found to be required for epiboly of the blastoderm. The blastoderm maintains epithelial integrity and the transmission of tension across the EVL. They further demonstrate that dynamin maintains EVL and epiboly progression by countering RhoA activity [37].

4.3. Epiboly regulation via the interaction of adhesion molecules and F-actin

Desmocollin (Dsc) and desmoglein a (Dsga), cadherin superfamily proteins, are desmosomes mediating intercellular junction adhesion [38]. Desmosomes appear in the mid-gastrula embryos of *F. heteroclitus* and zebrafish and are expected to play roles in early development [39,40]. The MO knockdown of Dsc and Dsga expression produce similar defects in epiboly, axis elongation, and somite formation, and is associated with abnormal desmosomes or reduced desmosome numbers [41]. With the tight link between desmosomes and sub-membrane F-actin [42], the knockdown of Dsc and/or Dsga should have an impact on the F-actin cytoskeleton, but this remains to be examined.

Claudins are the major transmembrane proteins of tight junctions. One of the claudins, claudin E, has restricted expression in the EVL. The MO knockdown of *claudin e* induces epiboly delay with high penetrance. The EVL margin attached to the YSL has reduced tension in *claudin e* morphants. Local variation in the strength of the EVL–YSL attachment in *claudin e* morphants might result in the slow and uneven advancement of the EVL and blastoderm during zebrafish epiboly [43].

Syntenin, a small intracellular scaffold protein, contains two PSD-95 proteins, discs large, and ZO-1 (PDZ) domains for interacting with the C-terminal tails of transmembrane receptors [44]. It binds to the syndecan family of heparan sulfate proteoglycans [45], occurs at the cell surface, and functions as a co-receptor for different adhesion molecules and growth factors like FGFs and Wnt proteins [46]. Syntenin is rate-limiting for the recycling of endosomal syndecans to the plasma membrane [47]. This recycling is important for cell spreading and depends on the activation of the small GTPase ADP-ribosylation factor 6 (Arf6), which recruits phosphatidylinositol-4-phosphate 5-kinase on perinuclear endosomes, and on the ability of syntenin to directly interact with phosphatidylinositol 4,5-bisphosphate (PIP2). Lambaerts et al. found that epiboly relies on the networking of syntenin with syndecan heparan sulfate proteoglycans [48]. The interaction of syntenin with PIP2 and with Arf6 can regulate the endocytic recycling of syndecan and is necessary for epiboly progression. Syntenin is required for the autonomous vegetal expansion of the YSL and rearrangement of the actin cytoskeleton in extra-embryonic tissues, but not in embryonic cell fate determination.

Heparan sulfate proteoglycans (HSPGs) are cell surface and extracellular matrix proteins with the covalent glycosaminoglycan (GAG) chain. The modulation of cellular responses by HSPGs is partially controlled by GAG chain modification [49]. O-sulfotransferases catalyze the transfer of a sulfate to heparan sulfate GAG chains. Cadwalader et al. showed that 2-O-sulfotransferase (2-OST) is essential for canonical Wnt signaling in zebrafish development [50]. Embryos deficient in 2-OST have reduced GAG chain sulfation and are not responsive to exogenous Wnt8 overexpression. 2-OST morphants have normal activation of selected zygotic mesoderm,

endoderm, and ectoderm genes, but exhibit decreased deep cell adhesion and cannot initiate epiboly. These defects in 2-OST-deficient zebrafish embryos are associated with decreased β-catenin and E-cadherin protein levels at cell junctions, and can be rescued by stabilized β-catenin or dominant-negative Gsk3 but not by overexpressing the Wnt8 ligand. Thus, 2-OST appears to act downstream of the Wnt ligand and is upstream of intracellular Gsk3 and β-catenin.

Tay et al. knocked down Cdc42 and Chp (Cdc42 homologous protein) and found that Chp is essential for zebrafish epiboly. Chp/RhoV is a Cdc42-like GTPase. It binds a number of effector kinases including PAKs [51,52]. The over-expressed Chp protein promotes rapid turnover of PAK1 in mammalian cells [51], but its role in embryogenesis is unclear. Chp is required for the stabilization of the E-cadh/bcatenin complex at adherens junctions. Cell adhesion during epiboly is perturbed without Chp. PAK activation at cell–cell junctions occurs downstream of Chp during this process [53].

