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Review

# $Ca^{2+}$ signaling and early embryonic patterning during the Blastula and Gastrula Periods of Zebrafish and *Xenopus* development

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**Abstract**

It has been proposed that $Ca^{2+}$ signaling, in the form of pulses, waves and steady gradients, may play a crucial role in key pattern forming events during early vertebrate development [L.F. Jaffe, Organization of early development by calcium patterns, BioEssays 21 (1999) 657–667; M.J. Berridge, P. Lipp, M.D. Bootman, The versatility and universality of calcium signaling, Nat. Rev. Mol. Cell Biol. 1 (2000) 11–21; S.E. Webb, A.L. Miller, Calcium signalling during embryonic development, Nat. Rev. Mol. Cell Biol. 4 (2003) 539–551]. With reference to the embryos of zebrafish (*Danio rerio*) and the frog, *Xenopus laevis*, we review the $Ca^{2+}$ signals reported during the Blastula and Gastrula Periods. This developmental window encompasses the major pattern forming events of epiboly, involution, and convergent extension, which result in the establishment of the basic germ layers and body axes [C.B. Kimmel, W.W. Ballard, S.R. Kimmel, B. Ullmann, T.F. Schilling, Stages of embryonic development of the zebrafish, Dev. Dyn. 203 (1995) 253–310]. Data will be presented to support the suggestion that propagating waves (both long and short range) of $Ca^{2+}$ release, followed by sequestration, may play a crucial role in: (1) Coordinating cell movements during these pattern forming events and (2) Contributing to the establishment of the basic embryonic axes, as well as (3) Helping to define the morphological boundaries of specific tissue domains and embryonic structures, including future organ anlagen [E. Gilland, A.L. Miller, E. Karplus, R. Baker, S.E. Webb, Imaging of multicellular large-scale rhythmic calcium waves during zebrafish gastrulation, Proc. Natl. Acad. Sci. USA 96 (1999) 157–161; J.B. Wallingford, A.J. Ewald, R.M. Harland, S.E. Fraser, Calcium signaling during convergent extension in *Xenopus*, Curr. Biol. 11 (2001) 652–661]. The various potential targets of these $Ca^{2+}$ transients will also be discussed, as well as how they might integrate with other known pattern forming pathways known to modulate early developmental events (such as the Wnt/$Ca^{2+}$pathway; [T.A. Westfall, B. Hjertos, D.C. Slusarski, Requirement for intracellular calcium modulation in zebrafish dorsal–ventral patterning, Dev. Biol. 259 (2003) 380–391]).


*Keywords:* $Ca^{2+}$; Blastula Period; Gastrula Period; Zebrafish; *Xenopus*

## 1. Introduction

### 1.1. Embryonic $Ca^{2+}$ signaling: an overview

With the development of less invasive live imaging techniques, evidence has been steadily accumulating to support the hypothesis that $Ca^{2+}$ signaling, in the form of pulses, waves, and steady gradients, may play a crucial role in key pattern forming events during early development [1–3]. Webb and Miller [8] have suggested that in zebrafish, the complexity of embryonic $Ca^{2+}$ signaling mirrors that of the developing embryo. Thus, early Zygotic and Cleavage Period $Ca^{2+}$ signaling events take the form of relatively simple intracellular waves. Then during the Blastula to early Gastrula Periods (BP and GP, respectively), there is a transition from intracellular to more complex localized intercellular $Ca^{2+}$ wave generation. This is followed, as gastrulation proceeds, by the generation of more extensive (including pan-embryonic) intercellular $Ca^{2+}$ waves, which reflect the more wide-ranging morphological events occurring during this period, such as epiboly, convergent extension and subsequent axis and germ layer formation. Evidence is beginning to accumulate to suggest that these GP intercellular $Ca^{2+}$ waves might help to regulate the coordinated movement of cells during gastrulation in both fish [5,9,10] and amphibian [6] embryos. Once the basic embryonic body plan

*Abbreviations:* BP, Blastula Period; GP, Gastrula Period; MTs, microtubules; MFs, microfilaments

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has been established during gastrulation, it has been proposed that embryonic $Ca^{2+}$ signaling then returns to more localized, intercellular events that are associated with the generation of specific structures such as the somites, tail, and various organ anlagen [8]. In this review, with reference to the embryos of zebrafish and *Xenopus*, we consider the generation and possible function of $Ca^{2+}$ transients reported during the BP and GP, a developmental window crucial to the establishment of essential embryonic complexity.

### 1.2. The blastula and gastrula periods in zebrafish and Xenopus

It is during the BP and GP that embryos initiate their remarkable transition from a simple ball of cells to complex form. It is not the purpose of this review, however, to consider in detail the epigenetic processes that constitute these essential patterning periods during zebrafish and *Xenopus* development. For such an enterprise, we recommend that our readers consult the numerous excellent texts and reviews on early developmental processes, such as the classic "Normal Table of *Xenopus* Development" by Nieuwkoop and Faber [11] and the "Stages of Embryonic Development of the Zebrafish" by Kimmel et al. [4], as well as the excellent recent volume titled – "Gastrulation" – edited by Claudio Stern [12]. In order, however, to put the $Ca^{2+}$ signaling events during the BP and GP into a comparative developmental context (with respect to zebrafish and *Xenopus*), we thought it necessary to include a brief description of the key developmental events that occur during this developmental window in these two vertebrate models.

#### 1.2.1. Zebrafish blastula period

The BP in zebrafish development has been described as a stage of "regional specifications" [13]. Four major developmental events occur during the BP: (1) Enveloping Layer Formation: at around the 256-cell Stage (i.e., during interphase of the 9th cell division cycle) the outermost layer of blastomeres begin to thin-out and form the enveloping layer, or EVL (see Fig. 1, panel B). (2) Mid-blastula Transition: at the 512-cell Stage, the embryo undergoes the mid-blastula transition (MBT; see Fig. 1, panel C) where the zygotic gene transcription

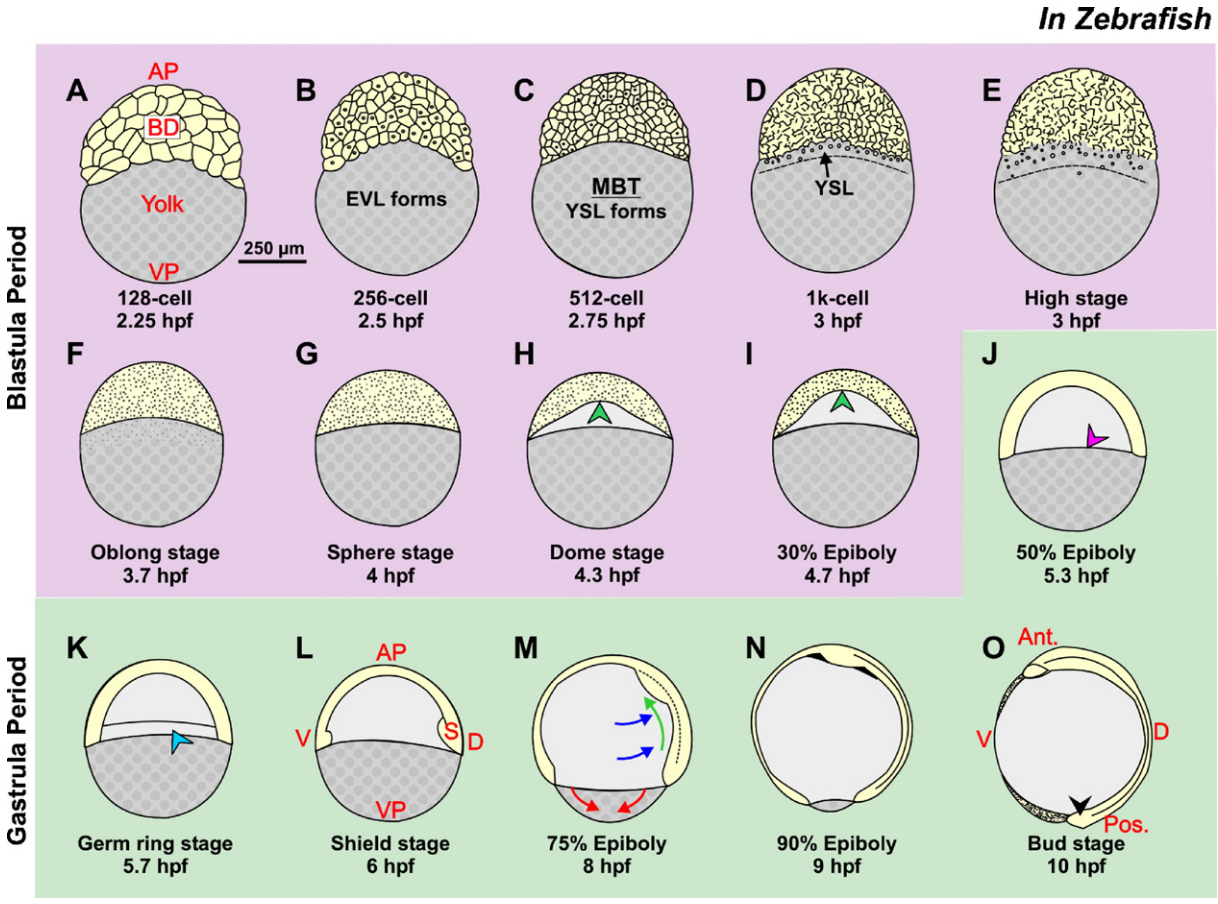



Fig. 1. Schematic representation of zebrafish development during the Blastula and Gastrula periods. (A–I) The Blastula Period runs from the 128-cell Stage at 2.25 hpf to 30% epiboly at 4.7 hpf, whilst (J–O) the Gastrula Period starts where the Blastula Period ends and runs through to Bud Stage at 10 hpf. All these images are of zebrafish embryos in a lateral orientation. The yolk, blastoderm (BD), animal pole (AP) and vegetal pole (VP) are labeled in panel A and the yolk syncytial layer (YSL) is labeled in panel D. The process of yolk cell doming can clearly be seen by 4.3 hpf and is indicated by green arrowheads in panels H and I. The leading edge of the blastoderm is indicated by a pink arrowhead in panel J. Panel K illustrates the formation of the germ ring, indicated by a blue arrowhead. The dorso-ventral axis can first be distinguished morphologically at Shield Stage (L). D, V, and S are dorsal, ventral and shield, respectively. The simultaneous movements of epiboly (red arrows), convergence (blue arrows) and extension (green arrow) are shown in panel M. In panel O, Ant. and Pos. are anterior and posterior, respectively and the tail bud is indicated by a black arrowhead. Schematics are modified from Kimmel et al. [4].

commences. (3) Yolk Syncytical Layer Formation: at approximately the same time (i.e., the 512-cell to 1K-cell transition), the yolk syncytial layer (YSL) forms (see Fig. 1, panel D), and (4) Commencement of Epiboly: At the Dome Stage, epiboly begins (see Fig. 1, panel H).

During the early stages of the BP, near-synchronous (or metasynchronous) cell division occurs, but the orientation of the cleavage planes is indeterminate and the daughter cells are much less regularly arranged than they were during the previous Cleavage Period [4]. The cell cycle then lengthens and this marks the onset of the MBT. In zebrafish, the MBT begins during the 10th cell cycle, two cycles earlier than in *Xenopus* [14,15], but otherwise the essentials of the MBT have been reported to be the same in the two species [16]. The MBT also marks the start of blastomere motility and thus the beginning of morphogenetic movements. Despite the apparent uniformity of the blastomeres during the BP, careful observation reveals differences among cells in terms of cell division, cell adhesion, and cell motility that define three mitotic domains: (1) The yolk cell. As the early cell divisions during the preceding CP are meroblastic, the yolk cell remains undivided and is initially cytoplasmically continuous with all the blastomeres above. The yolk cell only becomes separated from the overlying tiers of blastomeres by subsequent horizontal cell division. There always remains, however, a ring of blastomeres at the yolk-blastoderm margin that are still cytoplasmically continuous with the yolk. These marginal blastomeres eventually collapse and release their cytoplasm and nuclei into the adjoining cytoplasm of the yolk cell to form the yolk syncytical layer (YSL). When it first forms, the YSL is a narrow ring around the edge of the blastoderm, called the external YSL, or E-YSL, but within a few rounds of cell division, it spreads underneath the blastoderm to additionally form a complete internal syncytium layer, called the internal YSL, or I-YSL [4]. After the YSL forms, the nuclei in this region continue to undergo karyokinesis for several rounds of division, then mitotically arrest. Evidence is emerging to suggest that the YSL, and the E-YSL in particular, may also play an important role in the establishment of the basic body plan in the zebrafish embryo during the BP, by acting as the source of signals that may induce mesoderm, neuroectoderm, the trunk organizer and the notochord [17]. (2) The second mitotic domain is the Enveloping Layer (EVL), and is formed from cells that make up the outermost layer of the blastoderm. These peripheral cells thin-out to form an embryonic epithelium, or periderm, and acquire a long asynchronous cell cycle. When cell division does occur in the EVL, the daughter cells remain within the plane of the periderm. The EVL is an extra-embryonic structure, as these cells do not contribute to the embryo-proper, and are later replaced by a true ectodermal–mesodermal derived skin [18]. An important aspect with regards to our later consideration of the $Ca^{2+}$ transients generated during this period is that the cells of this lineage-restricted periderm adhere tightly to one another to prevent both the intrusion of the extra-embryonic medium into the embryo, as well as the escape of the more motile blastomeres from the underlying blastoderm. Furthermore, during the subsequent doming of the yolk cell (see later, and Fig. 1, panels H and I), considerable upward mechanical forces are exerted on the overlying blastoderm, and the EVL thus serves to resist this pressure and maintain embryonic integrity. (3) Sandwiched between the overlying EVL and the underlying YSL, is the third mitotic domain, known as the deep cells (DCs). These go on to form the embryo proper. Following the MBT, these cells become motile, extruding projections and moving in a seemingly random manner, thus resulting in an extensive mixing of the DC population. There is a high degree of extracellular space in the DC blastoderm during this period, thus facilitating cell motility and perhaps providing an environment suitable for the secretion and diffusion of extracellular signaling molecules. What is important to note, is that at this stage it is not possible to distinguish the dorsal side of the embryo via any morphological criteria. This can be done, however, at least by the mid-blastula period, through the use of molecular markers such a β-catenin, which enters the nuclei of the dorsal region of the yolk cell, a process consistent with its role in the formation of the Nieuwkoop center of the embryo (i.e., the embryonic shield in zebrafish; [19]). During the later stages of the BP, the process of establishing the dorsal organizer is well under way from a molecular (if not morphological) viewpoint. The study of mutant phenotypes, in combination with the use of *in situ* hybridization using a variety of probes that mark both the dorsal and ventral regions, clearly indicate that during the BP, large regional domains of the embryo are being established.

