Calcium Signaling during Convergent Extension in *Xenopus*

**John B. Wallingford*, Andrew J. Ewald,
Richard M. Harland*,** and **Scott E. Fraser†,**

Division of Biology and Beckman Institute (139-74)
California Institute of Technology
Pasadena, CA, 91125

*Department of Molecular and Cell Biology
401 Barker Hall,
University of California
Berkeley, CA 94720-3204

Abstract

Background: During *Xenopus* gastrulation, cell intercalation drives convergent extension of dorsal tissues. This process requires the coordination of motility throughout a large population of cells. The signaling mechanisms that regulate these movements in space and time remain poorly understood.

Results: To investigate the potential contribution of calcium signaling to the control of morphogenetic movements, we visualized calcium dynamics during convergent extension using a calcium-sensitive fluorescent dye and a novel confocal microscopy system. We find that dramatic intercellular waves of calcium mobilization occur in cells undergoing convergent extension in explants of gastrulating *Xenopus* embryos. These waves arose stochastically with respect to timing and position within the dorsal tissues. Waves propagated quickly and were often accompanied by a wave of contraction within the tissue. Calcium waves were not observed in explants of the ventral marginal zone or prospective epidermis. Pharmacological depletion of intracellular calcium stores abolished the calcium dynamics and also inhibited convergent extension without affecting cell fate. These data indicate a direct role for calcium signaling in the coordination of convergent extension cell movements.

Conclusions: The data presented here indicate that intercellular calcium signaling plays an important role in vertebrate convergent extension. We suggest that calcium waves may represent a widely used mechanism by which large groups of cells can coordinate complex cell movements.

Introduction

A wide variety of morphogenetic cell movements are required for normal development of vertebrate embryos. These include not only migrations of small populations of cells, such as germ cells, but also massive, coordinated rearrangements of entire tissues, such as folding of the neural plate to form a tube. Another example of such large-scale, coordinated cell movement is the process of convergent extension, in which cell intercalation elongates embryonic axes in both invertebrate and vertebrate embryos [1-7]. In some animals, this process is relatively simple; only about 40 cells intercalate in the ascidian notochord [4]. In contrast, convergent extension of the presumptive notochord of *Xenopus* embryos involves the intercalation of many hundreds of cells [8].

The process by which convergent extension occurs in the amphibian dorsal mesoderm has been very well described [8-15]. Intercalation is accomplished by a complicated suite of cellular behaviors, as individual cells polarize in the mediolateral axis, orient and stabilize lamellipodia, and exert traction on neighboring cells [10-12]. These behaviors propagate through the tissue from anterior to posterior as gastrulation proceeds [8, 11]. The need for reliable coordination of these cell behaviors suggests that several mechanisms must work in concert to control this process. For example, molecules which specify cell fates in the mesoderm, such as the transcription

factor brachyury, influence convergent extension [16]. In addition, convergent extension is also modulated by adhesion factors such as cadherins and protocadherins [17, 18]. Finally, signaling via Wnt pathways is also critical, as inhibition of Wnt, Frizzled, or Dishevelled activity suppresses convergent extension and cell intercalation [15, 19-24]

Since certain Wnt ligands and Frizzled receptors have been shown to signal through protein kinase C and elicit calcium release [25], it is possible that calcium signals may provide an additional level of regulation for convergent extension. Interestingly, intercellular calcium waves have been reported to occur in the marginal zone of gastrulating zebrafish embryos [26, 27]. Though the function of these waves remains to be elucidated, intercellular calcium waves allow rapid communication across large cell populations and have been shown to be involved in a wide variety of biological processes [28]. In this report, we describe intercellular waves of calcium mobilization which occur during the initiation of convergent extension in the dorsal marginal zone of *Xenopus*. Furthermore, using depletion of calcium from the endoplasmic reticulum, we show that calcium signaling is critical to the process of convergent extension, but is dispensable for dorsoventral cell fate specification in the mesoderm during gastrulation. These data indicate a direct role for calcium signaling in the control of convergent extension. We suggest that intercellular calcium waves represent a novel mechanism by which large groups of cells can coordinate complex morphogenetic movements during vertebrate development.
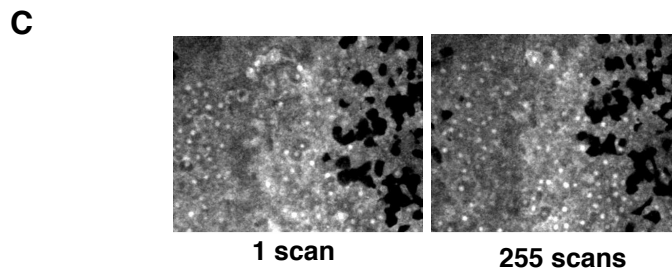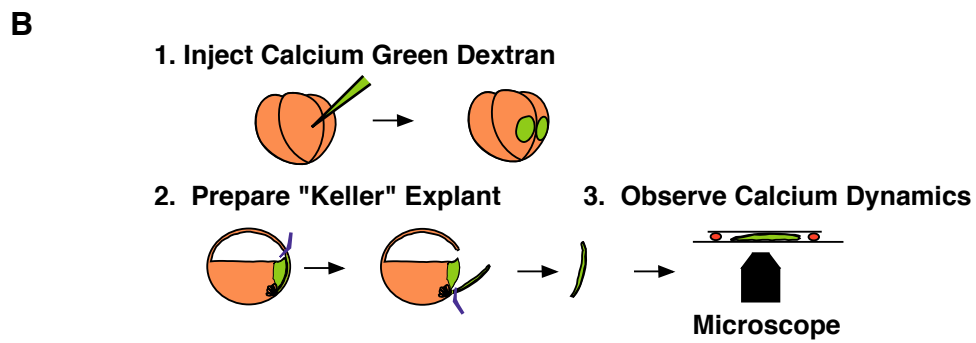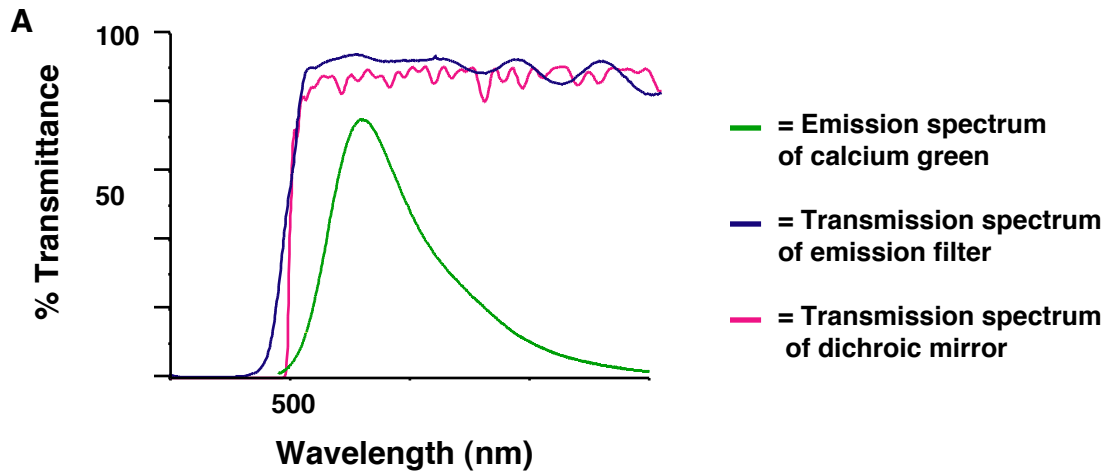
Results