4.4. Epiboly regulation via calcium regulation

Contraction of the actin-myosin ring is the driving force for the progression of epiboly. Calcium is a key regulator of actin ring contraction. After 50% epiboly, a transiently high concentration of calcium appears at the blastoderm margin where the actin ring originates [54,55]. Blockage of the calcium transient inhibits actin ring formation [13]. On the other hand, the zebrafish homolog of the serine-threonine kinase mitogen-activated protein kinase-activated protein kinase 2 (MAPKAPK2) and its regulator p38 MAPK are associated with premature ring contraction and ectopic calcium levels increasing in the blastoderm [56]. These facts suggest that intracellular calcium needs to be tightly regulated for contraction of the actomyosin ring and epiboly progression.

Nrz, a member of the bcl-2 family of apoptosis inhibitors, is specifically expressed in the YSL [57]. Knockdown of Nrz induces premature actin ring contraction and embryonic mortality [58]. This premature contraction is triggered by increasing calcium levels at the margin between the blastoderm and the YSL even before the initiation of epiboly. Calcium presumably activates the calcium-dependent protein calmodulin and downstream myosin light chain kinase (MLCK), leading to increased myosin phosphorylation and subsequent actin-myosin contraction. Nrz is found in the endoplasmic reticulum (ER) and interacts with inositol 1,4,5-trisphosphate receptor type 1 (IP3R1) to regulate ER calcium release. Nrz binds to the IP3R1 BH4 domain to control calcium fluxes inside the YSL and regulates actin–myosin ring formation during epiboly. Together, this series of studies suggest that Nrz is a central mediator for regulating calcium signaling and actin dynamics during epiboly progression.

Nrz contains four BH (Bcl-2 homology) domains, numbered BH4, BH3, BH1, and BH2, from the N to the C terminus, respectively. Bcl-2 interacts with IP3R1 via its BH4 domain. Histamine can elicit an IP3-induced Ca^{2+} release (IICR). Bonneau et al. found that histamine-induced increased cytosolic Ca^{2+} is significantly reduced by overexpressing Nrz BH1, BH3, and BH4 domains (Nrz1-94Cb5), but not Nrz BH3 and BH4 domains (Nrz1-67Cb5), suggesting that both BH4 and BH1 domains are required for IICR [59]. They also found that BH4 and BH1 domains are required for the inhibition of IICR for actin remodeling and epiboly in zebrafish. Furthermore, they demonstrated that phosphorylation on Nrz Ser31 by an unknown kinase is critical for inhibiting the interaction between Nrz and IP3R1 and promoting IICR.

Different protein kinases or phosphatases have been reported to modify IP3R and modulate its channel activity via phosphorylation or dephosphorylation at well conserved sites among species that include zebrafish. In general, IP3R is activated by phosphorylation and inactivated by dephosphorylation. Bcl-2 can modulate the phosphorylation status of IP3R by direct interaction with calcineurin [60] and calcium/calmodulin-dependent phosphatase, which are both known to be phosphatases for IP3R [61]. Calcineurin specifically regulates increased IICR induced by the protein kinase C (PKC) phosphorylation of IP3R. Thus, Bcl-2 may recruit calcineurin to trigger dephosphorylation of IP3R and inhibit IICR. Bcl-2 could also be phosphorylated by PKC at the Ser70 site, a residue located in an unstructured loop at the N-terminus of the protein between the BH4 and BH3 domains [62]. Collectively, these studies all point to the critical regulation of IP3R phosphorylation status and activity by Bcl-2. Bcl-2 phosphorylation on serine or threonine residues of IP3R is associated with its inactivation since mutation of these phosphorylated sites enhances the anti-apoptotic effect of Bcl-2 [63,64]. More importantly, a non-phosphorylatable Bcl-2 was shown to be more efficient for decreasing IICR [65], suggesting that a phosphatase is required for its activation and that calcineurin, a Blc-2 interacting phosphatase, should be a favorable candidate. By the same token, Nrz also contains an unstructured loop between its BH4 and BH3 domains, and this loop has two serine residues and one threonine residue as does Bcl-2. It was thus hypothesized that Nrz may be similarly regulated by phosphorylation [66].