Epiboly, the spreading of the blastoderm to encompass the yolk cell, begins during the late BP. The early stage of epiboly, during which the blastoderm covers the animal hemisphere of the yolk cell, is driven by the doming of the yolk cell upwards toward the animal pole (see Fig. 1, panels H and I). This results in the blastoderm changing from a high-piled mound of cells to a cup-shaped multilayer (initially ~5 cells thick) around the doming yolk [4]. When the blastoderm margin reaches the equator of the yolk cell (i.e., 50% epiboly), this represents the end of the BP and the beginning of the GP. Another important feature of the mid to late BP is a process termed "dorsal compaction". This results from deep cells compacting together at the dorsal margin, extending in an arc of about 30° from the dorsal midline. During the late BP, deep cell intercalations are radial and accompany the blastoderm thinning that is associated with epiboly. This is in contrast to later intercalations that occur during the GP and which tend to be mediolateral, and thus play an important role in convergent extension resulting in a narrowing and elongation of the primary embryonic axis [20].

### *1.2.2. Zebrafish gastrula period*

During the GP, epiboly continues and, in addition, the major morphogenic cell movements of involution, convergence, and extension occur, with the beginning of involution defining the onset of gastrulation [4] (see Fig. 1, panels J to K). All four types of cell movement contribute to generating the primary germ layers and the embryonic axes. With the exception of epiboly, these cell movements primarily involve the DCs, as during the GP the EVL cells neither divide nor involute, nor do they appear to converge to any great extent [4]. Also during the GP, the EVL cells continue the flattening process initiated

during the BP and they also continue to relocate, via the process of epiboly, toward the vegetal pole. Initially, between the BP and the GP, the vegetal pole-ward advance of the blastoderm stops for a short time at the equator. This coincides with the formation of a structure called the germ ring (see Fig. 1, panel K), which takes the form of a thickened ring of cells that appears near simultaneously around the blastoderm margin. The germ ring results from the involution of the marginal deep cells under the leading edge of the blastoderm to form a new layer called the hypoblast. This process thus changes the embryo from a single to a two germ layered structure, comprised of an inner hypoblast and an outer epiblast. Involution in zebrafish has been reported to be similar to involution in *Xenopus* [21]. The hypoblast is fated to become endoderm and mesoderm [18]. On the ventral and lateral edges of the blastoderm, the first cells to involute tend to become endoderm, and the later involuting cells become mesoderm, although at this early stage of the GP there are no obvious morphological differences between these cells [22].

It is possible to begin to construct a zebrafish fate map, which at the 50% Epiboly Stage overtly resembles the gastrula fate map of *Xenopus*, i.e., cells at the blastoderm margin go on to form both mesodermal and endodermal structures, and cells away from the margin develop into endodermal structures alone. Cells on the dorsal side of the blastoderm make axial structures and tend to contribute more to anterior structures, while those on the ventral side make lateral structures and tend to contribute more to posterior structures. The superficial similarities in the fate maps when comparing early zebrafish and *Xenopus* development may help us to determine the possible functional role of regional $Ca^{2+}$ signaling associated with this period of development.

Appearing with the germ ring is the embryonic shield, which is a thickening of the ring that is homologous to the dorsal lip in *Xenopus* and Hensen's node in the chick (see Fig. 1, panel L). Formation of the embryonic shield visibly marks the future dorsal side of the embryo. The first cells to involute at the shield, stream in an anterior direction, and have been reported to correspond to precursors of the tetrapod prechordal plate. Cells of the notochord rudiment, the chorda mesoderm, then begin to involute at the dorsal midline only shortly after these earliest prospective prechordal plate cells, as the embryonic shield becomes more visible. Convergence and extension, involving cell mixing and anterior-ward cell migration, are prominent within the embryonic shield and the surrounding region (see Fig. 1, panel M).

Following the germ ring and shield-formation hiatus, which lasts for around 60 min, the EVL and the YSL continue to spread over and around the remaining vegetal hemisphere of the yolk cell, until by the end of the GP, they completely cover the yolk cell. During this process, the yolk cell membrane (YCM) is progressively absorbed by a process of endocytosis [23]. The progression of the blastoderm and the YSL toward the vegetal pole, as well as the endocytosis of the YCM, is thought to involve a complex interaction between microtubules (MTs; [24,25]) and microfilaments (MFs; [23]) as well as other cytoskeletal components. It has also been suggested that $Ca^{2+}$ signals reported during epiboly may serve to orchestrate these cytoskeletal components via $Ca^{2+}$-sensitive elements. In *Xenopus*, which has no distinct EVL and no YSL, epiboly still occurs to a lesser extent, where cell movement is thought to be mediated initially by radial intercalation [26], and later by a little-understood purse-string-like movement [13]. Both of these processes may also involve $Ca^{2+}$-based modulation.

The mid-gastrula period is also a time of cell convergence toward the dorsal midline. Although involution continues during the mid-gastrula period, some form of convergence is found in every cell movement within the embryo [13]. Cells of the epiblast thin into a single-cell-thick epithelium as they begin to converge to the dorsal side. The dorsal epiblast is fated to become central nervous system [27], and this region of the epithelium undergoes extensive convergence and extension as it forms the neural keel [28]. The ventral side of the epiblast is fated to become epidermal cells, and the cells of this region flatten into a thin epithelium of cells as they cover the forming embryo. During this period, the germ layers of the hypoblast also become morphologically distinguishable and also begin to move dorsally. Endodermal cells sort into a lower layer of flat thin cells against the surface of the yolk cells, and mesodermal cells form a loose layer of rounded cells that are located between the epiblast and the endoderm [22].

With regards to the following sections dealing with $Ca^{2+}$ signaling during the GP, it is worth mentioning the essential role played by cell-to-cell adhesion molecules, such as members of the cadherin family, in the convergence process. Evidence is beginning to accumulate to suggest that a possible down-stream target of some of the BP and GP $Ca^{2+}$ signals might be modulating the cell adhesion properties governing the motility of populations of cells. This proposal will be expounded in the following sections.

Epiboly, and the GP come to a close at the Bud Stage, during which the blastoderm fully covers the yolk cell and the tail bud develops (see Fig. 1, panel O). The latter is a distinct swelling generated at the caudal end of the embryonic axis just dorsal to the site of yolk plug closure. It has been proposed that the tail bud develops into a secondary organizing center, and plays a key role in the development of the posterior trunk [29]. This region has also been reported to show distinct $Ca^{2+}$ signaling activity [5,8]. Along the dorsal side, anterior to the tail bud, the neural plate is now thickened along the entire embryonic axis, its more posterior cells will contribute to the trunk spinal cord. The thickening of the embryonic axis is most prominent near the animal pole, in the prospective head region. At this stage, the GP is considered to be over, and the embryo is now ready to enter into the Segmentation Period.

### *1.2.3. Xenopus blastula period*

After around 3.5 h post fertilization (hpf), the Stage 6.5 *Xenopus* morula develops into a Stage 7 Large-cell Blastula Stage (see Fig. 2, panels A and B). The formation of the blastocoel in the center of the animal hemisphere around this time is often taken to indicate the beginning of the BP. A compete network of junctions around the exterior cell margins seals the blastocoel from the external bathing medium, while

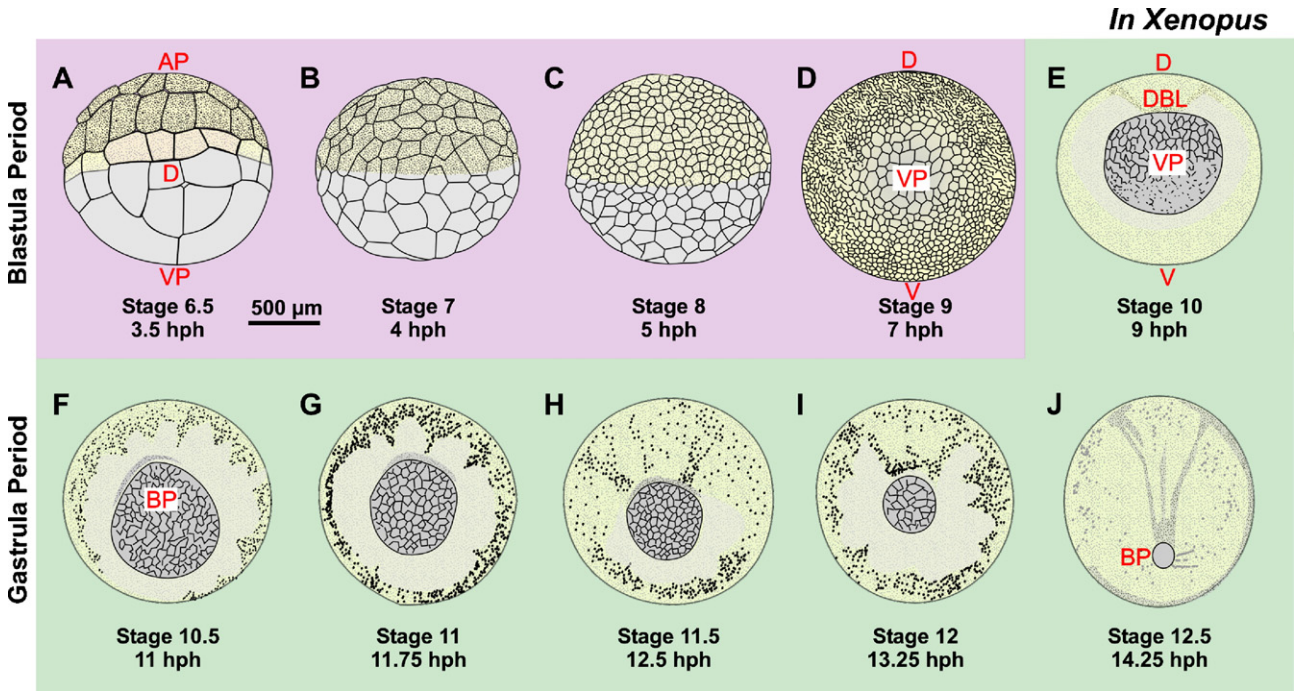



Fig. 2. Schematic representation of *Xenopus laevis* development during the Blastula and Gastrula periods. (A–D) The Blastula Period runs from around Stage 6.5 at 3.5 hpf through to Stage 9 at 7 hpf. (E–J) The Gastrula Period then starts where the Blastula Period ends and runs through to around Stage 12.5 at 14.25 hpf. Images A to C show a dorsal view of an embryo, whilst images E to J show vegetal pole views of the embryo with the dorsal side to the top. AP, VP, D and V are animal pole, vegetal pole, dorsal and ventral, respectively. In panel E, DBL indicates the dorsal lip of the blastopore, the site of the *Xenopus* organizer, a region equivalent to the embryonic shield in zebrafish. In panels F and J, BP indicates the blastopore at the vegetal pole. Schematics are modified from Nieuwkoop and Faber [11].

internal cells are connected by junctions that are readily disrupted in $Ca^{2+}$-free media. During this period, tangential holoblastic cleavage continues and pregastrulation movements of deeper blastomeres are noticeable for the first time. A slight epibolic extension of the animal and equatorial areas of the blastula has also been described [30]. Stage 8 embryos (see Fig. 2, panel C) are referred to as being at the Medium-cell Blastula Stage, where a distinct single-cell thick outer layer develops, leaving an inner cell layer between 1 to 4 cells thick between the outer cell layer and the blastocoel. Furthermore, the first indication of differences between animal, marginal and vegetive regions of the embryo becomes visible. Stage 9 represents the Fine-cell Blastula Stage (see Fig. 2, panel D). Embryos now display a sharp delimitation of the outer cell layer in the animal and equatorial regions, where the outer layer becomes segregated into presumptive ectodermal and endodermal areas. The inner blastomeres also become more sharply segregated into presumptive ectodermal (animal), mesodermal (equatorial) and endodermal (vegetative) areas. As mentioned previously, this low resolution fate map superficially resembles the zebrafish fate map at this developmental stage. As in zebrafish, cell division during the BP of *Xenopus* is initially synchronous. However, as the BP proceeds, synchrony is lost, the cell division rate slows, and zygotic transcription commences, thus marking the start of the *Xenopus* MBT [14,15]. As mentioned previously, the MBT occurs around two cell cycles later than in zebrafish [16]. Furthermore, as the BP progresses, blastomeres begin to compact more intensively, as intercellular adhesion increases. As a result, during the BP, the *Xenopus* blastula changes in its overall appearance, from a knobbly, multicellular ball to a smoother looking sphere.

