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Review

Calcium signaling in developing embryos: Focus on the regulation of cell shape changes and collective movements

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ABSTRACT

During morphogenesis tissues significantly remodel by coordinated cell migrations and cell rearrangements. Central to this problem are cell shape changes that are driven by distinct cytoskeletal reorganization responsible for force generation. Calcium is a versatile and universal messenger that is implicated in the regulation of embryonic development. Although calcium transients accrue clearly and more intensely in tissues undergoing rearrangement/migration, it is far from clear what the role of these calcium signals is. Here we summarize the evidence implicating calcium participation in tissue movements, cell shape changes and the reorganization of contractile cytoskeletal elements in developing embryos. We also discuss a novel hypothesis that short-lived calcium spikes are required in cells and tissues undergoing migration and rearrangements as a fine tuning response mechanism to prevent local, abnormally high fluctuations in cytoskeletal activities.

Contents

1. Introduction

Variations in calcium concentration play a pivotal role, as calcium is a ubiquitous and versatile signaling molecule participating in a wide range of developmental processes from fertilization to organ formation [1].

This review focuses on the role of calcium-mediated signals in morphogenetic events in the early embryo, with special emphasis on cell shape changes and tissue rearrangements. We will in particular emphasize the link between calcium transients and intracellular forces. The reader interesting in the molecular pathways and broader description of calcium signaling during embryonic patterning are referred to the reviews of Slusarski and Pelegri [2], and Webb and Miller [3].

In the past decades advances in the development of calcium imaging techniques prompted scientists to study the endogenous calcium activity in early embryonic development. Fluorescent

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calcium probes used in embryonic imaging include chemical dyes such as Calcium Green [4] and Fura [5], proteins such as Cameleon [6] and G-CamMP [7] and bioluminescent aequorins [6]. Dyes that are injected in embryos at early stages spread in the injected cell that after several cleavages became the multicellular tissue loaded with calcium sensing probes. Calcium dyes are retained inside the cells, which allow long term in vivo imaging of intracellular calcium levels.

At the end of blastula and at the onset of the gastrula stages embryos already consist of several thousands of cells, however the number of different tissues is still small and tissues geometries are rather simple [8]. Also in contrast to later stages such as organogenesis, cell movements and rearrangements occur near the surface of the embryo. Thus, the blastula and gastrula stages are excellent in vivo systems that are amenable to biophysical approaches and imaging techniques.

During development, tissues organize in a variety of forms by precise spatiotemporal modification of cell movements and cell shape changes. Internally-generated forces produce changes in cell behaviors, which can integrate at the level of tens to hundreds of cells to produce macroscopic tissue movements or tissue rearrangements [9]. A first example of such a movement is convergent extension. During this process the tissue narrows in one direction and elongates in another. Convergent extension relies on cell intercalation, which results either from polarized cell junction remodeling or polarized cell migration. A second example of tissue reorganization is tissue invagination that takes place during gastrulation in flies and neural tube formation in vertebrates. Apical constriction of the cells is important for tissue invagination. These two types of movements yield amazingly complex topological changes in the geometry of the embryo such as epiboly, gastrulation and somite formation. Convergent extension movements and tissue invagination rely on local forces, yet coordination at the multicellular level is also required. Several lines of evidence indicate that calcium might play role in the coordination of these tissue movements.

In this review we will first describe the principles of calcium signaling. We then present the evidence for the role of calcium in convergent extension, gastrulation, epiboly and somitogenesis in different model organisms. And finally we show processes where calcium signals are related to cell shape changes and cytoskeleton reorganization in developing embryo.

2. Mechanisms of calcium signaling

Calcium is an ion that in the form of divalent cation, Ca^{2+}, is present in the cell cytosol, intracellular organelles and the extracellular space of all organisms. Calcium concentration is about 100 nM inside the cell at rest, while in extracellular space and cellular organelles it is about 2 mM. The difference in concentration across the membranes is thus as large as 20,000 times. This concentration gradient is achieved by the constant activity of calcium pumps that remove calcium ions from the cytosol. Thus opening of calcium channels leads to massive calcium influx into the cell simply by passive diffusion along the existing concentration gradient. Other abundant cations, such as potassium and sodium, have only up to 30-fold differences between their intracellular and extracellular concentrations and their fluxes are less pronounced. The steep gradient of calcium concentration makes the calcium ion unique for inducing precise and rapid signaling responses.

The Ca^{2+}-signaling system has a very large toolkit of signaling molecules [1,10]. The following description is by no means exhaustive but it summarizes many of the calcium related pathways (Fig. 1A). The molecules upstream of calcium signaling are families of G-protein-coupled and tyrosine-kinase-linked receptors.

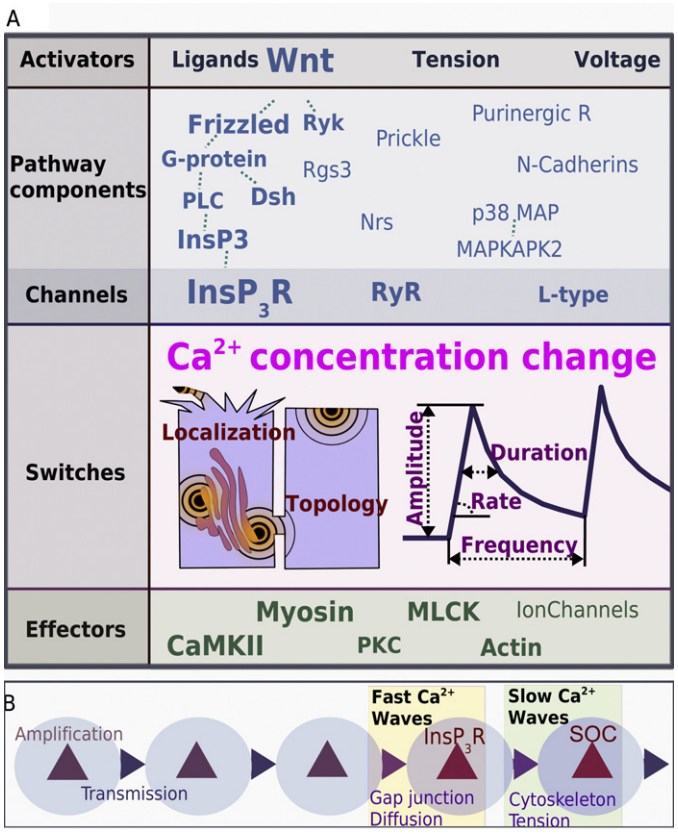

Fig. 1. (A) Mechanisms of calcium signaling. Regulators and effectors of calcium signaling during morphogenesis in early embryonic epithelium. The font size of molecules reflect number of studies of this molecule in literature in the context of early calcium signaling during morphogenesis. (B) Scheme of calcium wave propagation.