Imaging of calcium dynamics in open-face explants of the dorsal mesoderm

To assess the role of calcium signaling in convergent extension we first examined calcium dynamics in the dorsal marginal zone of *Xenopus* embryos. We have previously used time-lapse confocal microscopy to examine cell behavior during *Xenopus* gastrulation over short periods of time (~15 minutes) [15]. However, photobleaching of fluorescent reagents and phototoxicity both limit prolonged imaging of living cells. To overcome these difficulties, we have developed a new confocal system where improved light harvesting allows long-term live imaging of cell behaviors.

To increase the efficiency of light collection from green emitting fluorophores, we used specially designed, high-Q dichroic mirrors and emission filters (Chroma Technology) which allow a very large fraction of the emission spectrum of calcium green to be collected (Fig. 4-1, A). We gained further benefit from the use of a high numerical aperture (0.75) Zeiss 20x Fluar objective. These improvements allow high-resolution images to be captured in a short time using very low excitation intensities, which is significant because photobleaching and phototoxicity increase disproportionately with excitation intensity.

Xenopus mesodermal cells involute into the embryo during gastrulation. Imaging of these internalized cells during convergent extension in intact

Figure 4-1: Imaging of calcium dynamics. **A.** Emission filters and dichroic mirrors used here were designed to reflect laser light efficiently at 488 nm, but transmit 90% of light at 505 nm, collecting a large fraction of the emission spectrum of calcium green. **B.** Experimental design for observation of calcium dynamics in the *Xenopus* DMZ. Embryos were injected with calcium green dextran at the 4-cell stage (1), and cultured to early gastrula stages. Open-face DMZ explants were then prepared (2) with deep cells apposed to coverglass (3). **C.** The confocal system used allows effective, long-term confocal time-lapse of living cells. Left panel shows a field of cells from a calcium green dextran-labeled DMZ explant after one 8-second scan; right panel shows the same field of cells after 254 additional 8-second scans over 85 minutes; mean fluorescent intensity was reduced by only 6%.

A

% Transmittance

100

50

500

Wavelength (nm)

= Emission spectrum
of calcium green

= Transmission spectrum
of emission filter

= Transmission spectrum
of dichroic mirror

B

1. Inject Calcium Green Dextran

2. Prepare "Keller" Explant   3. Observe Calcium Dynamics

Microscope

C

1 scan   255 scans

Xenopus gastrulae is not possible due to the opacity of the yolk contained in each cell. However, open-face "Keller" explants of the dorsal marginal zone (DMZ) of *Xenopus* gastrulae provide a well-defined system in which to examine the cell behaviors involved in convergent extension [8, 10-12, 15] Briefly, the dorsal marginal zone of a *Xenopus* embryo is manually removed and cultured between coverslips separated by clay feet such that the deep cells which undergo convergent extension are directly apposed to the coverglass (Fig. 4-1, B). To examine calcium dynamics in the DMZ, presumptive dorsal cells of *Xenopus* embryos were loaded with calcium green dextran by microinjection at the 4-cell stage. At gastrula stages, open-face explants were prepared (Fig. 4-1, B) and time-lapse confocal microscopy produced high-resolution images with no significant attenuation of signal even after hundreds of scans (Fig. 4-1, C).

Intercellular calcium waves during convergent extension

Time-lapse microscopy of calcium green dextran-labeled deep cells during early gastrula stages revealed dramatic intercellular waves of calcium mobilization in the DMZ. Most striking was the occurrence of long-range waves (Fig. 4-2; MOVIES 1 & 2; available at http://bicsnap1.caltech.edu/jwetal/ jwetal.html) These large waves initiated in 2-4 adjacent cells and propagated 15-20 cell diameters from the initiation point at a rate of about 5 microns sec^{-1}. More common were smaller waves that also initiated in about 2-4 cells and

propagated radially 5-10 cell-diameters at a slower rate of approximately 2-3 micron sec^{-1} (MOVIES 3 & 4; Fig. 4-3). Waves appeared to arise stochastically

FIGURE 4-2 and Movies 1 & 2:

Long-range intercellular calcium waves in the dorsal marginal zone. **A)** Individual frames from confocal time-lapse of the large propagating calcium wave in MOVIE 1. Time points are indicated in white. Cell mixing produces a mosaic pattern of labeled (bright) and unlabeled (black) cells. A large calcium wave initiates (t= :00), travels approximately 20 cell diameters (t= 1:00), and subsides over approximately eight minutes (t= 8:00). Scale bar = 50 microns in this and all subsequent figures. **a')** Plot of DF/F$_0$ for each of the areas shown in the colored boxes in the last panel of A. Plot shows the propagatory nature of the rise in calcium levels and the more even recovery. **MOVIE 1)** This movie shows the patterns of calcium release in a DMZ explant labeled on the left side with calcium green dextran; the right side is unlabeled and is black, though cells are present in the field of view. In this and all DMZ movies, the dorsal lip of blastopore is at the bottom of the screen and the dorsal midline runs vertically through the middle of the screen; the mediolateral axis is horizontal. As the movie begins, several small flashes of calcium release can be observed throughout the explant (described below in Fig. 4-4, B). About halfway through the movie, a small intercellular calcium wave arises near the midline of the explant, followed closely by another, much larger calcium wave. Still frames depicting the large wave in this movie are presented above in Fig. 4-1, A. **MOVIE 2)** This movie shows a DMZ explant in which all the cells are labeled

with calcium green dextran. This explant undergoes a small wave, then a larger wave, then another small wave. These waves are less dramatic than those in MOVIE 1, possibly due to less effective calcium green dextran loading.