### *1.2.4. Xenopus gastrula period*

The GP of *Xenopus* development, similar to that of the zebrafish, is one of extensive morphological movements. It has been proposed that the forces responsible for driving gastrulation are mainly provided by a belt of tissue around the equator of the embryo called the Marginal Zone [30]. Although some autonomous expansion of the animal hemisphere and some dorsal convergence of the marginal zone have already occurred during the BP (referred to in the previous section as pregastrulation movements), the conventional start to gastrulation is marked by the appearance of a pigmented depression in the dorsal vegetal quadrant. This is the dorsal lip of the blastopore. The blastopore lip becomes elongated laterally, and soon becomes a complete circle (see Fig. 2, panel E). When the circle is complete, the dorsal segment is referred to as the dorsal lip while the ventral segment is referred to as the ventral lip. The blastopore is the site of invagination of the superficial marginal zone of the embryo, which results in the formation of the archenteron (i.e., the primitive gut). Although invagination occurs all around the blastopore, it is much more extensive on the dorsal side, where it proceeds until the leading edge of the invaginating tissue is well past the animal pole, with the archenteron roof being closely apposed to the overlying ectoderm. At the lateral and ventral portions of the blastopore, however, invagination is less extensive. The dorsal region of the blastopore, and the developmental processes that are associated

with it, are correlated with the generation of distinct $Ca^{2+}$ transients. These will be discussed in the following sections. Deep marginal zone cells also involute and then extend toward the animal pole. Dorsal involution is again more extensive, while the ventral involution is slight, and the whole involution process is accompanied by a general shift of cells in a dorsal direction. Simultaneously with the movements of the marginal zone, the animal cap tissue spreads vegetally so that the blastopore becomes a smaller and smaller ring as it approaches the vegetal pole and eventually narrows to a small opening (see Fig. 2, panels E to J). The exact nature of the motive mechanism responsible for the invagination and involution movements of the deep marginal zone cells are still a matter of considerable debate. It is clear, however, that two components contribute to this movement: convergence and extension. Convergence involves the packing together of the cells to shrink the total marginal zone circumference and it occurs equally around the marginal zone. Extension refers to an active process of cell intercalation leading to elongation in the animal to vegetal direction, and this occurs only in the dorsal region [31,32].

By the end of gastrulation the former animal cap ectoderm has covered the whole external surface of the embryo and the yolky vegetal tissues have become a mass of endoderm in the interior. In addition, the former marginal zone has become a layer of mesoderm, which extends from the now slit-shaped blastopore, reaching a considerable distance to the anterior end on the dorsal side of the embryo, but only a short distance on the ventral side. Thus, as in zebrafish, by the end of the GP, the three classic germ layers, ectoderm, mesoderm and endoderm have achieved their final trilaminer arrangement. In *Xenopus*, the archenteron has become the principal cavity (at the expense of the blastocoel) and the embryo has rotated so that the dorsal side is uppermost. It now also has a true anterioposterior axis, which runs from the leading edge of the mesoderm to the blastopore.

#### *1.2.5. Summary*

In the introductory sections above, we have outlined the basic morphological features and developmental events (in both zebrafish and *Xenopus*) during the BP and GP. These characteristics and processes are relevant to the following sections, where we describe the $Ca^{2+}$ signaling events that have been associated with these essential pattern-forming periods of development. This will allow the reader to relate any of the $Ca^{2+}$ signals described to a specific developmental process or period, as well as being able to make a comparison of similar processes in zebrafish and *Xenopus*, without necessarily having to refer back to the original publications.

## 2. ZEBRAFISH: a review of the $Ca^{2+}$ signaling events that have been reported in the Blastula and Gastrula Periods during zebrafish development

With regards to $Ca^{2+}$ signaling, the BP marks the beginning of a transition in the nature of the $Ca^{2+}$ signals reported from zebrafish embryos. During the preceding Zygote and Cleavage Periods, intracellular $Ca^{2+}$ transients were exclusively observed [9,33–37], whereas, during the BP we begin to see the development of intercellular $Ca^{2+}$ signaling [38] (see Fig. 3). It has been suggested that this transition represents the beginning of the combination of basic elements of intracellular $Ca^{2+}$ signaling into higher order signaling networks that are necessary to keep pace with the increase in developmental complexity [3].

From the 128-cell Stage onwards (i.e., the beginning of the BP), an increasing number of highly localized transient increases in intracellular $Ca^{2+}$ (referred to by some authors as $Ca^{2+}$ spikes) are generated almost exclusively in the outermost embryonic cell layer [8,38] (see Fig. 3). As mentioned in Section 1.2.1, this population of cells develops epithelial characteristics and differentiates into the enveloping cell layer (EVL) that protects the embryo from trauma and helps establish intraembryonic conditions distinct from the surrounding environment. It is important to note that such $Ca^{2+}$ spiking has not been reported from deep cell blastomeres, i.e., the population of cells that goes on to form the embryo proper, but that it remains restricted to the EVL cells until the end of the BP. Reinhard et al. [38] reported that as the BP progresses, $Ca^{2+}$ spikes in neighboring EVL cells started occurring in the same short time interval, thus indicating that perhaps small groups of EVL cells were beginning to synchronize their signaling activity. The maximum distance across the EVL with synchronized $Ca^{2+}$ spikes was not seen to exceed 100 μm (i.e., up to ~5 cells), and the time required to propagate the signal across this distance was rarely greater than 5 s. This suggests that the individual $Ca^{2+}$ spikes in a single cell could develop into intercellular $Ca^{2+}$ waves and propagate across the EVL with velocities of ~20 μm/s.

Reinhard et al. [38] also demonstrated that the appearance of the EVL $Ca^{2+}$ spiking and intercellular wave generation nicely coincided with an increase in phosphatidylinositol (PI) cycle activity, resulting in a rise in $IP_3$ levels within the embryo. This suggests that the $Ca^{2+}$ transients visualized in the EVL might be generated via $IP_3$-mediated $Ca^{2+}$ release. Such activation of the PI cycle has been reported to be involved with other developmental processes such as the cell compaction process in mouse embryos [39], and the establishment of the dorsal–ventral axis, mesoderm induction, and patterning in the *Xenopus* embryo [40–43]. Furthermore, it has also been reported that during the BP there is an increase in the number of gap junctions in the EVL of zebrafish [44]. Thus, the increase in levels of $IP_3$ combined with an increase in the numbers of gap junctions may provide the means to initiate and propagate the intercellular $Ca^{2+}$ waves in the EVL at this time, in a manner proposed by Berridge et al. [45].

It has also been reported that the frequency of $Ca^{2+}$ spiking in EVL cells can be modulated by Wnt-5A [7,46]. Wnts are secreted proteins that are thought to play a role in a variety of developmental events such as axis formation, morphogenic cell movements, mesoderm induction, and neural patterning [47]. Some Wnt proteins have been shown to work by interaction with members of the *frizzled* gene family of Wnt receptors to stimulate the PI signaling pathway [48,49]. Thus, this interaction directly links modulation of $Ca^{2+}$ signaling patterns with members of the *frizzled* gene family. The role of the Wnt/

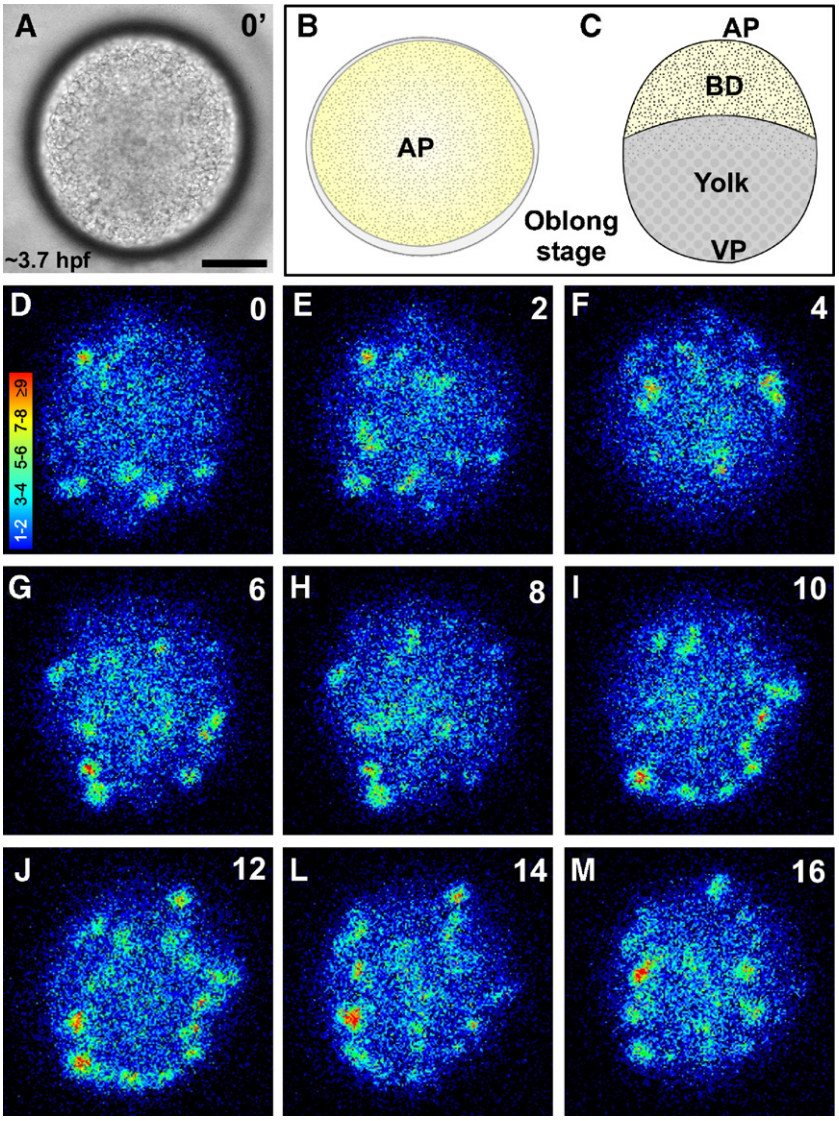



Fig. 3. Representative example of the localized $Ca^{2+}$ signaling events that occur during the Blastula Period of zebrafish development. (A) A bright-field image of an Oblong Stage embryo viewed from the animal pole, which was acquired just prior to the aequorin-generated image in panel D. (B and C) Schematics of the embryo from (B) animal pole (AP) and (C) lateral views to show the morphology of the Oblong Stage embryo more clearly (for reference, see Fig. 1). BD and VP are blastoderm and vegetal pole, respectively. (D–M) The patterns of aequorin-generated light that are emitted by groups of interconnected EVL cells within the blastoderm of the Oblong Stage embryo shown in panel A. Restriction of the $Ca^{2+}$ transients to the EVL cells alone has been confirmed by confocal microscopy [38]. Each panel represents 240 s of accumulated luminescence with a 120 s gap between each image. Color scale indicates luminescence flux in photons/pixel. Scale bar is 200 μm.

$Ca^{2+}$ signaling pathway in regulating morphological events during *Xenopus* gastrulation are described in Section 3.3.

With regards to the possible function of the EVL $Ca^{2+}$ transients, it has recently been reported that cell contact might play an important role during the determination of the EVL cells in zebrafish. It has been shown that by the late Blastula Stage, the outer cells alone are specified to express the EVL-specific marker, *cyt1*. Following blastoderm dissociation, cells did not express *cyt1*, suggesting that EVL specification requires cell-to-cell contact [50]. We propose, therefore, that the EVL-restricted $Ca^{2+}$ transients, via their interaction with $Ca^{2+}$-sensitive cytoskeletal elements, may play a role in establishing the essential cell-to-cell contact necessary for subsequent EVL specification. This is supported by the observation that the generation of $Ca^{2+}$ transients in the outermost blastomeres precedes the formation of the EVL (i.e., $Ca^{2+}$ transients are seen to begin at the 128-cell Stage, while the first morphological sign of EVL formation – a flattening of the outermost cell layer – begins to become apparent at the later 256-cell Stage). Sagerström et al. [50] also proposed that the zebrafish EVL and the *Xenopus* embryonic epithelial layer are potentially homologous structures, however, there have been no reports to date of $Ca^{2+}$ transients visualized during the specification of the epithelial layer in *Xenopus*.