These receptors activate G proteins and numerous regulators of G-protein signaling. Hydrolysis of a membrane-bound inositol lipid is initiated that generates diacylglycerol and inositol-1,4,5-trisphosplate (InsP3). Diacylglycerol stimulates protein kinase C, while InsP3 promotes the release of calcium from intracellular stores. The most studied channels on the internal cellular structures are the inositol trisphosphate (InsP3R) and ryanodine (RyR) receptors. InsP3R is activated by inositol trisphosphate but also by calcium itself. InsP3R action results in massive calcium release from internal stores known as calcium-induced calcium release. The channels expressed on the cell plasma membrane include voltage operated channels (CaV, L-type, T-type and others), receptor operated channels (NMDA, nACh and others), second messenger operated channels (SOCs), store-independent Orai channels, transient receptor potential (TRP) channels and many others.

The versatility of calcium signaling relies on the one hand on numerous calcium-sensing proteins [1,10,11], among which we describe MLCK and CaMKII below. Intracellular rises of calcium concentrations are decoded into signaling via calcium-sensing proteins In the cytosol numerous calcium-sensing proteins are able to bind calcium with different affinities. Upon calcium binding calcium-sensing proteins undergo conformational changes and then bind to proteins involved in signaling cascades. As the affinities of these proteins to calcium vary, the increase of intracellular calcium concentration to levels, which cover the whole range from resting calcium up to micro molar concentration, will efficiently activate a subset of calcium-sensing proteins. Thus the different levels of calcium concentration may result in the activation of different signaling pathways.

On the other hand the versatility of calcium signaling relies on its spatial patterns, duration of calcium signals and on the rates and frequencies of calcium concentration changes. Short-time activation of a single or a small number of calcium channels results in short local calcium increase. Higher entry of calcium can activate calcium sensing channels on the intracellular organelles and then causes calcium increase in the whole cell due to positive feedback loops. Channels then are inactivated after a certain amount of time or when calcium concentration reaches certain levels, dampening calcium influx. Concomitantly calcium buffer proteins capture free calcium and pumps transport calcium outside of the cytosol. Thus the calcium increases and decreases are transient. Even if such transients can be frequent in excitable cells the basal level of calcium is kept constant in most physiological contexts. Calcium signaling has a wide temporal range varying from ultrashort of few milliseconds pulses to long transients up to minutes and even hours long. The spatio-temporal decoding of calcium concentration provides an effective mechanism to activate different subsets of events.

The important phenomenon of calcium signaling intracellular and intercellular calcium waves propagation require Transmission and Amplification mechanisms (Fig. 1B). Transmission mechanisms relocate calcium from one cell to another while Amplifier mechanisms increase calcium concentration. Absence of Transmission mechanisms prevents propagation of calcium waves, at the cell–cell interface for example, while absence of Amplification mechanisms results in the decrease of wave amplitude on the scale of few micrometers. For the propagation of fast intercellular calcium waves (10–40 μm/s) calcium-induced calcium release from endoplasmic reticulum through InsP3R is a part of an Amplification mechanism. Gap junctions serve as a Transmission mechanism that allow diffusion of calcium through gap junction pores that connect cytosol of adjacent cells [12]. For the propagation of slow intercellular calcium waves (0.1–1 μm/s) opening of stretch-activated calcium channels contributes to the Amplification mechanism causing increase of intracellular calcium concentration due to massive influx from extracellular space. The transmission of calcium waves from one cell to another relies on the contraction of the cytoskeleton in response to calcium [13]. The contraction of the cell is a Transmission mechanism as it stretches neighboring cells which in turn activates the Amplification mechanism in the stretched cell and so the following wave propagation.

It is tempting to speculate that the versatility of calcium signaling in time and space mirror the diversity of events that occurs during morphogenesis.

3. Calcium regulation of cell motility

Numerous studies have shown that calcium has a role in cell motility [14]. Crawling cells on a coverslip are flat and polarized in the direction of movement. At the leading edge, actin polymerizes constantly, and produces traction force by pulling on adhesion complexes, whereas active actomyosin arcs help the retraction of the cell. Round chemotactic cell on the coverslip responds to the chemoattractant by increasing of its intracellular calcium level. This calcium increase triggers cell flattening [15]. During migration endogenous calcium spikes accompany cell crawling. These spontaneous transients correlate temporally and spatially with traction and retraction phases and presumably participate in the accumulation of actomyosin arcs at the back of the cell [16,17]. Migrating cells in zebrafish embryos increase calcium in coordination with their movements. Myosin is activated by this calcium increase and generates the forces required for the cell protrusive activity [18]. Thus in crawling cells the role of calcium is related to the activation of the cytoskeleton.

In motile cells several targets were found to be downstream of calcium transients, which include actin and adhesion regulators [16]. Several actin binding proteins such as villin and gelsolin have calcium binding sites that regulate their activity. Depending on the calcium concentration the villin severing of actin filaments reduces or increases [19]. Calcium activated potassium channels can regulate motility by interacting with integrins, which form cell-extracellular matrix adhesion sites. Stretch-induced activated channels have also been proposed to regulate cells shape changes in epithelia and cell motility [20–22].

4. Basal calcium level and early tissue morphogenesis

Numerous studies indicate that proper calcium signaling is required for tissue elongation, cell reorganization and folding. Experimentally induced changes of physiological intracellular calcium concentrations to high or low values perturb the elongation of the egg chamber in *Drosophila* [23], convergent extension movements during gastrulation in *Xenopus* [4] and zebrafish [24], epiboly progression in zebrafish [25,26] and newt [27], cells rearrangements in somitogenesis in zebrafish [28] and chicken [29], tissue folding during sea urchin gastrulation [30] and neural fold formation in *Xenopus* [31].

The general concept of calcium signaling assumes a constant and low calcium concentration (about 100 nM) in the resting state. However in the early embryonic development intracellular calcium levels change over time. These changes occur in a precise manner in conjunction with developmental steps. It has been observed for different species that calcium starts to increase at the end of the blastula stage and reaches peak levels during early gastrulation, when the first collective cell movements start. Such calcium increases have been observed in *Drosophila* [32], *Xenopus* [6], zebrafish [33] and *Amblystoma punctatum* [34]. In *Xenopus* calcium increases five-fold in the dorsal part of the embryo where convergent extension progresses [33] while no increase in calcium is observed in the ventral ectoderm, mesoderm or endoderm. Interestingly, calcium increases during somite patterning, where cell rearrangements also take place [6]. Thus in vertebrates and invertebrates global calcium increases precede and accompany the initiation of coordinated cell movements.