FIGURE 4-3 and MOVIES 3 & 4:

Short-range intercellular calcium waves in the DMZ. **A)** A small wave arises (t= :00) propagates approximately 10 cell diameters (t= 1:00), then dissipates over the following two minutes (t= 3:20). Scale bar = 50 microns **a')** Plot of DF/F_0 for each of the areas shown in the colored boxes in the last panel of A. **MOVIE 3)** This movie shows the wave from Fig. 4-3, A. **B)** A small wave arises (t= :00), propagates approximately 10 cell diameters (t= 1:00), then dissipates over the following four minutes (t= 5:00). **MOVIE 4)** This movie shows the wave from Fig. 4-3, B. **b')** Plot of DF/F_0 for each of the areas shown in the colored boxes in the last panel of B. As with the larger waves, the plot shows a propagatory increase in calcium levels and a more even recovery.
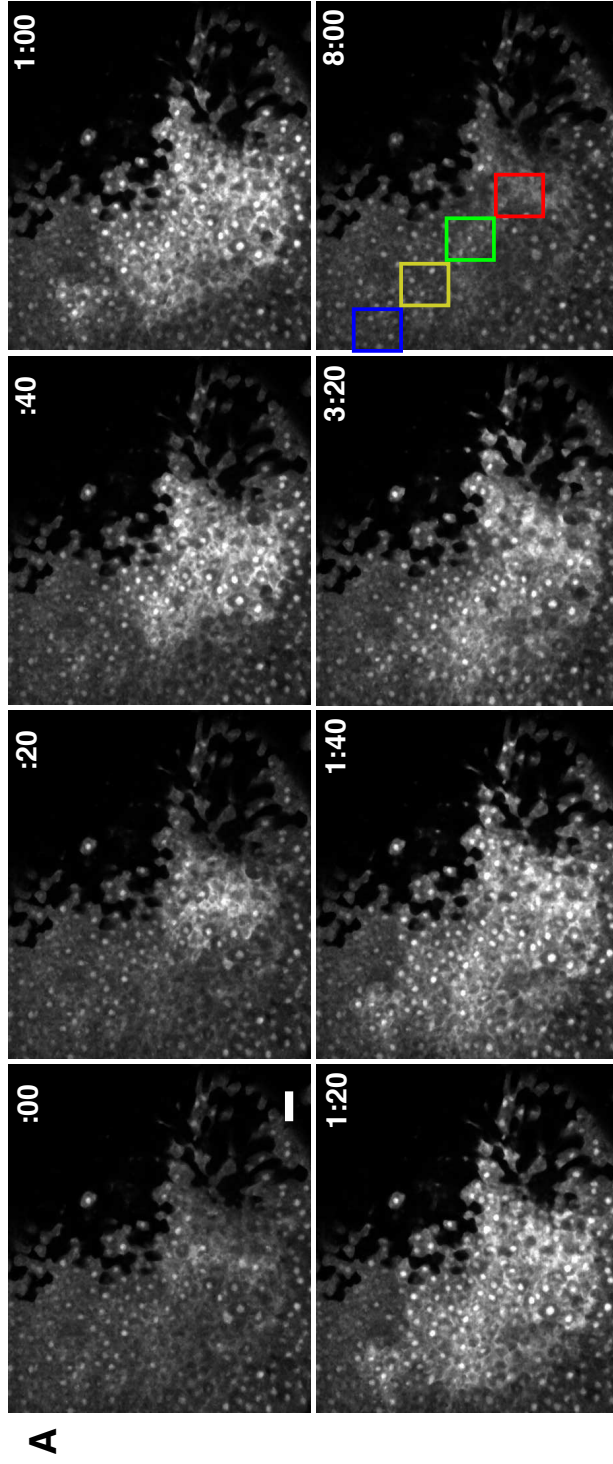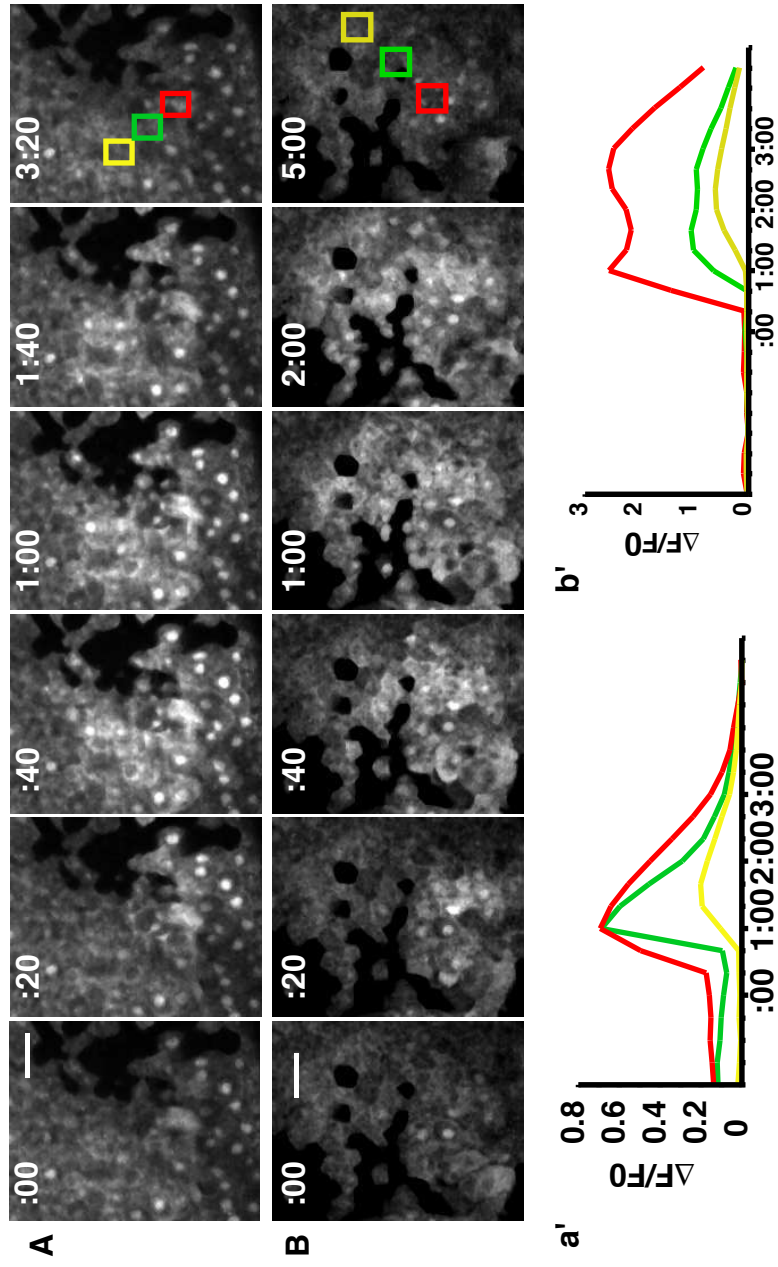
with respect to timing and location within the filmed DMZ explants. Most waves dissipated uniformly and simultaneously in all involved cells. Following wave propagation, calcium levels returned to baseline within a few minutes; in smaller waves, recovery was somewhat faster. Most calcium waves were accompanied by a wave of contraction within the tissue (Fig. 4-4, A; MOVIE 5).
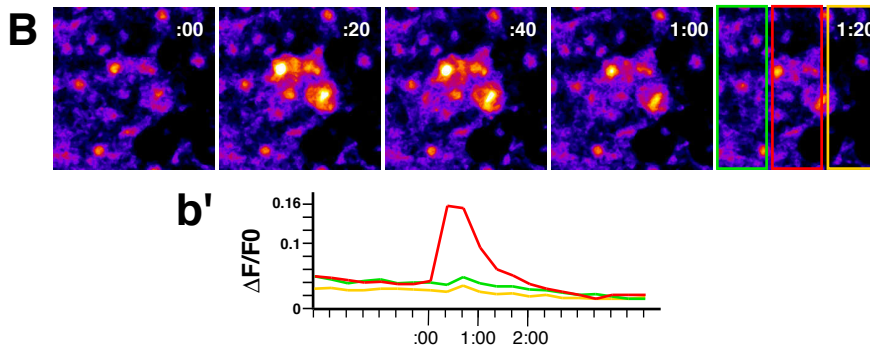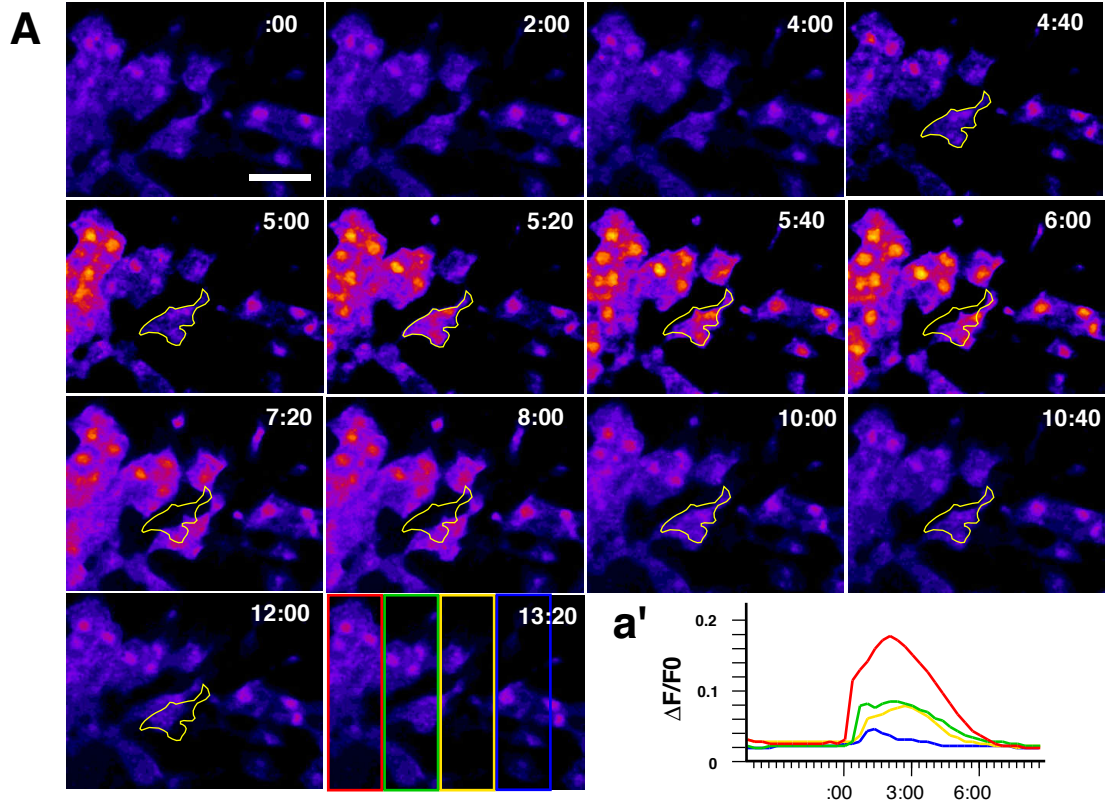
The DMZ explant contains both dorsal mesoderm and posterior neural ectoderm, and both tissues undergo convergent extension [8, 10]. Most of the observed waves clearly arose in the mesoderm, near the dorsal lip of the blastopore; however, we also observed calcium waves in the neural ectoderm of DMZ explants (not shown), consistent with results obtained by imaging the externally visible neural plates of intact *Xenopus* embryos [29].