It has also been suggested that a second form of BP $Ca^{2+}$ signaling may play a role in both the formation and the function of the YSL [8]. For example, during the formation of the YSL (i.e., from the 512 to 1K-cell Stage) distinctive elevated

domains of $Ca^{2+}$ have been reported from the marginal blastomeres [8]. A distinctive form of $Ca^{2+}$ transients continue to be generated at the YSL/blastoderm margin during the GP as this embryonic domain begins to assume a leading role in the process of epiboly. This is described in more detail below.

As the embryo enters the GP, epiboly continues and the morphogenetic cell movements of involution, convergence, and extension begin, producing the primary germ layers and the embryonic axis. Thus, the vertebrate body plan emerges through an orchestrated pattern of cellular rearrangements, inductive interactions, and gene expression. Several recent reports have clearly indicated that during this period the embryo generates a number of distinct $Ca^{2+}$ signaling events. These include transient non-propagating localized domains of elevated $Ca^{2+}$ [5,9] (see Fig. 4), as well as a rhythmic series of pan-embryonic intercellular $Ca^{2+}$ waves [5,10] (see Fig. 4). The latter either circumnavigate the advancing blastoderm margin, or propagate along the forming anterior/posterior axis of the embryo. They are generated with an average frequency and velocity of around 7 waves per hour and 5 μm/s, respectively, up to the time of yolk plug occlusion. Once again, we suggest that the intercellular transmission of these long-range $Ca^{2+}$ waves could be mediated by diffusion of either $IP_3$ or $Ca^{2+}$ through gap junctions [45]. This suggestion is supported by the report that in Gastrula Stage embryos there is an increase in the permeability of gap junctions in a circular zone around the blastoderm margin [51]. This correlates with the initiation time and propagation path of the pan-embryonic marginal waves [5,10].

Morphogen gradients and propagating second messenger waves have long been proposed to be carriers of putative long-range coordinating messages, which are essential for metazoan development [45]. These GP $Ca^{2+}$ waves might represent the first visualization of such a phenomenon in a developing vertebrate. Once established, the $Ca^{2+}$ waves are generated by and propagate out from what was described as a "pacemaker" region located in the embryonic shield [5], an organizer region of prime developmental significance in zebrafish, generally considered to be comparable to the Spemann organizer region of amphibian embryos [52]. It was suggested that the rhythmic $Ca^{2+}$ waves generated by the shield might serve a variety of possible developmental functions. One of these could be to provide positional information to which cells in the process of involuting, converging and extending can relate and respond. It has been reported that when comparing the attributes of propagated waves, and standing concentration gradients that

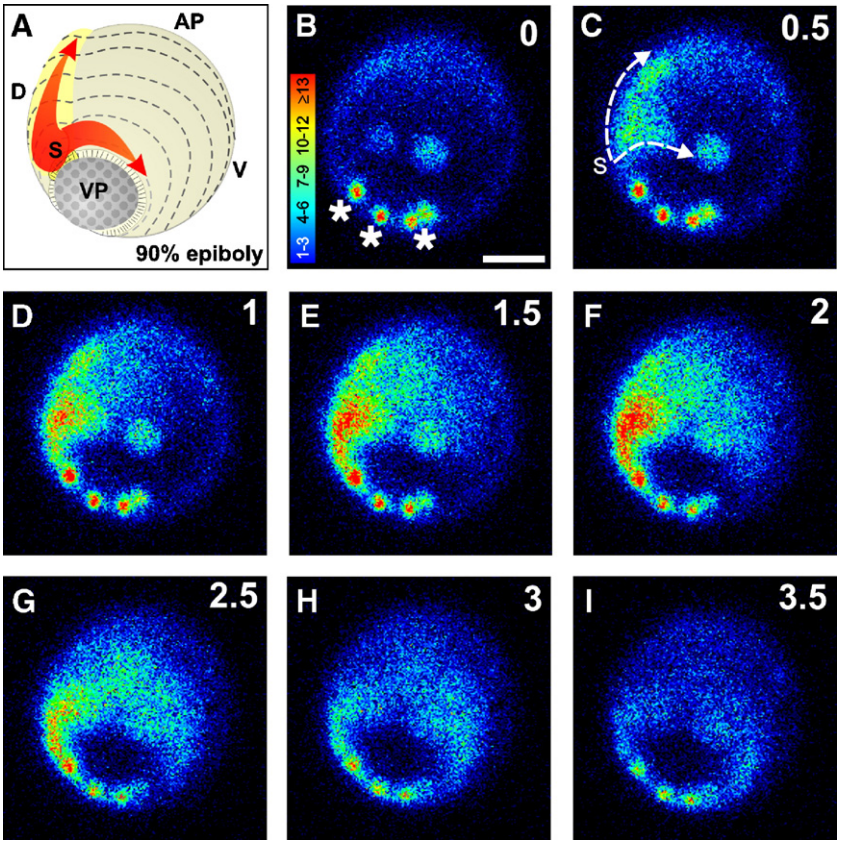



Fig. 4. Representative example of the non-propagating localized domains of $Ca^{2+}$ and the pan-embryonic intercellular $Ca^{2+}$ waves that occur during the Gastrula Period of zebrafish development. (A) Schematic representation of the embryo illustrated in panels B–I, to show its orientation and the direction of propagation of the $Ca^{2+}$ waves more clearly. AP, VP, D, V and S are animal pole, vegetal pole, dorsal, ventral and shield, respectively. (B–I) A representative example of two pan-embryonic intercellular $Ca^{2+}$ waves at ~90% epiboly. These waves are initiated at the shield, marked with an 'S' in panel C, and propagates both around the blastoderm margin and along the embryonic axis in an anterior direction. Examples of non-propagating localized domains of elevated $Ca^{2+}$ are marked with asterisks in panel B. Each panel represents 120 s of accumulated luminescence with a 30 s gap between each image. Color scale indicates luminescence flux in photons/pixel. Scale bar is 200 μm.

are set up by diffusion, the former has a two major virtues. The first, is that a pulsile signal is more efficient in terms of the drain on metabolic resources as well as the all-or-nothing nature of the signal that makes it unambiguous, whilst the second major advantage is that waves can cover greater distances than standing gradients within a fixed time period [53]. It was proposed, therefore, that propagated waves may be an essential aspect of global developmental control in the large teleost embryos such as those of the zebrafish. Thus, as the shield becomes a $Ca^{2+}$-signaling center, the rhythmic waves propagating out from it, constantly "remind" other cells where the organizing center is. Distant cells have, therefore, a positional point of reference for their global morphogenetic movements, as well as the ability to respond to local signals for more specific developmental instructions.

A second important function of these $Ca^{2+}$ waves might be to synchronize and coordinate cellular responses [54]. Many aspects of gastrulation require the coordinated movement of large populations of cells. Considering epiboly for example, blastomeres migrate over the yolk cell toward the vegetal pole (VP) in a coordinated manner. A mechanical model for epiboly in teleosts has been proposed that is based on two force generating mechanisms: MT motors in the YSL and an actin-mediated contraction at the blastoderm margin [23–25,55]. The model postulates that motors positioned in the YSL use MTs, which extend from the external YSL into the yolk cytoplasmic layer as tracks, thereby pulling the blastoderm toward the VP. Once the equator is crossed, constrictions of the blastoderm margin are initiated, aiding in its progression toward the VP. It has been suggested, therefore, that one of the developmental functions of the circumarginal gastrulation waves might be to coordinate and modulate the contraction of an actin-based network in the blastoderm margin [23]. Supporting this proposition is the fact that circum-marginal $Ca^{2+}$ waves are not seen until after the margin of the blastoderm crosses the embryonic equator. Thus, aspects of this epiboly model correlate well with the $Ca^{2+}$ signaling pattern observed during this time.

Recently, the activity of Fyn, a src-family protein tyrosine kinase, has been linked to the initiation of epiboly, involving the migration of both the YSL nuclei and the overlying marginal blastomeres. $Ca^{2+}$ imaging studies indicated that Fyn kinase exerts its effect, at least in part, through $Ca^{2+}$ signaling within the leading edge of the blastoderm [56]. The function of Fyn kinase during zebrafish development through the BP and GP was investigated through the use of dominant negative constructs designed to suppress the function of zebrafish c-Fyn [56]. Injection of mRNA encoding dominant-negative $Fyn^{K299M}$ just before the first mitotic division had no significant effect during cleavage and did not inhibit the formation of the YSL. Smoothing of the EVL at the MBT was also reported to occur normally and the expression of various zygotic transcription factors indicated that activation of the zygotic genome also occurred. Signaling pathways involved with axis determination such as β-catenin, activin, and nodal appeared to function normally, as evidenced by the expression of the *boz*, *goosecoid*, and *mezzo* genes. However, while the formation of the YSL was normal, it was reported that the marginal blastomeres failed to

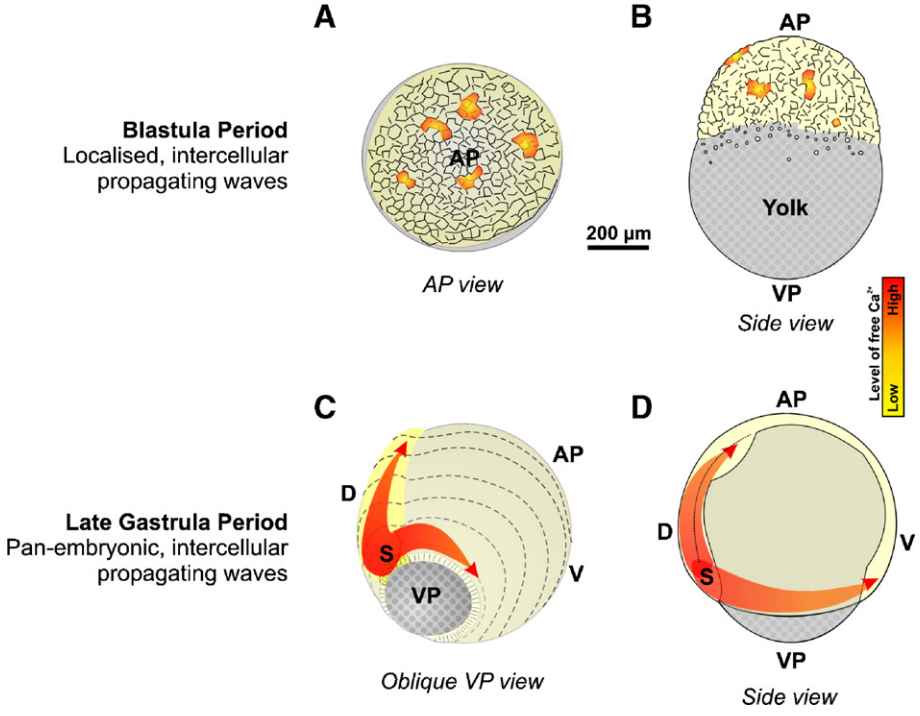



Fig. 5. Summary of the $Ca^{2+}$ signaling events observed during the (A and B) Blastula and (C and D) Gastrula Periods of zebrafish development. Embryos are shown from two orientations for each period in order to show the $Ca^{2+}$ signals more clearly. These images illustrate the switch from localized intercellular to pan-embryonic intercellular $Ca^{2+}$ signaling with the increasing morphological complexity of the embryo. The Blastula Period signals follow a period of cell proliferation (during the Cleavage Period) in which the cell size decreases as the overall number of cells increases. The switch from localized intercellular to pan-embryonic intercellular $Ca^{2+}$ signaling occurs during the transition from the Blastula to the Gastrula Periods, the latter being a time of major morphological change within the developing embryo.

migrate toward the vegetal pole and epiboly thus did not occur. This block to epiboly was elegantly prevented by co-injection of c-Fyn mRNA along with the dominant-negative construct, thus indicating that it was a specific effect. Analysis of the pattern of $Ca^{2+}$ signaling in experimental and control embryos demonstrated that the elevated $Ca^{2+}$ characteristic of the marginal blastomeres, was suppressed. These observations suggest that Fyn kinase plays an important role in epiboly, possibly through its effects on $Ca^{2+}$ signaling [56].

Some aspects of the $Ca^{2+}$ signaling seen in zebrafish appear to correspond to reports from other teleosts. The rhythmic $Ca^{2+}$ waves could be related to the propagating $Ca^{2+}$ waves that accompany the rhythmic contraction of the stellate layer of medaka [57–59]. However, in the case of zebrafish the pacemaker region generating these waves becomes established in the embryonic shield at the dorsal midline of the blastoderm margin, whereas in medaka the equivalent region appears to be located on the opposite side of the embryo [58].