The downstream mechanisms that are activated by transient calcium increases remain unknown; yet, the effects of calcium on morphogenesis are insightful.

The inhibition of intracellular calcium signaling abolishes coordinated movements in all studied model organisms [4,29,35,26,27]. Inhibition of intracellular calcium signaling is routinely performed by injection of calcium chelators into the cell, which capture free calcium ions and inactivate them. The concentration of chelators is high enough to capture all new calcium ions that enter into the cytosol from the extracellular space or intracellular store. A second way to perturb calcium signaling is to apply a drug (often thapsigargin), that removes all calcium from intracellular stores; thus the cells lack resources to increase calcium even if some calcium activated processes take place. In *Xenopus* thapsigargin treatment results in convergent extension defects [4]. In zebrafish [25] and in newt [27] such treatments arrest epiboly progression. Partial reduction of calcium activity results in the retardation of epiboly movements in zebrafish [26,35]. The formation of somites in chick is blocked in calcium free medium [29]. Thus calcium signaling is required for cell rearrangements and coordinated movements in vivo.

Extremely high calcium levels can be experimentally induced in tissues with the use of ionophores. Ionophores insert in the plasma membrane and increase the membrane permeability to calcium, so

Table 1
Duration of endogenous calcium spikes in early embryos.

Stage	Species	Tissue/region	Calcium sensor	Duration sec	Reference
Blastula (2–3hpf)	Zebrafish	Animal pole	F-aequorin	23 ± 0.3	Ma et al. [38]
Blast u la	Zebrafish	EVL	Calcium Green NuCa-green	20–50	Reinhard et al. [37]
Blastula	Zebrafish	EVL	F-aequorin Calcium Green	30–100	Zhang et al. [35]
Blastula (stage 4–10.5)	*Xenopus*	Dorsal ectoderm	Aequorin, f-aequorin	38 ± 11	Leclerc et al. [33]
Gastrula (stage 18)	Medaka	Stellate cells, periderm tissue	Fluo3-AM	15–30	Simon and Cooper [74]
Gastrula (65% epiboly)	Zebrafish	Yolk cells	F-aequorin	45–90	Gilland et al. [46]
Gastrula (stage 10.5–16)	*Xenopus*	Dorsal ectoderm	Aequorin, f-aequorin	175 ± 55	Leclerc et al. [33]
Gastrula	*Xenopus*	Chordamesoderm and lateral mesoderm	G-CaMP4.1 Fluo-4 AM	10–20	Shindo et al. [7]
Gastrula (50% epiboly)	*Xenopus*	Dorsal explants	Calcium Green	<90	Wallingford et al. [4]
Segmentation (1–8 somites)	Zebrafish	Paraxial mesoderm	F-aequorin	10–140; mean 42	Leung et al. [28]
Segmentation (> 11 hpf)	Zebrafish	Hindbrain, trunk, tail	R-aequorin h-aequorin	10–200	Creton et al. [32]
Somites patterning	Zebrafish	Somites	Bis-Fura-2 Fura-2	−15	Freisinger et al. [5]
Segmentation (stages 30–33; 23/24)	*Xenopus*	Posterior mesoderm in tailbud	Fluo3-AM	62 ± 5	Ferrari and Spitzer [69]
Late embryogenesis (neural crest cells migration)	Chicken	Neural crest cells	GcaMP3	0.2–60	McKinney and Kulesa [73]

that extracellular and intracellular concentrations tend to equilibrate. Even though such treatment induces strong perturbations to calcium homeostasis, which is vital for the cell, short lived and low dose treatments can give clues to the role of calcium during morphogenesis. In different organisms such treatments were shown to accelerate coordinated movements and to advance their initiation. Increase of intracellular calcium promotes tissue elongation in the *Drosophila* egg chamber [23], resulting in increased chamber elongation in comparison to normal conditions. Somitogenesis occurs several times faster in high calcium conditions in chick embryos [29]. High intracellular calcium activates gastrulation in sea urchin prior to the physiological onset of gastrulation [30]. Other examples of calcium-activated tissue movements include the triggering of neural fold formation in *Xenopus* [31] and the optical cup formation in chick [36]. To be effective, such treatments have to be applied in specific temporal windows, which suggest that cells need to be competent to respond.

Overall increases or decreases in calcium levels in the whole tissue are a rather unspecific modulation of calcium signaling, as it does not address the precise spatio-temporal changes in calcium levels. As a result numerous potentially active calcium-dependent processes are perturbed. However, such treatments gave clear evidence of the importance of calcium in morphogenesis. The data on the endogenous calcium activity described below will give more detailed insights into how calcium signaling is involved in morphogenesis.

5. Endogenous calcium activity during blastula and gastrula stages

In the late blastula and gastrula stages stochastic calcium transients occur in single cells or in groups of a few cells. The duration of transients is short with respect to the duration of cell reorganization and is therefore referred to as spikes (Table 1) in the following. Spikes were found in zebrafish [37], *Xenopus* [4] and Medaka [74] embryos.

As embryos advance in development calcium signals become more organized spatially and temporally: in many cases transient and localized spikes are followed by long range calcium waves. These waves occur during convergent extension, epiboly and somites formation. Cell rearrangements accompany all these processes. We describe below the features of endogenous calcium signals observed in different species during gastrula and blastula stages.

5.1. Zebrafish

Calcium spikes have been mostly studied in zebrafish embryos. A seminal study on calcium spikes in zebrafish blastula showed that spikes occur within single cells or groups of a few cells [37]. The frequency, occurrence and duration of the calcium spikes do not correlate to those of the cell cycles and spikes are not involved in the cell-cycle regulation [38].

Slusarski et al. found non-canonical Wnt signaling to be an upstream regulator of calcium spikes in zebrafish [39]. We summarize briefly the main upstream activators found by this group [2]. Wnt-5A increases the frequency of calcium spikes in zebrafish blastula [2,5,40,41]. Wnt-5A acts through the activation of G-protein coupled receptor Frizzled as well as through tyrosine kinase receptor [42]. Frizzled activate G-proteins subunits [39] and disheveled. Disheveled activates calcium flux in a PTX-insensitive manner [43]. Calcium is released from intracellular stores through InsP3R which are activated by InsP3 [39]. Additionally to Wnt pathway it has been found that the frequency of calcium spikes in zebrafish is also increased by Prickle, which is a component of planar cell polarity pathway (PCP) [44]. Therefore, Prickle links PCP to Wnt/Ca pathway.