In addition to the intercellular waves, we also observed small flashes of calcium mobilization occurring simultaneously in a handful of adjacent cells in the DMZ (Fig. 4-4, B; MOVIE 6). These flashes initiated in 2-4 adjacent cells and occasionally spread to one or two additional adjacent cells, but no further. Such flashes were not accompanied by contraction. Flashes initiated stochastically in time and position, and were very short-lived, with calcium levels routinely increasing and recovering in less than 90 seconds (Fig. 4-4, B).

Calcium dynamics in DMZ explants were variable; 15 of 21 explants filmed displayed one or more waves, and 20 of 21 displayed calcium flashes. On average, calcium waves occurred in the dorsal marginal zone at a rate of 0.71/hour (Fig. 4-5; n=21). Calcium waves also varied in amplitude with some waves reaching DF/F_0 values of over 3 (Fig. 4-2, B and Fig 4-3, b'), while others

FIGURE 4-4 AND MOVIES 5 & 6:

Calcium waves and calcium flashes. **A)** High-magnification view of cells involved in the wave described in Fig. 4-2. During the four minutes preceding the wave, very little movement is observed (t= :00 - 4:00). The yellow outline at t= 4:40 indicates the position of two cells just prior to the calcium wave. The calcium wave propagates from left to right across the field of cells between t= 5:00 and t= 5:40. As the wave moves across and begins to dissipate, cells move dramatically (compare initial position to cells at t= 7:20). As the calcium levels recover, the cells move dramatically in the opposite direction beyond their original position (t= 8:00 - 12:00). Scale bar = 50 microns. **a')** Plot of DF/F_0 for each of the areas shown in the colored boxes in the last panel of A shows that local changes in calcium levels reflects the pattern of calcium release and recovery in the overall wave (compare with Fig 4-2, a'). **MOVIE 5)** This movie shows the cells in Fig. 4-4, A. **B)** High power view of cells involved in a calcium flash. Three cells dramatically increase calcium levels between t= :00 and t= :20. By t= 1:00 calcium levels are decreasing and return to baseline by t= 1:20. **b')** The plot of DF/F_0 for each of the areas shown in the colored boxes in the last panel show that the rise in calcium levels does not propagate to other cells in the frame. **MOVIE 6)** This movie shows the calcium flash in Fig. 4-4, B.