In summary, through the Gastrula Period we observe an elaboration of the simple intercellular $Ca^{2+}$ signaling patterns established during the Blastula Period (see Fig. 5). This results in patterns of long-distance intercellular signaling that we suggest provide a mechanism for coordinating the spatial and temporal regulation of both global and highly localized developmental processes across the whole embryo.

## 3. *XENOPUS*: a review of the $Ca^{2+}$ signaling events that have been reported in the blastula and gastrula periods during *Xenopus* and other amphibian development

### 3.1. Direct evidence for changes in $Ca^{2+}$

One of the first reports suggesting that $Ca^{2+}$ might play a role in the early development of amphibian embryos was from Stableford in 1967 [60], who measured the $Ca^{2+}$ content of the blastocoel and capsule fluids as well as total $Ca^{2+}$ content in Blastula, Gastrula and Neurula Stage embryos of the salamander, *Ambystoma punctatum*. He demonstrated that there was an overall increase in the $Ca^{2+}$ content of the embryo during gastrulation and suggested that this rise might correlate with the changes in cell shape and cell movements, which occur during invagination, perhaps though mediating contraction or cell adhesion [60]. More recently, Komazaki [61] examined the cells of the blastopore forming region during gastrulation in the newt *Cynops pyrrhogaster* using electron microscopy, cytochemistry and X-ray microanalysis. He demonstrated that at the start of blastopore formation (at early Stage 11) tubular-shaped smooth-surfaced ER (sER) was apparent in these cells and that $Ca^{2+}$ accumulated in the tubular sER. He suggested that this organelle might be important for the regulation of the intracellular concentration of $Ca^{2+}$ during the formation of the blastopore [61].

There are relatively few reports in the literature that describe the direct visualization of $Ca^{2+}$ signals during the BP of amphibian development. Muto et al. [62] briefly mentioned that when embryos of the frog *Xenopus laevis* were loaded with the fluorescent $Ca^{2+}$ reporter, calcium green-1, then a series of $Ca^{2+}$ transients followed the formation of cleavage furrows from the first cleavage and "at least until" the Blastula Stage. However, no information was provided regarding the spatial or temporal characteristics of the $Ca^{2+}$ signals observed during the BP in this report. More recently, Leclerc et al. [63] demonstrated that distinct patterns of $Ca^{2+}$ signaling occurred during both the BP and the GP of *Xenopus* development. By injecting the bioluminescent $Ca^{2+}$ reporter, aequorin, into the dorsal micromeres of 8-cell Stage embryos, and imaging the embryos with a Photon Imaging Microscope, they reported that changes in $Ca^{2+}$ occurred specifically in the anterior dorsal part of the ectoderm, with no significant changes in $Ca^{2+}$ being visualized in the ventral ectoderm, mesoderm or endoderm. In the dorsal ectoderm there was a slow increase in the level of $Ca^{2+}$, which was observed to start during the BP at around Stage 8 (at 5 hpf), and then reach a peak during the GP at around Stages 10.5 to 11, after which it gradually declined until it returned to its resting level at around Stage 13 (i.e., just at the end of the GP). Superimposed on this slow rising component there was a series of rapid localized $Ca^{2+}$ transients, which also occurred in the anterior dorsal part of the ectoderm and which increased in both number and amplitude between Stages 9 and 11, (i.e., from the end of the BP at 7 hpf, up to the horse-shoe shaped blastopore Stage at 11.75 hpf in the GP). Additionally, at Stage 11, a series of intercellular $Ca^{2+}$ waves was observed, which propagated in the anterior dorsal part of the embryo both laterally and along the anterioposterior axis of the dorsal ectoderm (covering a distance of $\sim 1$ mm$^2$) and which traveled at a velocity of $\sim 10$ μm/s with the $Ca^{2+}$ being estimated to rise approximately threefold, to 1 μM [63].

Leclerc et al. [64] subsequently imaged the $Ca^{2+}$ dynamics observed during gastrulation in *Xenopus* embryos in further detail, this time using Keller open-faced explants in order to simplify their experimental system. In intact embryos, the dorsal ectoderm normally receives both planar and vertical signals from the mesoderm during gastrulation. Planar signals move from the anterior mesoderm to the posterior ectoderm, whereas the vertical signals move to the ectoderm from the underlying involuting mesoderm. In Keller explants, the system is simplified such that the isolated dorsal ectoderm receives only planar signals from the mesoderm, with the vertical signals being effectively eliminated. Again using aequorin, they demonstrated that in these explants (as for the intact embryos) there was a gradual increase in the overall level of $Ca^{2+}$, superimposed on to which was a series of rapid $Ca^{2+}$ transients. Whilst the discrete $Ca^{2+}$ transients appeared to arise in a random manner with respect to time and location in each explant, when each signal was plotted on an outline of that explant over the course of gastrulation, the majority of the signals appeared to be generated in the ectoderm/mesoderm border and in the non-involuting zone of the ectoderm [64].

Wallingford et al. [6] also visualized $Ca^{2+}$ waves and localized transients in Keller explants prepared from gastrulating *Xenopus* embryos. Using the fluorescent $Ca^{2+}$ reporter, calcium green dextran, in combination with a custom built confocal microscopy system, they demonstrated that during the early GP stages, a series of both long-range and short-range

waves of intercellular $Ca^{2+}$ occurred in the dorsal marginal zone that were frequently accompanied by a wave of contraction. The authors described the waves as arising 'stochastically with respect to timing and position within the dorsal tissues'. While the majority of the waves that were observed arose near the dorsal lip of the blastopore in the mesoderm and traveled away from the blastopore, some were also visualized in the neural ectoderm of the explants. In addition, the large waves were described as propagating a distance of ~15 cell diameters at a rate of ~5 μm/s, whereas the smaller waves propagated a distance of just ~5 cell diameters at a rate of ~2 μm/s. Fig. 6 shows a representative example of an aequorin-loaded intact *Xenopus* embryo illustrating a series of non-propagating pulses (panels A to F and H) and a propagating wave (panel G) of $Ca^{2+}$ that occur around the blastopore lip, especially in the dorsal region of the lip and along the embryonic axis during the GP. The $Ca^{2+}$ wave propagates at a rate of ~4 μm/s, and thus resembles the long-range $Ca^{2+}$ waves generated in the Keller explants that were described by Wallingford et al. [6].

Thus, from the different spatial and temporal characteristics of the $Ca^{2+}$ signals visualized during gastrulation in both intact *Xenopus* embryos and in explants, it would appear that $Ca^{2+}$ may play a role in at least two different events during this period of development. Indeed, Leclerc et al. [63,64] proposed that the $Ca^{2+}$ signals in the anterior dorsal part of the ectoderm may play a role in neural induction and the formation of the anterior nervous system, whilst Wallingford et al. [6] suggested that the $Ca^{2+}$ signals that they observed in the dorsal lip of the blastopore might regulate convergent extension. Both of these groups have published additional evidence to support their suggestions. This is described in more detail in the following sections.

### 3.2. Source of the $Ca^{2+}$ signals

There is evidence to suggest that the $Ca^{2+}$ signals visualized by Leclerc et al. [63,64] and Wallingford et al. [6] come from different sources. For example, Wallingford et al. [6] demonstrated that the intercellular $Ca^{2+}$ waves were blocked, when embryos were treated with thapsigargin or 2,5-di(t-butyl)-1,4-benzohydroquinone (BHQ; both inhibitors of the endoplasmic reticulum $Ca^{2+}$-ATPase), which suggests that these $Ca^{2+}$ signals arose via release from intracellular stores. They also showed that thapsigargin treatment resulted in a failure of convergent extension as determined by *in situ* hybridization to the muscle-specific marker *MyoD*, which exhibited a restricted lateral extension of expression on either side of the notochord.

On the other hand, Leclerc et al. [63] demonstrated that in intact embryos the $Ca^{2+}$ transients in the anterior dorsal part of the ectoderm could be blocked by treatment with the L-type voltage-sensitive $Ca^{2+}$ channel (LT-VSCC) inhibitor, R(+) BayK 8644. Another LT-VSCC inhibitor (nifedipine) was applied to Keller explants and again the $Ca^{2+}$ signals were inhibited [64]. This group suggested that in both the intact embryo and the explant system, the $Ca^{2+}$ signals observed are generated from an extracellular source and enter the cells via these plasma membrane $Ca^{2+}$ channels.

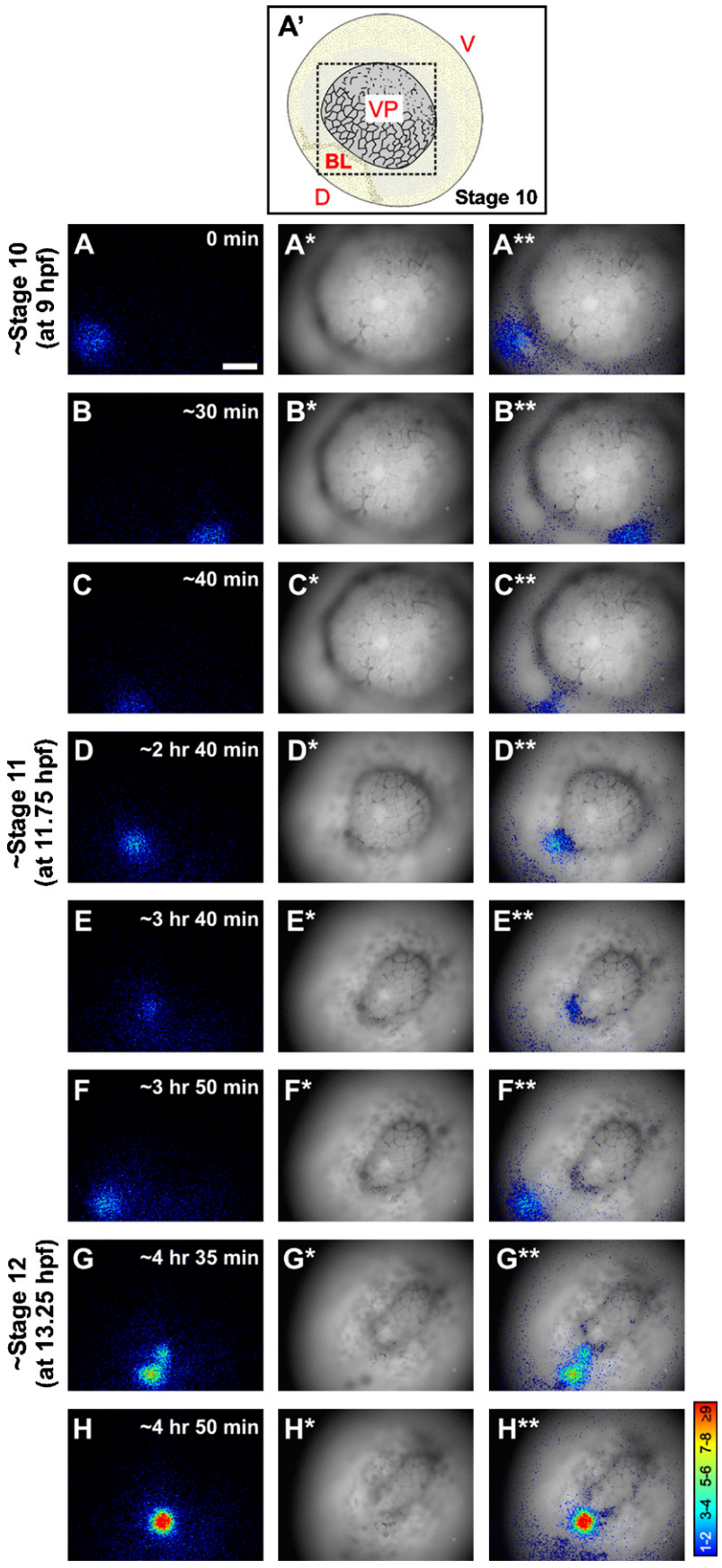
Further evidence that VSCC play a role in mediating gastrulation in *Xenopus* embryos comes from Palma et al. [65]. This group demonstrated that at the early Gastrula Stage (i.e., Stage 10), VSCC are normally expressed in the dorsal mesoderm above the blastopore lip. They also demonstrated that when VSCC were overexpressed in explants prepared from the dorsal marginal zone (DMZ), there was an increase in the level of intracellular $Ca^{2+}$, which they visualized through the use of the AM ester of the fluorescent $Ca^{2+}$ reporter, Fluo-3, in conjunction with confocal microscopy. In addition, the over-expression of VSCC in intact embryos resulted in an expansion of the dorsal territories, whilst their inhibition, with calciclu-dine, resulted in an inhibition of convergent extension. Palma et al. [65] suggested that $Ca^{2+}$ entering the DMZ via these channels might regulate the patterning of the mesoderm by specifying the dorsal–ventral identity of the mesodermal cells.