During the blastula period of zebrafish in addition to non-propagating calcium transients in single cells transients of calcium elevations that spread to 2–5 adjoining cells were described [6]. Propagation of calcium transients did not exhibit a preferred direction and the spreading speed is ∼9.2 μm/s [38]. Spikes last tens of seconds and their duration has broad distribution [37]. The first calcium transient in any given cell is always the larger one in amplitude than the subsequent ones [35].

All cells of the outermost enveloping layer (EVL) of the zebrafish embryo produce calcium spikes, while underlying deep cells does not show any calcium activity [35,37,38]. EVL cells are precursors for the embryonic envelope. At the blastula stage EVL cells cover deep cell layers. During the blastula stage, cells of the enveloping layer undergo a thinning process in the apico-basolateral direction, while deep cells keep a relatively round morphology (Fig. 2A). EVL cells show a direct relationship between the amount of cell thinning and calcium concentration. While keeping calcium concentration to the basal levels inhibits cell thinning, increase of calcium concentration causes the enveloping cells to thin prematurely and to a larger extent than in untreated control embryos. The specific activation of spiking activity by application Wnt-5A increases spike frequency and causes the EVL cells to thin prematurely and significantly. At the same time Wnt-5A has no effect on the shape of deep

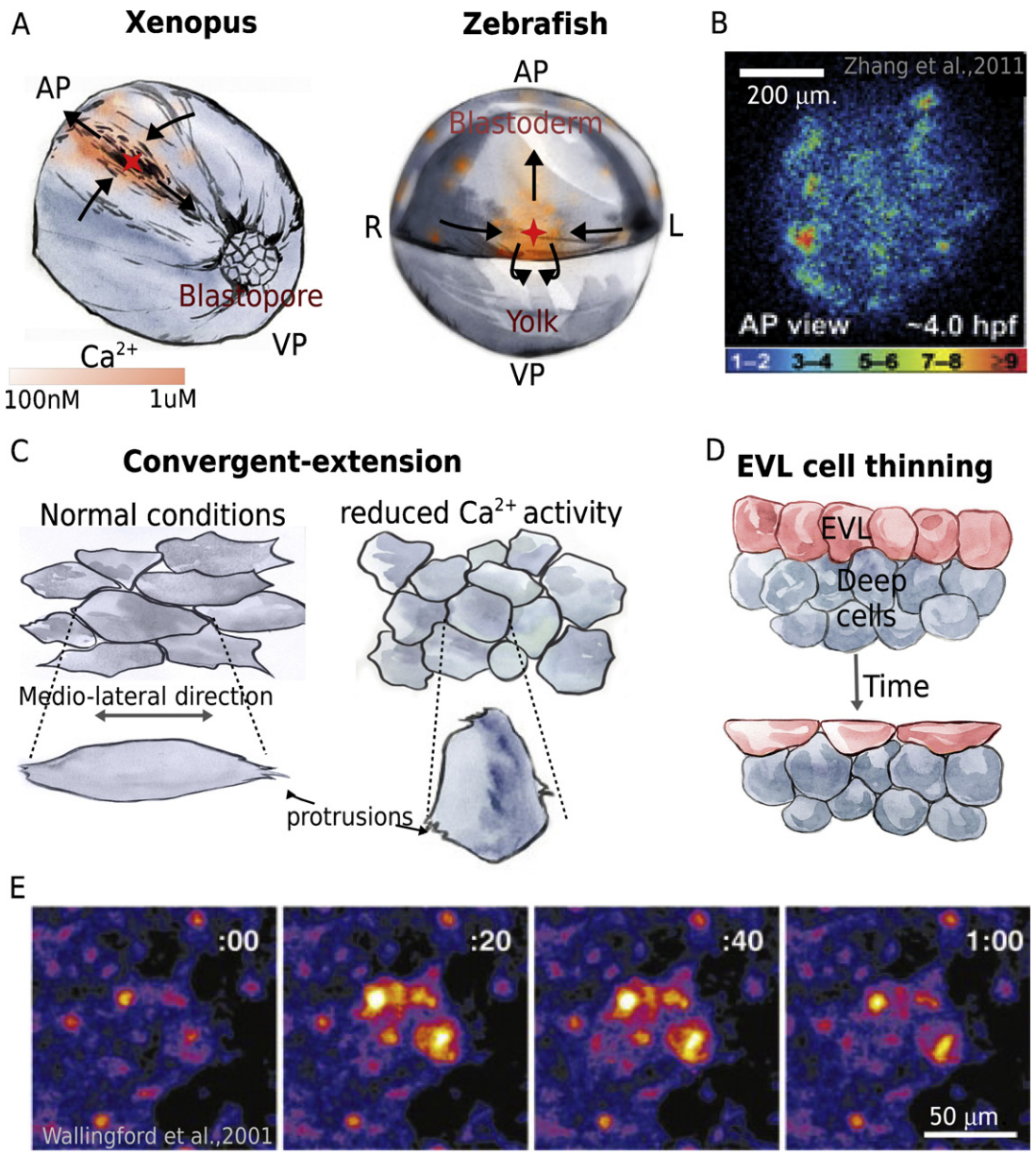

Fig. 2. Cell shapes and endogenous calcium activity at blastula–gastrula stages. (A) Cartoon of gastrula stage of *Xenopus* and zebrafish embryo. Zebrafish embryo is at 50% epiboly stage. Starts indicate regions of convergence-extension. Arrows indicate the direction of cell movements. VP: vegetal pole; AP: animal pole. Orange colors show spatial distribution of the Ca^{2+} transients during the blastula period. (B) Experimental data showing representative pattern of aequorin-generated luminescence, illustrating the localized Ca^{2+} transients observed at zebrafish blastula during. Animal pole view (Zhang et al. [35]). (C) Cartoon of cell tissue during convergent extension in *Xenopus* and zebrafish in normal conditions and under conditions where calcium activity is reduced. (D) Thinning of cells within the enveloping layer (EVL). (E) Experimental data showing calcium spikes in *Xenopus* explants (Wallingford et al. [4]).

cells [35]. These data indicate that calcium spikes are implicated in the EVL cell shape changes.