A

:00 2:00 4:00 4:40 5:00 5:20 5:40 6:00 7:20 8:00 10:00 10:40 12:00 13:20

a'

ΔF/F0

:00 3:00 6:00

B

:00 :20 :40 1:00 1:20

b'

ΔF/F0

:00 1:00 2:00

reach values of only about 1 (Fig 4-3, a'). Though also variable, local changes in calcium levels were of similar amplitude in both waves and flashes (Fig. 4-4, a', b'). It is important to note that variation in the intracellular concentration of calcium green may account for some variation in wave amplitude, due to leakage of the reagent from the injection wound.

We also performed time-lapse observations on explanted ventral marginal zone and explanted animal cap ectoderm. The ventral marginal zone undergoes only very weak convergent extension [13] and the animal cap undergoes epiboly, a very different morphogenetic process [30].   No intercellular calcium waves were observed in either tissue (Fig. 4-5; MOVIE 7). These data correlate propagating calcium waves with the robust convergent extension cell movements of the dorsal marginal zone.

In many systems, intercellular calcium waves arise in response to mechanical stimulation or wounding [28]. In contrast, calcium waves in the *Xenopus* DMZ arose spontaneously, and several lines of evidence demonstrate that the observed waves in the *Xenopus* DMZ did not arise as a result of the surgical manipulation of the tissue. Most importantly, no calcium waves were observed in explanted ventral marginal zones or animal caps (Fig. 4-5). Likewise, some DMZ explants displayed no calcium waves (Fig. 4-5). Furthermore, waves observed in DMZ explants did not initiate preferentially at the cut edges of the explant, but more frequently arose in the middle of the tissue (MOVIES 1 & 2). Finally, the occurrence of similar calcium waves in the marginal zone of intact, gastrulating zebrafish embryos [26] and in the neural

FIGURE 4-5 AND MOVIES 7 & 8:

Frequency of calcium waves. The graph plots waves/hour for different groups of explants; each point represents a single filmed explant. Calcium waves arise in DMZs at an average rate of 0.71/hour (see MOVIES 1-4). No calcium waves were observed in explanted animal caps (see MOVIE 7, described below) or in ventral marginal zones (VMZ). Calcium waves were not inhibited by expression of Nxfz-8, though the frequency was reduced. Treatment with thapsigargin abolished calcium wave activity (see MOVIE 8, described below). ** Indicates difference from wild-type is statistically significant to $p < 0.05$ by the Mann-Whitney U test. **MOVIE 7)** This movie shows a representative time-lapse of an animal cap explant labeled with calcium green dextran. Small calcium flashes can be seen, but no calcium waves arise. **MOVIE 8)** This movie shows a representative DMZ treated with thapsigargin, no calcium waves are observed.
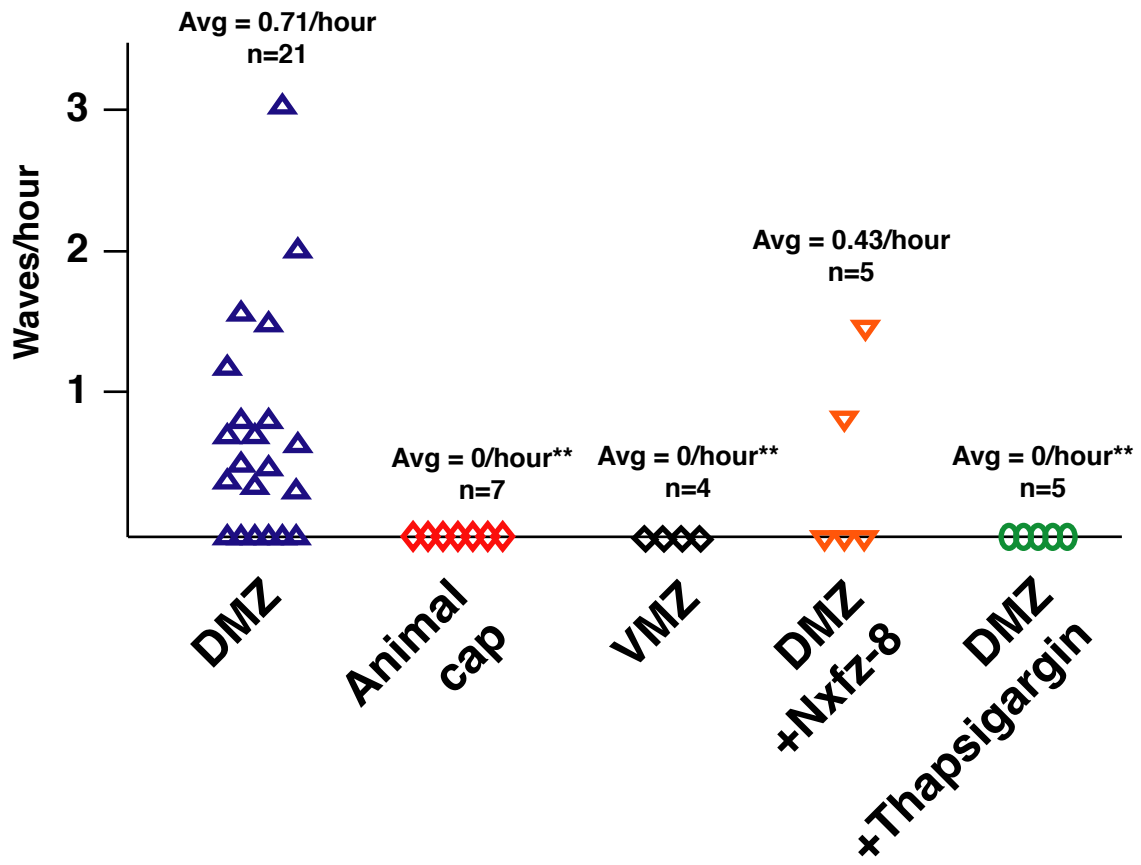
plates of intact *Xenopus* embryos [29] argues that these waves are part of normal development.

Calcium waves in the DMZ do not require Frizzled signaling

Non-canonical Wnt signals play a critical role in coordinating convergent extension. One non-canonical pathway signals via PKC to elicit calcium release [25, 31], raising the possibility that the observed calcium waves may require Wnt signals. To examine this possibility, we observed calcium dynamics in explants where Wnt signaling was compromised by expression of mutant Frizzled-8. An N-terminal fragment of *Xenopus* Frizzled-8 (Nxfz-8) inhibits both canonical and non-canonical Wnt signals and strongly suppresses convergent extension [20, 24]. Co-injection of 1 ng of Nxfz-8 mRNA did not inhibit calcium wave initiation or propagation, though the frequency was slightly diminished (Fig. 4-5).

Calcium waves in the DMZ require calcium from intracellular stores

Intercellular calcium waves in other systems often mobilize calcium from intracellular stores [28]. To determine the source of the released calcium in intercellular waves in the *Xenopus* DMZ, we treated DMZ explants with thapsigargin. Thapsigargin is a cell-permeable inhibitor of the calcium ATPase of the endoplasmic reticulum (ER) and effectively prevents regulated calcium release from internal stores [32-34]. Treatment of DMZ explants with 2 micromolar thapsigargin completely abolished the intercellular calcium waves

(Fig. 4-5, MOVIE 8) and dramatically suppressed calcium flashes (MOVIE 8), indicating that internal stores of calcium are required for these events.