There are several other reports that link elevated levels of $Ca^{2+}$ to the establishment of the dorsal–ventral axis in amphibian embryos [42,43,66]. However, these describe a ventral-to-dorsal gradient of $Ca^{2+}$, released from intracellular stores via activation of the phosphatidylinositol (PI) signaling pathway, as opposed to an elevated level of $Ca^{2+}$ entering the dorsal tissues from an extracellular source, as suggested by Palma et al. [65].

The PI pathway has long been suggested to play a role in the formation of the dorsal–ventral axis. This is largely following early reports of embryos being treated with lithium, which was suggested to block the recycling of $IP_3$ into inositol (reviewed by Berridge et al., [67]). For example, lithium treatment of *Xenopus* embryos during the BP resulted in the ventral mesoderm being converted to dorsal mesoderm [68,69]. Zebrafish embryos have also been shown to hyperdorsalize when treated with lithium [70].

More recently, Kume et al. [71] investigated both the expression of $IP_3$Rs and the localization of $IP_3$ in *Xenopus* embryos during early development. They showed that as early as Stage 8, $IP_3$Rs were localized in the yolk-free cytoplasm of the animal blastomeres, especially in the perinuclear region of these cells. Later, in Gastrula Stage embryos, $IP_3$Rs were localized in the presumptive neuroectoderm, in the epidermal

Fig. 6. Representative example of the $Ca^{2+}$ signaling events that occur during the Gastrula Period of *Xenopus* development. (A') Schematic representation of the embryo illustrated in panels A–H, to show its orientation more clearly. VP, D, V, and BL are vegetal pole, dorsal, ventral and blastopore lip, respectively. The black dotted box shows the region of the embryo shown in panels A–H. Representative patterns of luminescence (A–H), the corresponding bright-field images (A*–H*) and the superimposed luminescence/bright-field images (A**–H**) demonstrate a series of pulses of intercellular free $Ca^{2+}$ at the blastopore lip, especially on the dorsal side, as well as along the forming embryonic axis. This embryo was loaded with lucifer yellow into both blastomeres at the 2-cell Stage and with aequorin into the two dorsal blastomeres at the 4-cell Stage. Each panel represents 120 s of accumulated luminescence. The numbers in white in the upper right of each of panels A–H represent the time elapsed since the generation of the first $Ca^{2+}$ transient (taken as 0 min). Color scale indicates luminescence flux in photons/pixel. Scale bar is 200 μm.


A'
V
VP
BL
D
Stage 10
~Stage 10 (at 9 hpf)
~Stage 11 (at 11.75 hpf)
~Stage 12 (at 13.25 hpf)
A
0 min
A*
A**
B
~30 min
B*
B**
C
~40 min
C*
C**
D
~2 hr 40 min
D*
D**
E
~3 hr 40 min
E*
E**
F
~3 hr 50 min
F*
F**
G
~4 hr 35 min
G*
G**
H
~4 hr 50 min
H*
H**
1-2
3-4
5-6
7-8
≥9

ectoderm and in the involuted dorsal mesoderm. Kume et al. [71] also showed that a significant increase in $IP_3$ mass first occurred at around Stage 10.5, at the start of gastrulation and the onset of neural induction. At this stage, the $IP_3$ mass was significantly greater in the presumptive neuroectoderm than in the mesoderm and endoderm. From these results, the authors suggested that $Ca^{2+}$ signaling via $IP_3$ might be activated in the ectoderm during gastrulation and thus play a role in the regulation of neural induction or the morphogenetic movements that occur during this stage of development. In the same year, Kume et al. [42] demonstrated that when $IP_3R$ were blocked with specific blocking antibodies in *Xenopus* embryos, the inhibition of $Ca^{2+}$ release lead to a conversion of ventral mesoderm to dorsal mesoderm. The authors suggested from these results that the $IP_3$-$Ca^{2+}$ pathway plays an active ventralizing role in early embryos [42]. A summary of the different $Ca^{2+}$ signals that have either been visualized directly or else proposed (from indirect evidence) to occur during the GP in *Xenopus* embryos is illustrated in Fig. 7.

### *3.3. Possible interaction of $Ca^{2+}$ signaling with other morphogenetic signaling pathways*

At least two main signaling pathways have been identified, which are upstream of the PI signaling pathway, regulate changes in intracellular $Ca^{2+}$ during gastrulation, and play essential roles in the specification of the body plan during this process. These are the FGF and the non-canonical Wnt signaling pathways.

One branch of the FGF signaling pathway has long been known to be essential for mesoderm induction during gastrulation via its interaction with the Ras/MAPK pathway [72]. More recently, however, Nutt et al. [73] in *Xenopus* and Sivak et al. [74] in the diploid frog, *Xenopus tropicalis*, identified a second distinct FGF signaling pathway, which they demonstrate plays a role in convergent extension and involves a mobilization of $Ca^{2+}$ from intracellular stores.

As mentioned in Section 2, some members of the Wnt family stimulate the PI signaling pathway, interacting with members of the frizzled gene family. These Wnts stimulate the release of

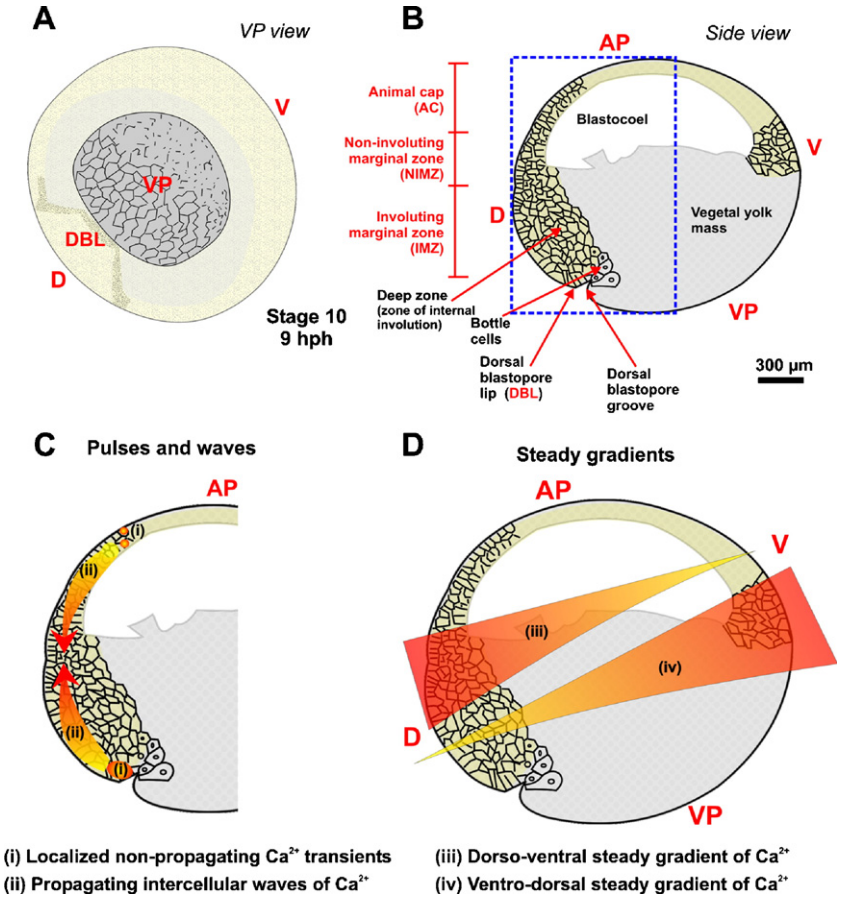



Fig. 7. Summary of the $Ca^{2+}$ signaling patterns observed both in intact *Xenopus* embryos, and extrapolated from signals recorded from explants, during gastrulation. Schematic representations of an embryo (A) from a vegetal pole (VP) view and (B) as a transverse section at Stage 10 (i.e., at around the start of gastrulation). (C) Schematic representation of the dorsal side of the embryo shown in panel B (see region in blue box) to illustrate the different $Ca^{2+}$ pulses and waves that have been reported to occur in the blastopore lip and in the dorsal ectoderm during gastrulation. These include (i) repetitive localized $Ca^{2+}$ transients at the blastopore lip (see Fig. 6) and in the non-involuting marginal zone [64] and (ii) intercellular waves of $Ca^{2+}$ emanating from the dorsal lip of the blastopore and within the dorsal ectoderm [6,63]. (D) Schematic representation of the entire transverse section in panel B, to show illustrate the steady gradients of $Ca^{2+}$ generated (iii) in the dorsal marginal zone [65] and (iv) in the ventral mesoderm [71]. Schematics are modified from (A) Nieuwkoop and Faber [11] and (B to D) the Frontiers in Bioscience: An Atlas of *Xenopus* Embryogenesis at http://www.bioscience.org/atlases/embryoge/embryoge.htm.

intracellular $Ca^{2+}$ from the ER via $IP_3Rs$ and activate $Ca^{2+}$-sensitive enzymes such as $Ca^{2+}$/calmodulin-dependent protein kinase II (CamKII), protein kinase C (PKC) and the $Ca^{2+}$/calmodulin-dependent protein phosphatase, calcineurin [49,66]. It has been suggested that Wnt/$Ca^{2+}$ signaling, like FGF signaling, might be involved in modulating convergent extension. For example, when Wnt-5A, which as described in Section 2 has been shown to stimulate the release of $Ca^{2+}$ in zebrafish embryos [46], is overexpressed in *Xenopus* blastula cap explants, the normal elongation of the explants (which can be induced by treatment with activin) is blocked but the differentiation of the dorsal and ventral mesoderm is unaffected [75]. In addition, Kühl et al. [76] more recently reported that the activation of the Wnt/$Ca^{2+}$ pathway might regulate convergent extension by blocking the Wnt/β-catenin signaling cascade via the inhibitory actions of both PKC and CamKII. It is interesting to note that while the $Ca^{2+}$ waves visualized by Wallingford et al. [6] were suggested to play a role in convergent extension, they were demonstrated not to be propagated via a Wnt signaling pathway as the signals continued to be initiated and propagated when both the Wnt/β-catenin and Wnt/$Ca^{2+}$ signaling pathways were blocked by the expression of mutant frizzled-8. It would be of interest to find out if these $Ca^{2+}$ waves are propagated via the FGF signaling pathway.

It has also been suggested that $Ca^{2+}$ signaling in conjunction with Frizzled-7 might regulate the adhesive properties of the mesoderm and ectoderm during gastrulation. Winklbauer et al. [77] demonstrated that when the function of the zygotic Frizzled-7 was inhibited, the normal separation that occurs between the involuted anterior mesoderm and the ectoderm was blocked, but convergent extension was not affected. This resulted in severe defects later on in development, such as spina bifida and defective head development [77].

Thus, $Ca^{2+}$ signaling appears to interact with multiple signaling pathways and be involved in regulating several distinct morphological events during the BP and GP of *Xenopus* development. These include neural induction, convergent extension and the specification of the mesoderm. Considerable effort is now underway to identify the possible downstream targets of the BP and GP $Ca^{2+}$ signaling events.

### *3.4. Suggested targets and functions of the Blastula and Gastrula $Ca^{2+}$ signals*

The importance of $Ca^{2+}$ in both cell locomotion and cell adhesion has long been recognized. A number of groups have reported that in *Xenopus* embryos, the disaggregation of blastula and gastrula cells is aided by $Ca^{2+}$-free medium. In addition, when the cells are maintained in $Ca^{2+}$-free medium, they continue to divide but remain in a dissociated state, sometimes for several hours. When $Ca^{2+}$ is added back into the medium, however, these cells immediately reaggregate [78–80].

In 1981, LeBlanc and Brick [81] investigated the role of $Ca^{2+}$ on the morphological and spreading responses of cells isolated from Blastula to late Gastrula-stage *Rana pipiens* embryos. They demonstrated that in $Ca^{2+}$-free media, only around 50% of the isolated cells attached to glass and those that did attach successfully did not either spread or form surface extensions. On the other hand, in media containing $Ca^{2+}$, the majority of cells attached to the glass and these exhibited a range of morphological characteristics including becoming flattened and extending numerous surface projections such as lamellipodia and filopodia. The authors suggested that $Ca^{2+}$ might affect the cells ability to spread and form protrusions, possibly via the regulation of microfilament activity.

More recently, the main focus of research has been on identifying the specific molecular targets of the $Ca^{2+}$ signals. For example, $Ca^{2+}$-dependent cell-to-cell adhesion in early embryos has been suggested to be mediated by members of the cadherin family of proteins [80]. Brieher and Gumbiner [82] examined the regulation of cell-to-cell adhesion and cadherin function during tissue morphogenesis using *Xenopus* animal caps (i.e., explants prepared from the animal hemisphere of Blastula Stage embryos). They demonstrated that $Ca^{2+}$-dependent adhesion between the blastomeres was mediated by C-cadherin. When the animal caps were treated with activin, the $Ca^{2+}$-dependent/C-cadherin mediated cell-to-cell adhesion was reduced and the cells appeared to mimic the cell rearrangements that normally occur during gastrulation [82].