Prior to the midblastula transition, calcium transients are generated in a homogeneous manner. Following the mid blastula transition, during a transitory window lasting about 1 h, the calcium spikes are generated dorsally with higher frequencies than in other parts of the embryo. Though direct evidence is missing, such heterogeneity might be related to dorsal-ventral patterning of the zebrafish embryos [38].

During the early gastrula stage of fish embryos, the blastoderm starts to flatten toward the yolk and then expands across the yolk cells by a process called epiboly. In zebrafish calcium waves first appear at about 65% epiboly and continue to arise for every 5–10 min up to at least the 16-somite stage [45]. After mid epiboly zones of elevated calcium appear on the blastoderm margin. These zones persist for various time intervals and served as initiation sites and sinks for a series of periodic, long-range propagating calcium waves. Waves propagate around the blastoderm margin in either a uni- or bi-directional manner with a speed of 4 μm/s. Later by 85% epiboly, the majority of the waves originate from the dorsal midline of the blastoderm margin. Waves travel up the main embryonic axis towards the animal pole and around the blastoderm margin [46]. At the end of epiboly much slower waves of 0.05 μm/s propagate along the forming trunk at the late gastrula [6].

5.2. Medaka

In the Medaka fish (*Oryzias latipes*) calcium spikes have been observed during early development after the onset of epiboly. Occasionally transient calcium spikes appear synchronously within a

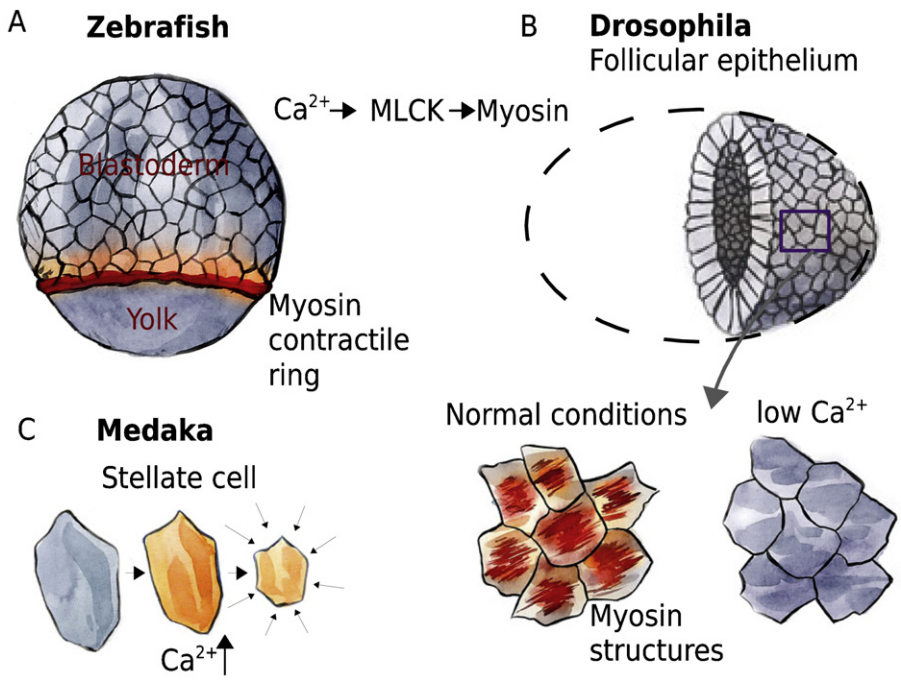

Fig. 3. Tensions, myosin accumulations and contractions. (A) 75% epiboly of zebrafish embryo. Accumulation of a myosin contractile ring and elevated calcium at the margin of the blastoderm. (B) *Drosophila* egg chamber. Myosin accumulates at the basal surface of cells in the follicular epithelium, in normal conditions and is absent at low calcium concentration. (C) Contraction of a stellate cell in Medaka embryo upon calcium activation.

group of neighboring cells. In other cases, a calcium spike first appears within individual cells and then spreads to one to five adjacent cells. Spikes typically last 15–30 s and precede cell contractions that occur 3–6 s after the start of rise of calcium concentration. However not all cells contract after the occurrence of spikes [74].

Shortly after the start of epiboly, a series of spontaneous rhythmic contractile waves begin to propagate across the blastoderm. The waves are initiated in a specific small population of cells located in the ventral blastoderm, called the pacemaker [47]. Actin filaments are involved in the contractions. Each rhythmic contraction is preceded or accompanied by calcium spike, which results from calcium entry from extracellular and an intracellular pools [48,74]

5.3. *Xenopus*

Similar calcium spikes are observed in *Xenopus* embryos during the blastula stage. Spikes occur in the dorsal marginal zone only. Spikes initiate in 2–4 adjacent cells and occasionally spread to one or two additional neighboring cells, but no further. The spikes initiate stochastically in time and position with calcium levels increasing and recovering in less than 90 s [4]. The mean duration of spikes is 38 s at blastula stage and increases to 175 s by the end of gastrulation [33]. The frequency of spikes increases from stage 10–11 by two-fold and then decreases at stage 13 [49].

In *Xenopus* explants calcium waves arise stochastically in space and time in the dorsal tissues. They initiate in 2–4 adjacent cells and propagate away from the initiation point to 5–20 cell diameters with rates of about 5 μm/s. Most waves dissipate uniformly and simultaneously in all involved cells. Waves are often accompanied by a wave of contraction within the tissue. After wave propagation, calcium levels return to basal levels within a few minutes [4]. Calcium waves are also abundant in the intact *Xenopus* embryo at the gastrulation stage and afterwards. They are generated in the dorsal region of the gastrulating embryo and spread then towards anterio-posterior and lateral directions of the embryo with a velocity of 10 μm/s [33,49].

The calcium transients in *Xenopus* require the presence of functional L-type voltage-sensitive calcium channels [33] and calcium from intracellular stores but do not require frizzled-8 signaling [4]. Calcium transients control expression pattern of neural gene *Zic3* [33,49] that do not influence on the expression of notochord specific probe *Xnot* [4].

6. Convergent extension in *Xenopus* and zebrafish

Convergent extension cells movements take place in *Xenopus* and zebrafish embryos during the gastrula stage. Cells elongate in the direction of tissue narrowing and intercalate (Fig. 2C). In *Xenopus* embryos convergent-extension occurs in the dorsal part of the embryo. In this region calcium spikes and waves are produced and calcium signaling is required for proper tissue remodeling [4].