Calcium release from intracellular stores is required during gastrulation for convergent extension, but not for cell-fate specification in the dorsal mesoderm

In the time-lapse observations of explants treated with thapsigargin (MOVIE 8), individual cells can be seen moving, demonstrating that thapsigargin did not completely suppress cell motility. Because thapsigargin effectively inhibited intercellular calcium dynamics (Fig. 4-5, MOVIE 8), it provides an effective tool with which to examine the possibility that calcium signaling plays a role in the coordination of convergent extension.

Convergent extension is a driving force in the closure of the blastopore during gastrulation [8, 11], and embryos exposed to thapsigargin during gastrulation fail to close their blastopores by stage 12 (Fig. 4-6). By this stage, control embryos displayed long, narrow notochords visible by *in situ* hybridization to the notochord-specific probe Xnot [35, 36] (Fig. 4-6, A, C). In embryos treated with thapsigargin, convergent extension was severely suppressed, and notochords remained short and broad (Fig. 4-6, B, G). In a few embryos, weaker suppression was observed (Fig. 4-6, B, far right). Treatment with thapsigargin did not change the total area of the Xnot expression domain (<3% difference), indicating that cell fate specification in the notochord was not inhibited.

FIGURE 4-6:

Thapsigargin treatment inhibits convergent extension but does not affect mesodermal cell fate. **A)** Control embryos at stage 12 have almost finished blastopore closure (dorsal view is shown, anterior at top). Xnot staining reveals long, narrow notochords. **B)** Embryos treated with thapsigargin fail to close their blastopores by stage 12. Xnot staining indicates that notochords have failed to converge and extend. In a few cases (far right embryo), some convergent extension occurs and the anterior notochord elongates, though the posterior notochord remains broad. **C)** Control embryos stained for Xnot. **D)** Embryos treated with BHQ fail to close their blastopores and notochords do not converge and extend, though Xnot is expressed strongly. **E)** Control embryos hybridized to MyoD; somites are elongated along each side of the notochord. **F)** Thapsigargin treated embryos express MyoD at normal levels, though somites fail to converge and elongate; MyoD expression domains remain short and broad. **G)** Quantitation of convergent extension by measurement of the length-to-width ratio (mean +/- standard error) of Xnot expression domains in control and thapsigargin-treated embryos. Total area of the Xnot expression domains differed by less than 3% between control and experimental embryos **H)** Length-to-width ratios of Xnot expression domains in control and BHQ-treated embryos. Slight differences in stages account for differences in control LWRs in THAP and BHQ experiments (see panels A and C, above)
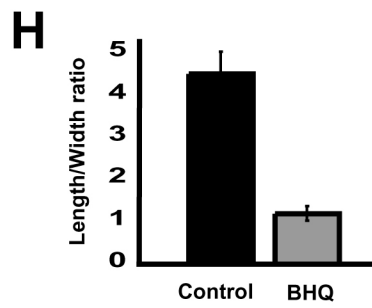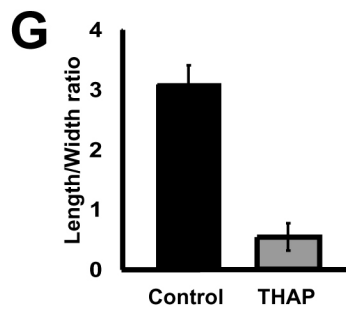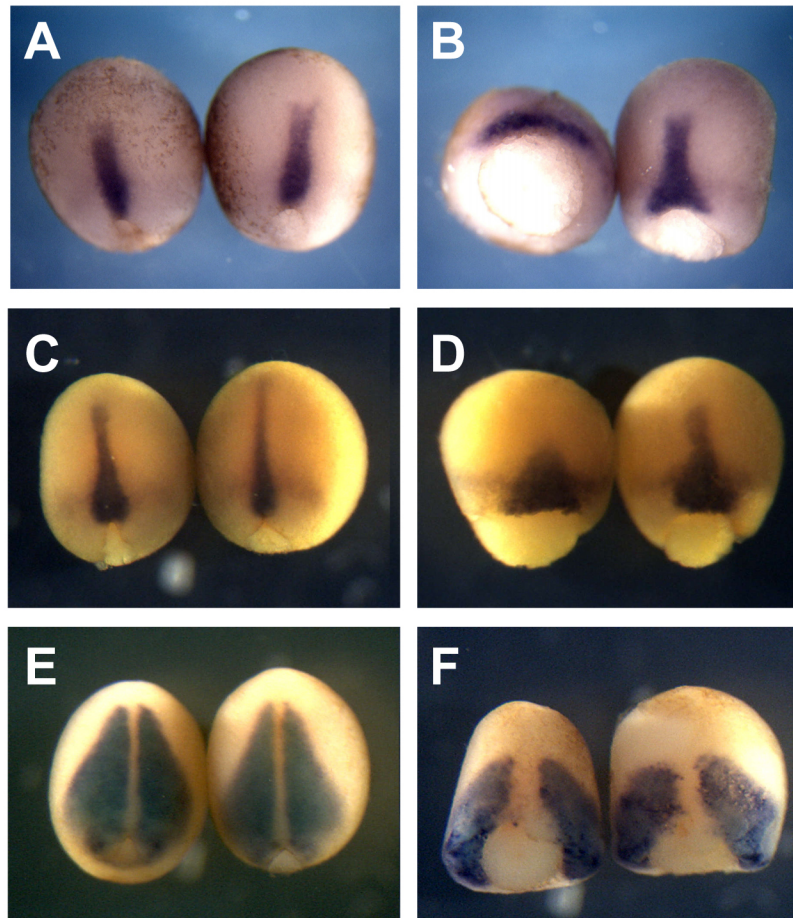
A different pharmacological inhibitor of the ER calcium ATPase, 2,5-di-(t-butyl)-1,4-benzohydroquinione (BHQ) [34], also suppressed convergent extension of the presumptive notochord without inhibiting expression of Xnot (Fig. 4-6, D, H). This result demonstrates that inhibition of convergent extension was not specific to thapsigargin, but is a common effect of depletion of calcium stores.

Convergent extension is most pronounced in the notochord, but also occurs in ventrolateral mesoderm such as somites [13], and identical cell behaviors are associated with convergent extension in both tissues [12]. At late gastrula stages, *in situ* hybridization to the muscle-specific marker MyoD confirmed that elongated arrays of somites flanked the notochord (Fig. 4-6, E). In thapsigargin-treated embryos, the somites remained short and broad, due to failure of convergent extension, though MyoD was expressed normally (Fig. 4-6, F).