As well as the classical cadherins, such as C-cadherin, members of the non-classical cadherins have been shown to play a role in gastrulation in amphibians [83]. For example, Paraxial Protocadherin (PAPC) has been shown to be expressed in the dorsal blastopore lip (Spemann's organizer) and in the paraxial mesoderm. In addition, PAPC has been shown to have cell adhesion activity and play a role in convergence extension. For example, in loss-of-function experiments with dominant-negative PAPC, convergence and extension movements were blocked, whereas in gain-of-function experiments, PAPC was shown to promote convergence extension and induce elongation of cells in open-faced explants [83]. It has recently been shown that as well as mediating cell adhesion, PAPC also has signaling functions and it interacts with the Frizzled-7 receptor to mediate the separation of tissues during gastrulation [84].

It is known from work on tissue culture cells that $Ca^{2+}$ can modulate the expression of some genes [85,86], with the frequency and amplitude of the $Ca^{2+}$ signals [87,88] being as important as the spatial pattern of $Ca^{2+}$ signaling. Reports are now starting to appear, which identify examples of $Ca^{2+}$-activated gene expression in intact embryos during development. For example, Leclerc et al. [63] demonstrated that the treatment of intact *Xenopus* embryos with BayK or another L-type channel inhibitor, nicardipine, lead to a decrease in the level of expression of the early neural genes, *Zic3* and *geminin* and resulted in the defective formation of the anterior nervous system. *Zic3* expression was also reduced in Keller explants when the $Ca^{2+}$ signals were inhibited with the $Ca^{2+}$ chelator, BAPTA-AM [64]. From these results, it was suggested that the $Ca^{2+}$ signals observed during gastrulation might play a role in the organization of anterior neural development in *Xenopus* and that the $Ca^{2+}$ may be involved in the regulation of expression of

early neural genes, such as *Zic3*. Most recently, Batut et al. [89] isolated and identified the arginine N-methyltransferase gene (*xPRMT1B*), which they demonstrated to be regulated by $Ca^{2+}$ and which in turn regulated the expression of *Zic3*. Furthermore, inhibition of *xPRMT1B* using an antisense approach resulted in impaired neural development. This report is perhaps the first example of $Ca^{2+}$ regulating gene expression in intact developing embryos. A more detailed description of the role of $Ca^{2+}$ signaling during early neural induction in *Xenopus laevis* embryos is reviewed by Leclerc et al. in this volume of BBA.

Regarding the identification of other possible targets of the $Ca^{2+}$ signals involved in other aspects of gastrulation, the calpain, XCL-2 has been shown to play a role in convergent extension in *Xenopus* embryos [90]. Calpains are intracellular cysteine proteases that are known to require $Ca^{2+}$ for their action. Cao et al. [90] demonstrated that when XCL-2 was overexpressed in embryos, the onset of gastrulation was normal but convergent extension was disrupted and the localization of various different neural and mesodermal markers was affected. Using RT-PCR, the zygotic transcription of *XCL-2* was first detected at the onset of gastrulation at Stage 10, and using *in situ* hybridization, the gene could first be distinguished close to the ventral blastopore lip at Stage 12.5. As calpains are known to be activated near to cell membranes and cleave both cytoskeletal and extracellular matrix proteins, the authors suggested that XCL-2 might regulate the cell movements that occur during gastrulation by changing the adhesive nature of cells through proteolytic cleavage of the proteins that are essential for morphogenesis [90].

Another possible target of some of the GP $Ca^{2+}$ signals in *Xenopus* embryos is the p21-activated kinase (PAK) 5 protein. PAK proteins are well characterized effectors of the GTPase, Cdc42 [91], which is itself known to be involved in Wnt/$Ca^{2+}$ signaling and also regulate convergent extension movements in *Xenopus* embryos [92]. PAK5 has been shown to bind to both MFs and MTs and is thought to regulate the cytoskeletal rearrangements that occur during cell motility [93]. Faure et al. [94] recently demonstrated that in *Xenopus* embryos undergoing gastrulation, X-PAK5 is expressed largely in the regions of the embryo where extensive convergent extension cell movements occur. They also demonstrated that this protein is located at cell-to-cell junctions and its activity is modulated by extracellular $Ca^{2+}$. The authors suggested that X-PAK5 might regulate convergent extension by mediating $Ca^{2+}$-dependent cell-to-cell adhesion.

In summary, several potential targets of the Blastula and Gastrula $Ca^{2+}$ signals, observed using both luminescent and fluorescent $Ca^{2+}$ reporters, have already been identified. These include $Ca^{2+}$-dependent genes, structural proteins and enzymes, which have been shown to mediate a range of different events that occur during these Stages of development.

## 4. Conclusions

In summary, as we progress through the BP and GP, we observe an elaboration of the simple intercellular $Ca^{2+}$ signaling patterns established during the early Blastula period. This results in patterns of long-distance intercellular signaling that we suggest provide a mechanism for coordinating the spatial and temporal regulation of both global and highly localized developmental processes across the whole embryo. Our current understanding results from the development of $Ca^{2+}$ imaging techniques, allowing us to extend our exploration of signaling events beyond the first few stages of embryonic development in both zebrafish and *Xenopus* embryos. What our early glimpses have revealed, is a fascinating cornucopia of as yet poorly understood signaling patterns that are now just starting to be explored with the array of molecular, genetic, and pharmacological tools that are at our disposal. In this review, we hope to have provided an overview of the $Ca^{2+}$ signaling during the BP and GP within a pair of developing vertebrate systems (see Figs. 5 and 7) that we feel are particularly suited to addressing some of the fundamental questions regarding signal transduction pathways in relation to developmental orchestration. We have proposed that $Ca^{2+}$-signaling pathways are built from fundamental processes, which combine together in step with the generation of embryonic complexity. This theme is summarized in Fig. 5. Assuming that $Ca^{2+}$-sensitive response elements originally evolved in unicellular systems, it would seem logical that during the development of metazoan forms, these response elements would be retained and intercellular $Ca^{2+}$ signaling pathways developed. We suggest that in a sense, this is what we see during embryonic development as one passes from a unicellular to multicellular form.

There is currently a lack of data that makes a definitive causal connection between many of the $Ca^{2+}$ transients observed and the developmental event they are associated with. However, there is accumulating evidence (certainly in *Xenopus*) that links the various $Ca^{2+}$ signaling pathways identified, with specific events during the BP and GP, through the identification and characterization of specific $Ca^{2+}$-dependent genes and proteins. We hope that the patterns of $Ca^{2+}$ signaling described in this review will provide a framework for investigators to continue to explore direct mechanistic connections and thus establish their developmental significance.

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## References

[1] L.F. Jaffe, Organization of early development by calcium patterns, BioEssays 21 (1999) 657–667.
[2] M.J. Berridge, P. Lipp, M.D. Bootman, The versatility and universality of calcium signaling, Nat. Rev., Mol. Cell Biol. 1 (2000) 11–21.
[3] S.E. Webb, A.L. Miller, Calcium signalling during embryonic development, Nat. Rev., Mol. Cell Biol. 4 (2003) 539–551.

[4] C.B. Kimmel, W.W. Ballard, S.R. Kimmel, B. Ullmann, T.F. Schilling, Stages of embryonic development of the zebrafish, Dev. Dyn. 203 (1995) 253–310.
[5] E. Gilland, A.L. Miller, E. Karplus, R. Baker, S.E. Webb, Imaging of multicellular large-scale rhythmic calcium waves during zebrafish gastrulation, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 157–161.
[6] J.B. Wallingford, A.J. Ewald, R.M. Harland, S.E. Fraser, Calcium signaling during convergent extension in *Xenopus*, Curr. Biol. 11 (2001) 652–661.
[7] T.A. Westfall, B. Hjertos, D.C. Slusarski, Requirement for intracellular calcium modulation in zebrafish dorsal–ventral patterning, Dev. Biol. 259 (2003) 380–391.
[8] S.E. Webb, A.L. Miller, Calcium signalling during zebrafish embryonic development, BioEssays 22 (2000) 113–123.
[9] R. Créton, J.E. Speksnijder, L.F. Jaffe, Patterns of free calcium in zebrafish embryos, J. Cell Sci. 111 (1998) 1613–1622.
[10] S.E. Webb, A.L. Miller, Imaging intercellular calcium waves during late epiboly in intact zebrafish embryos, Zygote 11 (2003) 175–182.
[11] P.D. Nieuwkoop, J. Faber, Normal Table of *Xenopus laevis* (Daudin), Amsterdam, North Holland, 1967.
[12] C.D. Stern (Ed.), Gastrulation: From Cells to Embryo, Cold Spring Harbor Press, New York, 2004.
[13] D.A. Kane, R.M. Warga, Teleost Gastrulation, In Gastrulation from Cells to Embryo, C.D. Stern (Ed.), Cold Spring Harbor Press, New York, 2004, pp. 157–169.
[14] J. Newport, M. Kirschner, A major developmental transition in early *Xenopus* embryos: I. Characterization and timing of cellular changes at the midblastula stage, Cell 30 (1982) 675–686.
[15] J. Newport, M. Kirschner, A major developmental transition in early *Xenopus* embryos: II. Control of the onset of transcription, Cell 30 (1982) 687–696.
[16] D.A. Kane, C.B. Kimmel, The zebrafish midblastula transition, Development 119 (1993) 447–456.
[17] E.A. Ober, S. Schulte-Merker, Signals from the yolk cell induce mesoderm, neuroectoderm, the trunk organizer and the notochord in zebrafish, Dev. Biol. 215 (1999) 167–181.
[18] C.B. Kimmel, R.M. Warga, T.F. Schilling, Origin and organization of the zebrafish fate map, Development 108 (1990) 581–594.
[19] S. Schneider, H. Steinbeisser, R.M. Warga, P. Hausen, Beta-catenin translocation into nuclei demarcates the dorsalizing centers in frog and fish embryos, Mech. Dev. 57 (1996) 191–198.
[20] R. Keller, P. Tibbetts, Mediolateral cell intercalation in the dorsal, axial mesoderm of *Xenopus laevis*, Dev. Biol. 131 (1989) 539–549.
[21] H. Ibrahim, R. Winklbauer, Mechanisms of mesendoderm internalization in the *Xenopus* gastrula: lessons from the ventral side, Dev. Biol. 240 (2001) 108–122.
[22] R.M. Warga, C. Nüsslein-Volhard, Origin and development of the zebrafish endoderm, Development 126 (1999) 827–838.
[23] J.C. Cheng, A.L. Miller, S.E. Webb, Organization and function of microfilaments during late epiboly in zebrafish embryos, Dev. Dyn. 231 (2004) 313–323.
[24] U. Strähle, S. Jesuthasan, Ultraviolet irradiation impairs epiboly in zebrafish embryos: evidence for a microtubule-dependent mechanism of epiboly, Development 119 (1993) 909–919.
[25] L. Solnica-Krezel, W. Driever, Microtubule arrays of the zebrafish yolk cell: organization and function during epiboly, Development 120 (1994) 2443–2455.
[26] R. Keller, The cellular basis of epiboly: an SEM study of deep-cell rearrangement during gastrulation in *Xenopus laevis*, J. Embryol. Exp. Morphol. 60 (1980) 201–234.
[27] K. Woo, S.E. Fraser, Order and coherence in the fate map of the zebrafish nervous system, Development 121 (1995) 2595–2609.
[28] M. Concha, R. Adams, Oriented cell divisions and cellular morphogenesis in the zebrafish gastrula and neurula: a time-lapse analysis, Development 125 (1995) 983–994.
[29] A. Agathon, C. Thisse, B. Thisse, The molecular nature of the zebrafish tail organizer, Nature 424 (2003) 448–452.
[30] J.M.W. Slack, From Egg to Embryo, Chapter 4, Cambridge Univ. Press, UK, 1991, pp. 67–110.
[31] R.E. Keller, M. Danilchik, R. Gimlich, J. Shih, The function and mechanism of convergent extension during gastrulation of *Xenopus laevis*, J. Embryol. Exp. Morphol. 89 (1985) 185–209.
[32] R. Keller, M. Danilchik, Regional expression, pattern and timing of convergence and extension during gastrulation of *Xenopus laevis*, Development 103 (1988) 193–209.
[33] D.C. Chang, C. Meng, A localized elevation of cytosolic free calcium is associated with cytokinesis in the zebrafish embryo, J. Cell Biol. 131 (1995) 1539–1545.
[34] S.E. Webb, K.W. Lee, E. Karplus, A.L. Miller, Localized calcium transients accompany furrow positioning, propagation, and deepening during the early cleavage period of zebrafish embryos, Dev. Biol. 192 (1997) 78–92.
[35] D.C. Chang, P. Lu, Multiple types of calcium signals are associated with cell division in zebrafish embryo, Micros. Res. Tech. 49 (2000) 111–122.
[36] K.W. Lee, S.E. Webb, A.L. Miller, $Ca^{2+}$ released via $IP_3$ receptors is required for furrow deepening during cytokinesis in zebrafish embryos, Int. J. Dev. Biol. 47 (2003) 411–421.
[37] K.W. Lee, S.E. Webb, A.L. Miller, Requirement for a localized, $IP_3R$-generated $Ca^{2+}$ transient during the furrow positioning process in zebrafish zygotes, Zygote 14 (2006) 143–155.
[38] E. Reinhard, H. Yokoe, K.R. Niebling, N.L. Allbritton, M.A. Kuhn, T. Meyer, Localized calcium signals in early zebrafish development, Dev. Biol. 170 (1995) 50–61.
[39] G.K. Winkel, J.E. Ferguson, M. Takeichi, R. Nuccitelli, Activation of protein kinase C triggers premature compaction in the four-cell stage mouse embryo, Dev. Biol. 138 (1990) 1–15.
[40] W.B. Busa, R.L. Gimlich, Lithium-induced teratogenesis in frog embryos prevented by a polyphosphoinositide cycle intermediate or a diacylglycerol analog, Dev. Biol. 132 (1989) 315–324.
[41] J.A. Maslanski, L. Leshko, W.B. Busa, Lithium-sensitive production of inositol phosphates during amphibian embryonic mesoderm induction, Science 256 (1992) 243–245.
[42] S. Kume, A. Muto, T. Inoue, K. Suga, H. Okano, K. Mikoshiba, Role of inositol 1,4,5-trisphosphate receptor in ventral signaling in *Xenopus* embryos, Science 278 (1997) 1940–1943.
[43] S. Kume, T. Inoue, K. Mikoshiba, Gαs family G proteins activate $IP_3$-$Ca^{2+}$ signaling via Gβγ and transduce ventralizing signals in *Xenopus*, Dev. Biol. 226 (2000) 88–103.
[44] J.D. Dasgupta, U.N. Singh, Spatio-temporal distribution of gap junctions in zebra fish embryo, Wilhelm Roux's Arch. 191 (1982) 378–380.
[45] M.J. Berridge, M.D. Bootman, H.L. Roderick, Calcium signaling: dynamics, homeostasis and remodeling, Nat. Rev. Mol. Cell Biol. 4 (2003) 517–530.
[46] D.C. Slusarski, J. Yang-Snyder, W.B. Busa, R.T. Moon, Modulation of embryonic intracellular $Ca^{2+}$ signaling by Wnt-5A, Dev. Biol. 182 (1997) 114–120.
[47] R. Nusse, H.E. Varmus, Wnt genes, Cell 69 (1992) 1073–1087.
[48] J. Yang-Snyder, J.R. Miller, J.D. Brown, C.J. Lai, R.T. Moon, A *frizzled* homolog functions in a vertebrate Wnt signaling pathway, Curr. Biol. 6 (1996) 1302–1306.
[49] D.C. Slusarski, V.G. Corces, R.T. Moon, Interaction of Wnt and a Frizzled homologue triggers G-protein-linked phosphatidylinositol signaling, Nature 390 (1997) 410–413.
[50] C.G. Sagerström, L.S. Gammill, R. Veale, H. Sive, Specification of the enveloping layer and lack of autoneuralization in zebrafish embryonic explants, Dev. Dyn. 232 (2005) 85–97.
[51] V. Bozhkova, D. Voronov, Spatial-temporal characteristics of intercellular junctions in early zebrafish and loach embryos before and during gastrulation, Dev. Genes Evol. 207 (1997) 115–126.
[52] L. Solnica-Krezel, Pattern formation in zebrafish–fruitful liaisons between embryology and genetics, Curr. Top. Dev. Biol. 41 (1999) 1–35.
[53] A. Robertson, Waves propagated during vertebrate development: observations and comments, J. Embryol. Exp. Morphol. 50 (1979) 155–167.
[54] T. Meyer, Cell signaling by second messenger waves, Cell 64 (1991) 675–678.