To examine the role of Nrz phosphorylation during epiboly, [59] first showed that Nrz is phosphorylated during early epiboly. They constructed several Nrz phosphomimetic mutant genes bearing mutations to prevent the phosphorylation required for binding to IP3R1. In cultured cells, wild-type Nrz but not Nrz with phosphomimetic mutations interacted with the IP3 binding domain of IP3R1, inhibited the binding of IP3 to IP3R1, and prevented histamine-induced increases in cytosolic Ca^{2+}. The functional importance of Nrz phosphorylation was validated by the expression of the Nrz phosphomimetic mutant in zebrafish embryos and observations of the disruption of cyclic Ca^{2+} transients on the assembly of the actin-myosin ring and epiboly arrest. These data suggest that Nrz phosphorylation is necessary for the generation of IICR and the formation of circumferential actin-myosin cables required for epiboly. Gillet and colleagues thus built a model for the control of IP3R1/Nrz interactions by reversible phosphorylation and dephosphorylation [66]. Before 50% epiboly, Nrz interacts and inhibits IP3R1 by a phosphatase like calcineurin or by the direct binding of Nrz to the IP3R regulatory domain. After the shield stage, an unidentified signal activates phospholipase C (PLC) to generate IP3 and diacylglycerol (DAG) for the subsequent activation of PKC [67]. PKC may then phosphorylate both Nrz and IP3R1, thus inhibiting Nrz and activating IP3R1, which allows the release of calcium from the ER upon IP3 binding. The PKC-dependent phosphorylation of IP3R is inhibited by calcium [68] to maintain low levels of calcium in the YSL. The Nrz-dependent release of calcium may activate calmodulin to interact with MLCK and promote MLC2 phosphorylation, thus inducing actin-myosin ring contraction. On the other hand, calcium and calmodulin may activate calcineurin, which could in turn dephosphorylate both Nrz and IP3R1 by interacting with Nrz and prevent channel opening. This feedback loop may be of major importance during epiboly since abnormal calcium levels in the YSL may induce unwanted actin ring contraction leading to the detachment of the blastoderm from the yolk and death of the embryo. This reversible phosphorylation of the Nrz/IP3R complex is critical for proper calcium signaling and subsequent actin cytoskeleton remodeling in the developing embryo. According to this model, a phosphorylation/dephosphorylation loop may be implicated in generating temporally controlled calcium transients and actin–myosin ring contraction, which drive epiboly progression. If confirmed, this hypothesis could give new insight into calcium signaling and cytoskeleton dynamics during early zebrafish development. Recently, the Gillet group further identified a newly characterized Bcl-2 family member, Bcl-wav, and demonstrated its

necessity for zebrafish gastrulation. Bcl-wav regulates the formation of actin protrusions during convergence and extension via mediating mitochondrial Ca^{2+} uptake. This finding further illustrates the diverse role of Bcl-2 protein functions.

4.5. Epiboly regulation via Rho GTPases

Rho is one master regulator for actin cytoskeleton-related cellular processes, including exocytosis, endocytosis, vesicle transport/secretion, cell migration, and cytokinesis [69]. Pharmacological inhibition of Rho by *Clostridium botulinum* C3-exoenzyme (C3) disrupts cytokinesis, epiboly, and gastrulation movements [70]. The effects of Rho are activated by Wnt5/Wnt11 and mediated via its downstream effector Rho-associate kinase and diaphanous-related formin 2 (Dia2) [70–72]. Rho signaling exerts its effect on actin dynamics during gastrulation via modulation of actin binding proteins profilin and cofilin [70–73]. Actin dynamics can also be regulated by PIP2/PIP3 balance via their catalyzing enzymes, PTEN and PI3K, respectively [74,75].

The Wnt/PCP pathway mediates mitosis by an unknown mechanism in dorsal epiblast cell divisions along the animal–vegetal (AV) axis during zebrafish gastrulation. A recent finding indicates that anthrax toxin receptor 2a (Antxr2a) localizes in a polarized cortical cap aligned with the embryonic AV axis and forecasts the division plane as that of a F-actin AV polarized cap. More interestingly, the formation of this F-actin cap depends on Wnt/PCP and its effectors RhoA and Rho-associate kinase 2. Antxr2a interacts with actin and RhoA in the cap to activate the diaphanous-related formin Dia2. It suggests that Antxr2a acts as a Wnt-dependent polarized determinant to exert torque on the spindle and align it with the AV axis via the activation of RhoA and Dia2 [76].

5. Conclusion

Gastrulation has the most striking morphological changes during early development. Those changes are accomplished by coordinated cellular movements to build germ layers and body axes to insure correct patterning for further development. Gastrulation cell movements mediated by the dynamic changes of cytoskeleton proteins, microtubules, and actin have been documented and the development of cell imaging technologies further improves our understanding over time and space. The power of molecular tools also unravels the functional necessity of genes and factors like calcium and its biding proteins involving in the regulation of microtubules and actins during gastrulation. However, many questions still remain to be answered, in particular the initiation of epiboly and the coordination between different cell movements during gastrulation.

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