We next examined the effects of thapsigargin on DMZ explants, which elongate *in vitro* as a result of convergent extension, accurately mimicking their morphogenetic behavior in intact embryos. These explants allow convergent extension to be assessed independently of other morphogenetic movements occurring in the embryo [8, 15]. In contrast to the dramatic elongation of control explants (Fig. 4-7 A, D), thapsigargin treated explants were strongly inhibited in elongation (Fig. 4-7, B, D). The shape of thapsigargin treated explants was variable, with some extending subtly; however, the difference in the mean

Figure 4-7:

Thapsigargin inhibits convergent extension of DMZ explants without affecting gene expression. **A)** Untreated control DMZ explants (n= 31) elongate significantly and change shape as a result of convergent extension, forming a rounded head and an elongate tail. **B)** DMZ explants treated with thapsigargin (2 mM) fail to elongate, though subtle narrowing of the mesoderm is sometimes observed (n= 33). This variability is consistent with that of treated whole embryos. **C)** BHQ treatment (10 mM) suppresses convergent extension (n=18). **D)** Quantitation of convergent extension of DMZ explants (mean LWR +/- standard error). **E)** RT-PCR demonstrates that thapsigargin treatment does not affect dorsoventral patterning of the mesoderm in DMZ explants cultured to stage 22. **F)** Thapsigargin does not affect dorsal cell fate specification in DMZ explants cultured to stage 12. (THAP = 10 thapsigargin-treated DMZs; CTL= 10 untreated DMZ explants; -RT = no reverse transcriptase control). EF1a and actin serve as loading controls.
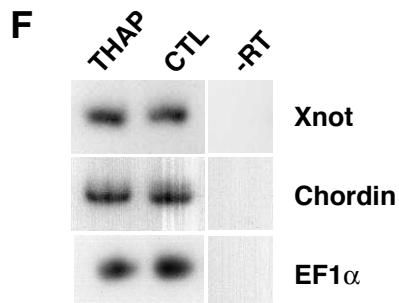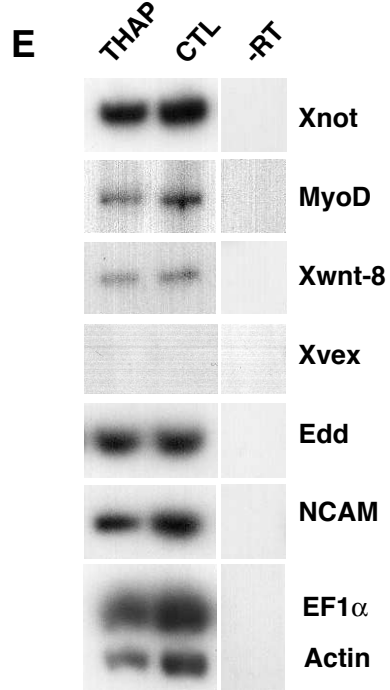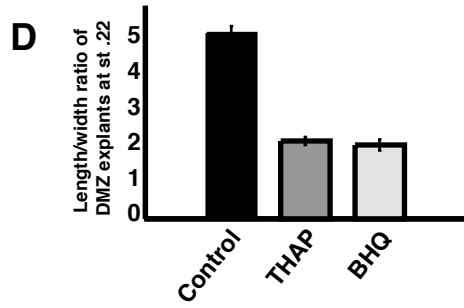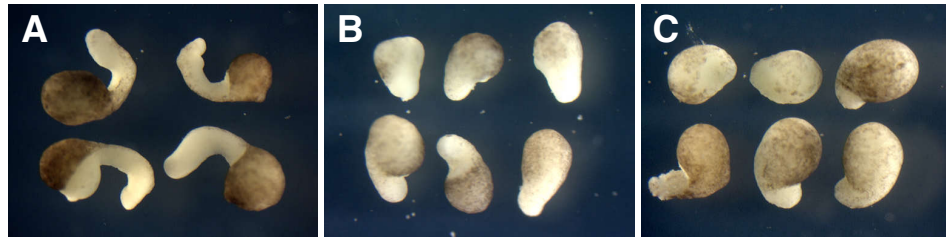
elongation of the two groups was highly significant (Fig. 4-7 D). BHQ also suppressed convergent extension in DMZ explants (Fig. 7, C, D).

Dorsoventral patterning of the mesoderm influences convergent extension [13], and calcium signaling has been implicated in the specification of cell fates in the *Xenopus* mesoderm prior to the onset of gastrulation [37-39]. As such, it is possible that thapsigargin treatment during gastrulation affected convergent extension indirectly by modulating cell fates in the DMZ. The strong expression of both Xnot and MyoD in thapsigargin- and BHQ-treated embryos (Fig. 4-6) argues against this possibility. RT-PCR analysis also demonstrated that thapsigargin treatment during gastrula stages did not alter cell fates in DMZ explants.

Treated DMZs cultured to tailbud stages expressed normal levels of the dorsal mesoderm marker Xnot, as well as the ventrolateral mesoderm markers, MyoD and Xwnt-8 (Fig. 4-7, E). Likewise, neither treated nor untreated explants expressed the ventral mesoderm marker Xvex (Fig. 4-7, E). Thapsigargin-treated DMZ explants cultured only to the late gastrula stages expressed normal levels of both Xnot and the dorsal mesoderm marker chordin (Fig. 4-7, F), demonstrating that thapsigargin did not transiently ventralize DMZs. Finally, the failure of convergent extension did not result from conversion of mesoderm to endoderm or neural ectoderm, as no increase in expression of NCAM or endodermin (Edd) was observed following thapsigargin treatment (Fig. 4-7, E). Together, these data strongly indicate that suppression of convergent extension following depletion of ER calcium stores is not

secondary to changes in mesodermal cell fate but is instead due to a direct effect on coordinated morphogenetic cell movements.

Discussion

Using an improved confocal system (Fig. 4-1), we have described intercellular calcium waves which occur during convergent extension in explants of the *Xenopus* dorsal marginal zone (Figs. 4-2, 4-3, 4-4). Calcium waves are correlated with convergent extension movements, and waves were not observed in explanted animal cap ectoderm or ventral marginal zone mesoderm (Fig. 4-5). These waves did not strictly require Frizzled signaling, indicating that they may function in parallel to Wnt signals in the control of morphogenesis.

Previous studies have implicated calcium signaling in the specification of ventral cell fates prior to gastrulation [37-39]. In this study we have used cell-permeable inhibitors of calcium release from the ER to examine the role of calcium signaling during gastrula stages. Inhibition of calcium dynamics with either thapsigargin (Fig. 4-5) or BHQ inhibited convergent extension but did not affect cell fate (Fig. 4-6, 4-7). Together, these data indicate an important role for calcium signaling in the control of convergent extension.