[55] T. Betchaku, J.P. Trinkaus, Contact relations, surface activity, and cortical microfilaments of marginal cells of the enveloping layer and of the yolk syncytial and yolk cytoplasmic layers of *Fundulus* before and during epiboly, J. Exp. Zool. 206 (1978) 381–426.
[56] D. Sharma, L. Holets, X. Zhang, W.H. Kinsey, Role of fyn kinase in signaling associated with epiboly during zebrafish development, Dev. Biol. 285 (2005) 462–476.
[57] R.A. Fluck, C.E. Killian, K. Miller, J.N. Dalpe, T.M. Shih, Contraction of an embryonic epithelium, the enveloping layer of the medaka (*Oryzias latipes*), a teleost, J. Exp. Zool. 229 (1984) 127–142.
[58] B. Barber, M.J.B. da Cruz, J. DeLeon, R.A. Fluck, M.P. Hasenfeld, L.A. Unis, Pacemaker region in a rhythmically contracting embryonic epithelium, the enveloping layer of *Oryzias latipes*, a teleost, J. Exp. Zool. 242 (1987) 35–42.
[59] J.Z. Simon, M.S. Cooper, Calcium oscillations and calcium waves coordinate rhythmic contractile activity within the stellate cell layer of medaka fish embryos, J. Exp. Zool. 273 (1995) 118–129.
[60] L.T. Stableford, A study of calcium in the early development of the amphibian embryo, Dev. Biol. 16 (1967) 303–314.
[61] S. Komazaki, Calcium-containing, smooth-surfaced endoplasmic reticulum and vacuoles in cells of the blastopore-forming region during gastrulation of the newt, *Cynops pyrrhogaster*, Anat. Embryol. 191 (1995) 369–376.
[62] A. Muto, S. Kume, T. Inoue, H. Okano, K. Mikoshiba, Calcium waves along the cleavage furrows in cleavage-stage *Xenopus* embryos and its inhibition by heparin, J. Cell Biol. 135 (1996) 181–190.
[63] C. Leclerc, S.E. Webb, C. Daguzan, M. Moreau, A.L. Miller, Imaging patterns of calcium transients during neural induction in *Xenopus laevis* embryos, J. Cell Sci. 113 (2000) 3519–3529.
[64] C. Leclerc, M. Lee, S.E. Webb, M. Moreau, A.L. Miller, Calcium transients triggered by planar signals induce the expression of *ZIC3* gene during neural induction in *Xenopus*, Dev. Biol. 261 (2003) 381–390.
[65] V. Palma, M. Kukuljan, R. Mayor, Calcium mediates dorsoventral patterning of mesoderm in *Xenopus*, Curr. Biol. 11 (2001) 1606–1610.
[66] T. Saneyoshi, S. Kume, Y. Amasaki, K. Mikoshiba, The Wnt/calcium pathway activates NF-AT and promotes ventral cell fate in *Xenopus*, Nature 417 (2002) 295–299.
[67] M.J. Berridge, C.P. Downes, M.R. Hanley, Neural and developmental actions of lithium: a unifying hypothesis, Cell 59 (1989) 411–419.
[68] J. Cooke, E.J. Smith, The restrictive effect of early exposure to lithium upon body pattern in *Xenopus* development, studied by quantitative anatomy and immunofluorescence, Development 102 (1988) 85–99.
[69] K.R. Kao, R.P. Elinson, The entire mesodermal mantle behaves as Spemann's organizer in dorsoanterior enhanced *Xenopus laevis* embryos, Dev. Biol. 127 (1988) 64–77.
[70] S.E. Stachel, D.J. Grunwald, P.Z. Myers, Lithium perturbation and *goosecoid* expression identify a dorsal specification pathway in the pregastrula zebrafish, Development 117 (1993) 1261–1274.
[71] S. Kume, A. Muto, H. Okano, K. Mikoshiba, Developmental expression of the inositol 1,4,5-trisphosphate receptor and localization of inositol 1,4,5-trisphosphate during early embryogenesis in *Xenopus laevis*, Mech. Dev. 66 (1997) 157–168.
[72] M. Whitman, D.A. Melton, Involvement of $p21^{ras}$ in *Xenopus* mesoderm induction, Nature 357 (1992) 252–254.
[73] S.L. Nutt, K.S. Dingwell, C.E. Holt, E. Amaya, *Xenopus* Sprouty2 inhibits FGF-mediated gastrulation movements but does not affect mesoderm induction and patterning, Genes Dev. 15 (2001) 1152–1166.
[74] J.M. Sivak, L.F. Petersen, E. Amaya, FGF signal interpretation is directed by Sprouty and Spred proteins during mesoderm formation, Dev. Cell 8 (2005) 689–701.
[75] R.T. Moon, R.M. Campbell, J.L. Christian, L.L. McGrew, J. Shih, S. Fraser, *Xwnt5A*: a maternal *Wnt* that affects morphogenetic movements after overexpression in embryos of *Xenopus laevis*, Development 119 (1993) 97–111.
[76] M. Kühl, K. Geis, L.C. Sheldahl, T. Pukrop, R.T. Moon, D. Wedlich, Antagonistic regulation of convergent extension movements in *Xenopus* by Wnt/β-catenin and Wnt/$Ca^{2+}$ signaling, Mech. Dev. 106 (2001) 61–76.
[77] R. Winklbauer, A. Medina, R.K. Swain, H. Steinbeisser, Frizzled-7 signalling controls tissue separation during *Xenopus* gastrulation, Nature 413 (2001) 856–860.
[78] K.W. Jones, T.R. Elsdale, The culture of small aggregates of amphibian embryonic cells *in vitro*, J. Embryol. Exp. Morphol. 11 (1963) 135–154.
[79] K. Shiokawa, K. Tashiro, T. Oka, K. Yamana, Contribution of maternal mRNA for maintenance of $Ca^{2+}$-dependent reaggregating activity in dissociated cells of *Xenopus laevis* embryos, Cell Differ. 13 (1983) 247–255.
[80] K. Nomura, M. Uchida, H. Kageura, K. Shiokawa, K. Yamana, Cell to cell adhesion systems in *Xenopus laevis*, the South African Clawed Frog I. Detection of $Ca^{2+}$ dependent and independent adhesion systems in adult and embryonic cells, Dev. Growth Differ. 28 (1986) 311–319.
[81] J. LeBlanc, I. Brick, Calcium and spreading behaviour of amphibian blastula and gastrula cells, J. Embryol. Exp. Morphol. 64 (1981) 149–168.
[82] W.M. Brieher, B.M. Gumbiner, Regulation of C-cadherin function during activin induced morphogenesis of *Xenopus* animal caps, J. Cell Biol. 126 (1994) 519–527.
[83] S.H. Kim, A. Yamamoto, T. Bouwmeester, E. Agius, E.M. De Robertis, The role of paraxial protocadherin in selective adhesion and cell movements of the mesoderm during *Xenopus* gastrulation, Development 125 (1998) 4681–4690.
[84] A. Medina, R.K. Swain, K.M. Kuerner, H. Steinbeisser, *Xenopus* paraxial protocadherin has signaling functions and is involved in tissue separation, EMBO J. 23 (2004) 3249–3258.
[85] D. Freyssenet, M. Di Carlo, D.A. Hood, Calcium-dependent regulation of cytochrome *c* gene expression in skeletal muscle cells. Identification of a protein kinase c-dependent pathway, J. Biol. Chem. 274 (1999) 9305–9311.
[86] D.M. Studzinski, R.E. Callahan, J.A. Benjamins, Increased intracellular calcium alters myelin gene expression in the N20.1 oligodendroglial cell line, J. Neurosci. Res. 57 (1999) 633–642.
[87] R.E. Dolmetsch, K. Xu, R.S. Lewis, Calcium oscillations increase the efficiency and specificity of gene expression, Nature 392 (1998) 933–936.
[88] W. Li, J. Llopis, M. Whitney, G. Zlokarnik, R.Y. Tsien, Cell-permeant caged $InsP_3$ ester shows that $Ca^{2+}$ spike frequency can optimize gene expression, Nature 392 (1998) 936–941.
[89] J. Batut, L. Vandel, C. Leclerc, C. Daguzan, M. Moreau, I. Neant, The $Ca^{2+}$-induced methyltransferase xPRMT1b controls neural fate in amphibian embryo, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 15128–15133.
[90] Y. Cao, H. Zhao, H. Grunz, XCL-2 is a novel m-type calpain and disrupts morphogenetic movements during embryogenesis in *Xenopus laevis*, Dev. Growth Differ. 43 (2001) 563–571.
[91] E. Manser, T. Leung, H. Salihuddin, Z.S. Zhao, L. Lim, A brain serine/threonine protein kinase activated by Cdc42 and Rac1, Nature 367 (1994) 40–46.
[92] S.C. Choi, J.K. Han, *Xenopus* Cdc42 regulates convergent extension movements during gastrulation through Wnt/$Ca^{2+}$ signaling pathway, Dev. Biol. 244 (2002) 342–357.
[93] J. Cau, S. Faure, M. Comps, C. Delsert, N. Morin, A novel p21-activated kinase binds the actin and microtubule networks and induces microtubule stabilization, J. Cell Biol. 155 (2001) 1029–1042.
[94] S. Faure, J. Cau, P. de Santa Barbara, S. Bigou, Q. Ge, C. Delsert, N. Morin, *Xenopus* p21-activated kinase 5 regulates blastomeres adhesive properties during convergent extension movements, Dev. Biol. 277 (2005) 472–492.