Mesenchymal cells intercalate medio-laterally by polarized protrusive activity and cell crawling [50]. Experiments with cell explants further suggest calcium is involved into cell polarization and thinning. Explants cells that undergo convergent extension movements produce endogenous calcium spikes [4]. Exhaustion of intracellular calcium stores induces a decrease in spikes frequency and affect normal cell orientations and shapes. In calcium inhibitor-treated explants the mesenchymal cells become more round and align randomly relative to the lateral direction, which impairs their crawling along the elongation axis [7].

In zebrafish, deep cells undergo convergent extension reorganization during gastrula stage by a mechanism similar to *Xenopus*. Elongation of ectodermal and mesendodermal cells in the medio-lateral axis is a prerequisite for convergent extension movements. Calcium waves propagate in the deep cells [3]. The mutants for the Wnt signaling pathway were shown to decrease calcium activity in zebrafish [39]. The Wnt5b mutants have problems with cells elongations. In wild type zebrafish embryos, ectodermal cells possess a significantly lower degree of roundness than cells in similar regions of mutant embryos. Additionally protrusions of wild type ectodermal cells extend preferentially in both medial and lateral directions while in the embryos in which calcium signaling was

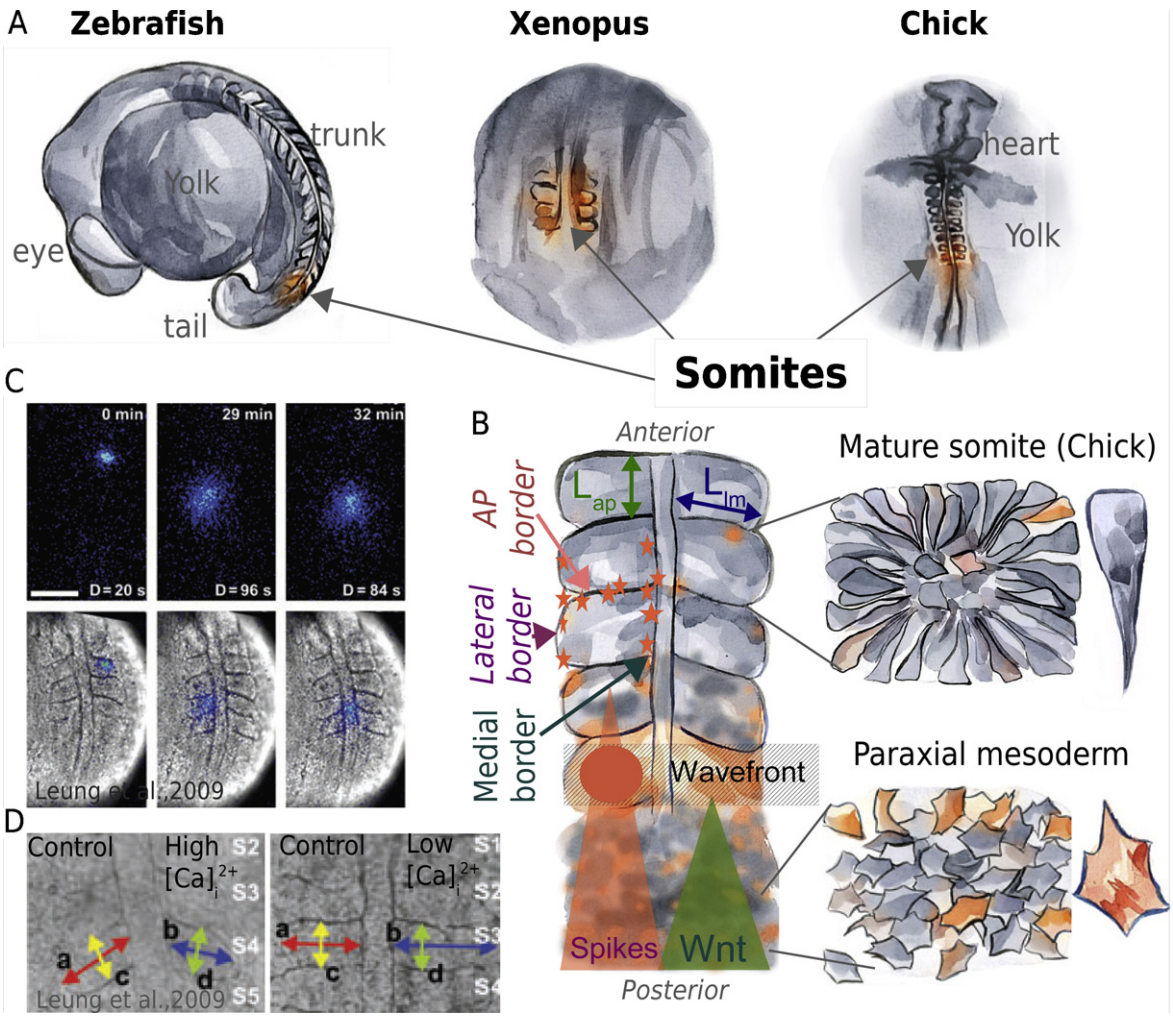

Fig. 4. Somitogenesis. (A) Cartoons of zebrafish, *Xenopus* and chick embryos at segmentation stage. Orange color mark regions with elevated calcium where new somites are formed. (B) Cartoon of calcium transients and cell organization in the paraxial mesoderm and formed somites. Orange color reflects elevated calcium concentration due to calcium wave and spikes. Circle indicates localization of calcium wave front. Stars indicate the regions in the somites where calcium spikes are more frequent. Lap is somite length along apico-posterior direction. Llm is somite length along medio-lateral direction. (C) Experimental data showing stochastic Ca^{2+} signals generated in formed somites and notochord during early somitogenesis in zebrafish (Leung et al. [28]). (D) Experimental data showing effect of calcium level on the length of somites along the medial-lateral axis (Leung et al. [28]).

perturbed protrusions are shorter, and less well aligned along the medio-lateral (ML) axis [51]. Blocking the activity of the calcium-regulated protein Ryk impair zebrafish cell motility as well as cell elongation in vitro. Cells without Ryk move slowly and have a more rounder shape than control cells. The exposure of Ryk expressing cells to Wnt results in the protrusion formation [42]. These observations suggest that calcium signals participate in the mediolateral elongation and orientation of cellular processes in ectodermal and mesendodermal cells at late gastrulation stages. Additionally CaMKII, which is a calcium sensing protein, is required for gastrulation in zebrafish [52], and motility of cells from the Japanese newt increase upon calcium elevation [27].