Variability of calcium events

The variability in frequency, extent, and amplitude of calcium dynamics in the *Xenopus* DMZ (Figs. 4-2, 4-3, 4-5) is notable, especially in light of the

variable effects of thapsigargin and BHQ on convergent extension (Figs. 4-6, 4-7). It is possible that this variability was due to a failure to capture episodic calcium events consistently or was a result of experimental manipulation. On the other hand, as calcium is but one of several potential regulators of convergent extension, the variability may indicate that this mechanism is not used to the same degree in each embryo. Intercellular calcium signaling may represent a tuning mechanism to ensure that convergent extension is reliably carried out in every embryo.

Mechanism of calcium wave propagation

The calcium waves in the DMZ use calcium from intracellular stores (Fig. 4-5), but the mechanism by which these waves propagate remains unclear. In other systems, the stimulus for calcium release from intracellular stores is propagated from cell to cell by a variety of mechanisms [28]. Some calcium waves propagate by release of ATP into the extracellular space, thereby activating purinergic receptors on neighboring cells, resulting in calcium release [40]. Other waves propagate by movement of IP_3 through gap junctions, which in turn stimulates calcium release in adjacent cells [41]. Interestingly, extracellularly-transduced calcium waves can propagate at rates comparable to those which propagate through gap junctions [42]. Previous examinations of the effects of gap-junction blocking agents on *Xenopus* development have not revealed specific defects in convergent extension [43, 44], which may suggest an extracellular route of propagation for the waves

observed in *Xenopus* embryos. On the other hand, many reagents commonly used to antagonize gap-junctional communication fail to eliminate gap-junction dependent intercellular calcium waves [45]. Finally, the observation of calcium waves in explants expressing Nxfz-8 argues that the initiation and propagation of these waves does not strictly require Frizzled signaling. It is important to note, however, that this does not preclude the possibility that the Wnt/Ca^{++} pathway functions to regulate convergent extension. Examinations of cell behaviors in embryos disrupted for Wnt/Ca^{++} function and in embryos treated with thapsigargin should shed light on this issue.

Calcium waves are a common feature of vertebrate convergent extension

Intercellular calcium waves have now been observed in the marginal zone of the zebrafish embryo [26], in the *Xenopus* neural plate [29], and in the *Xenopus* dorsal marginal zone (this report). All three of these tissues undergo convergent extension movements [6, 13] which involve grossly similar cell behaviors [12, 46, 47], and the velocity of calcium waves seen in each of these tissues is also roughly similar. Together, these findings suggest that calcium waves are a common characteristic of vertebrate convergent extension. Likewise, similar molecular mechanisms are involved in the control of convergent extension cell movements in all three of these tissues. For example, non-canonical Wnt signaling has been shown to be required for convergent extension of each of these tissues [14, 15, 23, 48, 49]. We have recently shown that non-canonical Wnt signals control the stability and polarity

of lamellipodia which drive convergent extension in *Xenopus* mesoderm [15]. It is tempting to speculate that calcium signaling may serve as an additional input into the machinery which coordinates cell polarity during convergent extension. In light of the role which intracellular calcium signals play in guiding migrations of single cells [50-52], we suggest that intercellular calcium waves may likewise provide a mechanism by which large groups of cells can coordinate complex morphogenetic movements during embryogenesis.

Materials and Methods

Embryos and microinjection: Female adult *Xenopus laevis* were ovulated by injection of human chorionic gonadotropin; eggs were fertilized *in vitro*, dejellied in 3% cysteine (pH 7.9) and subsequently reared in 1/3x MMR [53]. For microinjections, embryos were placed in a solution of 2.5% ficoll in 1/3x MMR, injected as described, and reared in ficoll + 1/3xMMR [53]. Calcium green dextran (10kD; Molecular Probes) was resuspended to 10mg/ml in sterile water and 5-10 nl was injected.

Imaging of calcium dynamics: For DMZ imaging, embryos were injected dorsally at the 4-cell stage and reared to gastrula stages as described above. At stage 10.25, 60-80° dorsal marginal zone explants were cut, centered on the midline of the dorsal blastopore lip. Eyebrow knives and forceps were used for dissections. Care was taken to remove involuted mesoderm, head

mesoderm, and endoderm. Explants were then placed with deep cells facing down in a culture chamber with a bottom made of coverglass for imaging with the inverted Zeiss 410 confocal modified as described in the text. A small fragment of coverglass supported by silicon grease or clay was used to hold the explant in place and prevent curling. For animal cap imaging, embryos were injected into the animal pole of 1-cell embryos and cultured until stage 9 in 1/3x MMR. Animal caps were removed using forceps at stage 9 and imaged as above. For VMZ imaging, embryos were injected ventrally at the 4-cell stage and reared to gastrula stage as described above. At stage 10.5, 60-80° ventral marginal zone explants were removed and imaged as DMZs, above. All explants were cut and reared in 1x Steinberg's solution [53]. All explants were imaged using eight-second scans every 20 seconds. Images were processed and DF/F_0 measured using NIH Image 1.62/fat.

Nxfz-8 expression: mRNA was prepared *in vitro* as described [53], mixed with calcium green dextran, and injected as above. Embryos from each injected batch were reared to tailbud stages to assess phenotypes and confirm that Nxfz-8 mRNA was functional.

Thapsigargin and BHQ treatments: Thapsigargin (Sigma) and BHQ (CalBiochem) were resuspended as 1000X stocks (2mM and 10mM, respectively) in ethanol and stored at -20° C. These doses were chosen

because both have been used effectively to inhibit calcium-regulated guidance of axons in culture [52].

For DMZ imaging, explants were cut as above and then transferred to a culture chamber containing 2 micromolar thapsigargin in 1x Steinberg's. Explants were cultured for approximately ten minutes before coverslipping to allow penetration of the thapsigargin, during which time explants were manually prevented from curling. Explants were then coverslipped in the same culture chamber allowing chronic exposure to thapsigargin under the coverslip during imaging.

For embryo treatments, vitelline envelopes were removed at stage 10+ and embryos were cultured in agarose wells in 2 micromolar thapsigargin or 10 micromolar BHQ in 1/3x MMR until stage 12, fixed in MEMFA, and processed for *in situ* hybridization [53]. Convergent extension of the Xnot expression domain was quantitated by measuring the length along the dorsal midline and the width along the blastopore lip.

For assessment of convergent extension, DMZ explants were prepared as above, but without coverslips. They were cultured in agarose wells in 1x Steinberg's plus 2$_m$M thapsigargin until stage 12, then rinsed and cultured in 1x Steinberg's alone or cultured continuously in 10 micromolar BHQ until scoring. At control stage 22, convergent extension was quantitated in DMZ explants by measuring the length of the longest aspect and width at the collar point where the mesoderm extends from the neural ectoderm. Measurements

were performed using NIH Image 1.62/fat. RT-PCR was performed as described [53].

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