7. Regulation of actomyosin activity by calcium

The forces driving cell movement and cell shape changes during tissue morphogenesis are produced by contractile elements, which are assemblies of cytoskeletal proteins. The cytoskeletal proteins involved in these processes include actin filaments, actin binding proteins and motors. In skeletal muscle, it has been known for a long time that calcium regulates acto-myosin complexes that are involved in force generation that underlies muscle contraction [53]. In smooth and non-muscle cells, contraction of acto-myosin assemblies is also mediated by the modulation of myosin activity. Regulation of myosin activity relies mainly on the phosphorylation of the myosin light chain by myosin light chain kinase (MLCK) [54], which is activated by calcium/calmodulin complexes. The other kinases that activate myosin act through the G-protein mediated Rho pathway and are calcium independent [55]. The activation of MLCK is directly related to the concentration of active calmodulin. Calmodulin itself is activated by specific calcium concentrations and can decode the frequency of temporal calcium changes. Thus calcium activates contractions of actomyosin assemblies in a concentration dependent way. Also transients of calcium reorganize actin networks [56]: increase in calcium concentration is correlated with reinforcement of acto-myosin fibers while decrease in calcium results in the shortening of actin filaments and loss of connected actin network in epithelial cells [21]. In turn actin cytoskeleton and actin binding proteins can also regulate calcium signaling [57]. Depolymerization of actin in starfish oocytes induces a massive release of calcium [58] that is thought to be mediated by activation of stretch-activated calcium channels of the endoplasmic reticulum [59] or direct calcium storing by actin [60].

After epiboly in zebrafish where more than 75% of the yolk is covered, an actomyosin ring forms at the margin of the extended tissue (Fig. 3). The contraction of this actomyosin ring facilitates the end phase of epiboly extension and blastopore closure. Calcium concentration is high at the margin of the epibolic tissue at this stage [61] and calcium waves propagate along the circumference of the margin [46]. These waves are presumably involved in the actomyosin ring formation. Inducing the increase of cytosolic calcium is able to trigger the accumulation of actomyosin at the margin in early stages of epiboly (even before it reach 50%), the formation of an actomyosin ring and the detachment of the epibolic tissue from the yolk following ring contraction [26,40]. Inhibition of calcium activity disrupts accumulation of actin at the margin and prevents epiboly progression [3]. Activation of MLCK was detected at the margin during epiboly and was shown to be required for the formation of the actin-myosin ring [26], which indicates that the mechanism of myosin accumulation is mediated in a similar way to the myosin activation in smooth and non-muscle tissue through the activation of MLCK.

While whole epithelial tissue movements are clearly visible, at the subcellular level the contractile actomyosin behavior that happens during coordinated tissue movements only recently attracted an attention of scientists. Such behavior occurs in parallel to larger tissue movements and has been shown to be required for the convergent extension movements during *Drosophila* germband elongation [62], mesoderm invagination [63], dorsal closure [64] and egg chamber elongation [23]. Recent evidence indicates that calcium is required for the accumulation of actomyosin and periodic contractions in epithelial cells in vitro and in vivo. The basal surfaces of *Drosophila* follicle cells undergo a series of directional, oscillating contractions driven by periodic myosin accumulation on a polarized actin network. Actomyosin networks form elongated bundled structures on the basal surface of follicle cells (Fig. 3). Proper cytosolic calcium levels are necessary for the assembly of the actomyosin network in these cells. Increases in calcium promote assembly of actomyosin while chelating of calcium have an opposite effect. Finally, the intensity of myosin that presumably relates to the amplitude of the observed oscillations was modulated by calcium levels, while the period of these oscillations was calcium independent [23]. Spontaneous periodic activity of the actin cytoskeleton has finally been observed in cultured epithelial cells, and the amplitude and frequency of such activity were strongly affected by intracellular calcium level changes [65].

8. Somitogenesis

During vertebrate development, somites are the first segmented structures to form (Fig. 4.). These morphological units emerge from the paraxial mesoderm (PAM), which reorganizes along the anterior–posterior embryonic axis [66]. Somites are precursors of dermis, skeleton and muscles. The visible changes of cell reorganization in the PAM occur at the end of gastrulation. In all vertebrates somites are formed one by one from the anterior to the posterior parts simultaneously either side of the notochord. The periodicity and length of somite formation is currently explained by the Clock and Wavefront mechanisms [67,68]. Periodic waves of gene expression, with a length of approximately one somite, drive somite formation. Such stripes form in the anterior PAM in response to a periodic clock signal at the level defined as the determination front, and it defines the future somitic AP boundaries. The determination front, Wavefront, is positioned by antagonistic gradients of FGF, Wnt and retinoic acid signaling. The Wavefront regresses posteriorly as the embryo elongates along the AP axis. Especially interesting in this context is the observation of slow calcium waves during zebrafish somitogenesis. The calcium wave moves posteriorly with a speed of 0.7 μm/s along with the formation of the somites [6,75].

The observation of calcium signaling during somitogenesis reveals the presence of endogenous transients in the form of calcium spikes. Cells spike individually but also simultaneously in groups of a few neighboring cells. These spikes last a few tens of seconds, which is comparable to those produced during gastrulation and blastula formation. The frequency and the duration of spikes correlate with the stage of somitogenesis. Spikes appear in the PAM before it reorganizes, but become more rare during somite formation and are not observed afterwards [69]. Wnt signaling is an upstream activator of calcium spikes in somitogenesis in zebrafish, while G-protein binding protein (Rgs3) inhibits Wnt5 induced calcium spikes during this process [5]. Wnt expression is high in the PAM and absent in formed somites behind the Wavefront line [67]. The duration and the frequency of spikes decreases with time; in pre-formed somites the duration of spikes is several times longer than in the formed ones [67]. The source of calcium that participates in the generation of calcium spikes varies from one species to another. In chicken extracellular calcium is crucial for spike generation [29] while intracellular organelles are the main source of calcium in *Xenopus* [69] and zebrafish [28].

Significant cell rearrangements take place during somitogenesis. Non-patterned PAM contains a mass of disorganized mesenchymal cells lacking preferential orientation. Cells of the PAM exhibit protrusive activity, which presumably mediates cell displacements by traction of one cell on another. Disorganized before somitogenesis, mature somites have a roughly rectangular shape. Cells inside mature chick somite embryo organize radially. Cells have pyramidal shapes, with wide basal surfaces exposed to the outside of the somite and constricted apices inside. A fraction of mesenchymal cells remain inside the somite, which are not polarized and retain random shapes [70].

Several lines of evidence indicate a direct participation of calcium signaling in the cell shape changes and reorganization in somitogenesis through its effects on cytoskeleton reorganization. Cytoskeletal and adhesion proteins are key players for cell movements and rearrangements. During somitogenesis, actin is involved in the constriction of apical cell surfaces, while microtubules are presumably associated with cell elongation. Inhibition of actin or microtubule polymerization abolishes somite patterning: cells become round and do not reorganize properly. Similar effects are observed by inhibition of the calcium sensing protein calmodulin as well as by direct inhibition of calcium signaling. These data suggest that calcium may regulate cytoskeleton reorganization required for somite patterning [29]. Myosin expression and activity increase during somitogenesis. Upon exposure to increased levels of calcium, preformed somites do not exhibit contractile activities, while formed ones do so [69]. This echoes observations in the development and differentiation of muscle cells, where elevated calcium transients are known to activate and reorganize actomyosin networks [71]. Experimentally induced increases of calcium during somitogenesis result in abnormal somite formation. Cells shrink giving somites a more compact and round shape, and causing the borders between somites to be impaired. The tails of developed embryos are kinked in the area where somitogenesis was perturbed by exposure to calcium. However, the expression level of myosin is unaltered and cells differentiate properly in perturbed somites [69], suggesting that calcium signal may reorganize myosin without perturbing either cell fate or myosin expression levels.

9. Spikes at tissue borders

The frequency of spikes at tissue boundaries was reported to be higher than inside the tissue in several systems. In *Xenopus*

embryos cells at the vicinity of the future somite furrow spike several times more than cells in the central region of somites [69]. The ectoderm/mesoderm border appears to generate most signals in *Xenopus* explants [49]. Numerous calcium spikes are generated at the boundary between mesodermal and chordamesodermal *Xenopus* explants when these tissues came into contact [7]. Additionally, in this system direct application of mechanical stress on tissue triggered calcium spikes. It has been recently shown that myosin-driven tension is high at the boundary of tissues in order to prevent tissue intermixing and keeps smooth borders [72]. Thus, as spikes occur more often at the tissue boundaries where tensions are elevated it is possible that spikes are activated by physical tensions.

Cells at the lateral and medial borders of somites in zebrafish also spike more than central ones [28]. Similarly, spikes are more numerous at the contact between explants from notochord and somite tissue of *Xenopus* [7]. While segmentation Clock and Wavefront mechanisms impose the length of somite along anterio-posterior axis, they have no influence on the length of somite along ML axis. Modulation of calcium level results in changes of ML somite length: at high calcium conditions mature somites are shorter in the ML direction then in control conditions as cells migrate less in ML direction. At low calcium cells in the formed somites migrate more resulting in longer somites in ML direction. In the chicken embryo, neural crest cells travel long distances towards the place where they form ganglia. Spontaneous calcium spikes are produced all along the cell trajectories. Cells that arrest movement have been reported be have a higher probability of spiking [73]. Additionally the calcium spikes observed during somitogenesis do not require actin polymerization and cell motility [69], indicating that spikes are not downstream of the cytoskeleton machinery in this system. Taking together these observations suggest that calcium spikes may act as a stop signal, acting upstream of the cytoskeleton to prevent the migration of cells out of somite along the ML direction.

10. The cell-help-hypothesis on the role of calcium spikes

Calcium signaling is important for morphogenesis of embryonic tissues in vertebrates and invertebrates. In a variety of animals calcium activity in form of spikes have been shown to have similar spatio-temporal characteristics. The duration of spikes last for a few tens of seconds and they occur in single, or in groups of a few cells in a stochastic way. Calcium spikes in single cells fluctuate clearly and more intensely in embryonic tissues undergoing cell rearrangements and migration. Motility of the cells in such tissues fully relies on the coordinated and elevated activity of the cytoskeleton. However it is not possible to conclude from the existing data whether calcium spikes are the cause or the consequence of cell shape changes induced by the cytoskeleton dynamics. No simple model can explain how calcium spikes in isolated cells globally affect hundreds of cells that comprise rearranging tissues. Based on the stochasticity of calcium spikes and their random appearance in the single cells, we propose an alternative view on the role of calcium spikes for morphogenesis, that we call the cell-help hypothesis (CHH). According to the CHH, spikes do not directly control cell shape changes per se but rather represent a system to locally control and regulate defects occurring in tissues undergoing massive reorganization. In any highly dynamic system, such as gastrulation or cell migration, there is intense fluctuations of activity of intracellular components. Fluctuations of apical concentrations of myosin motors are a striking example. The fluctuations of active molecules, such as force producing molecular motors, could eventually lead to local abnormalities in cell geometry, movements and tensions. To accomplish controlled morphogenesis, excessively active cells, for example, might be eliminated from the tissue due to rare but significant deviations from acceptable levels. We speculate a fine-tuning mechanism whereby spiking activity prevents and/or repairs such defective cell behaviors. Calcium signaling which is fast to establish and to stop could be efficient to prevent local, abnormally high, fluctuations in molecular (e.g. cytoskeletal) activities. Calcium spikes may thus help individual cells to survive in competitive situations with its neighbors.

11. Concluding remarks

Different methods using aequorin, fluorescent and genetically encoded calcium sensors show similar increase in calcium concentrations in early embryonic cells that are not usually considered as excitable cells such as neurons or muscles. Calcium signaling seems to be required for collective tissue movements and cell shape changes. In convergent extension, EVL cell thinning and somitogenesis, calcium signals are indispensable elements for the cell elongation and cell planar polarity. Calcium signaling is also required for the accumulation of myosin and cell contractions in early embryos. However whereas calcium spikes result in visible and detectable cell shape changes in some cases, such as cell contractions in Medaka and cell thinning in zebrafish, in other cases similar calcium transients have no visible effect on cell shapes. This suggests either that different signaling pathways may be activated by the same calcium transients, through different calcium sensing downstream molecules, or that the signaling output is modulated by differences in amplitude and temporal characteristics of the spikes, that have not been resolved yet. It remains to be established which components are missing in early tissues that prevent single cell spike propagation through the tissues as observed in later stages of development where multiple calcium waves take place. Yet the question of which is the cause or the consequence in the process of cell shape changes remains open. In particular the exact mechanistic connection between changes in calcium concentration and the downstream events is far from being understood. The simultaneous observation of tissue movements at the cellular level, calcium imaging and imaging of cytoskeleton rearrangements will be key to understanding the role of calcium in cell rearrangements during development.

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