REGULATION OF ANTERIOR-POSTERIOR AXIS PATTERNING AND CONVERGENT EXTENSION BY AQUAPORIN-3B DURING *XENOPUS* GASTRULATION.

by

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ABSTRACT

Convergent extension (CE) is a key morphogenetic process during gastrulation that drives anterior-posterior axis elongation through coordinated cell behaviors and signaling pathways, including noncanonical Wnt signaling. Our studies have revealed that the aquaglyceroporin *aqp3b* (*aqp3L*) gene plays a role in *Xenopus* gastrulation. Using morpholino oligonucleotides to inhibit *aqp3b* translation, we observed CE defects in Keller explants, which were rescued by Dvl, PKC and RhoA, but not Rac1 or Cdc42, implicating Aqp3b in noncanonical Wnt signaling. Rac1 and Cdc42 have been shown to promote cell migration in dorsal mesoderm cells. Their failure to rescue the absence of *aqp3b* suggested that Aqp3b may not promote active cell migration. Inhibition of *aqp3b* also impaired PKC localization to the cell membrane in animal caps. Other studies have shown that PKC can activate Cdc42 downstream of Dvl-independent Wnt/Ca2+ signaling, which promotes CE. Since PKC, but not Cdc42 rescued *aqp3b* morphants, Aqp3b may instead act upstream of Dsh-dependent PKC activation. Dorsal mesodermal cells in *Xenopus* embryos during gastrulation exhibit two distinct cell behavior patterns: convergent extension and cell migration in activin-treated animal caps. Aqp3b promotes tissue elongation: a hallmark for CE, while inhibition reduces elongation. A consequence of *aqp3b* misexpression revealed the downregulation of anterior markers (*goosecoid*, *otx2*, *noggin*) and upregulation of posterior markers (*Xbra*, *hoxB9*). Anterior genes promote cell migration rather than CE. Thus, these data are consistent with the rescue experiments and suggest that Aqp3b may inhibit active migration of posterior dorsal mesoderm cells. Finally, *aqp3b* induced FGF2 and eFGF expression in activin-treated animal caps. Studies have shown activation of *Xbra* and other posterior genes by FGF signaling. Thus, Aqp3b may modulate the FGF/Xbra signaling in promoting convergent extension. These findings suggest that Aqp3b may promote CE by antagonizing cell migration.

CHAPTER ONE

BACKGROUND

Xenopus laevis as a Model for Studying Gastrulation

Xenopus laevis, also known as African clawed frog, is an excellent model organism for studying embryonic development. Hormonal priming induces female *Xenopus* egg laying. These eggs are then fertilized artificially by the addition of *Xenopus* male sperm from the testes. Their eggs are large in quantity and size, with a diameter of 1.2 mm (Blum and Ott, 2018). Their accessibility from the zygotic stage (Figure 1) to adult stage makes chemical and physical manipulation easy and reproducible. Manipulations, such as microinjections and dissections can be easily done for analysis of gene function and expression patterns, individual cell behavior, and protein localization. The Nieuwkoop and Faber (NF) staging system (Figure 1) is widely used to describe the various developmental stages, enabling experimental reproducibility (Zahn et al., 2022). The conservation of cellular and molecular mechanisms, including gene conservation between *Xenopus* and other organisms, including humans, makes it a valuable model for biomedical research.

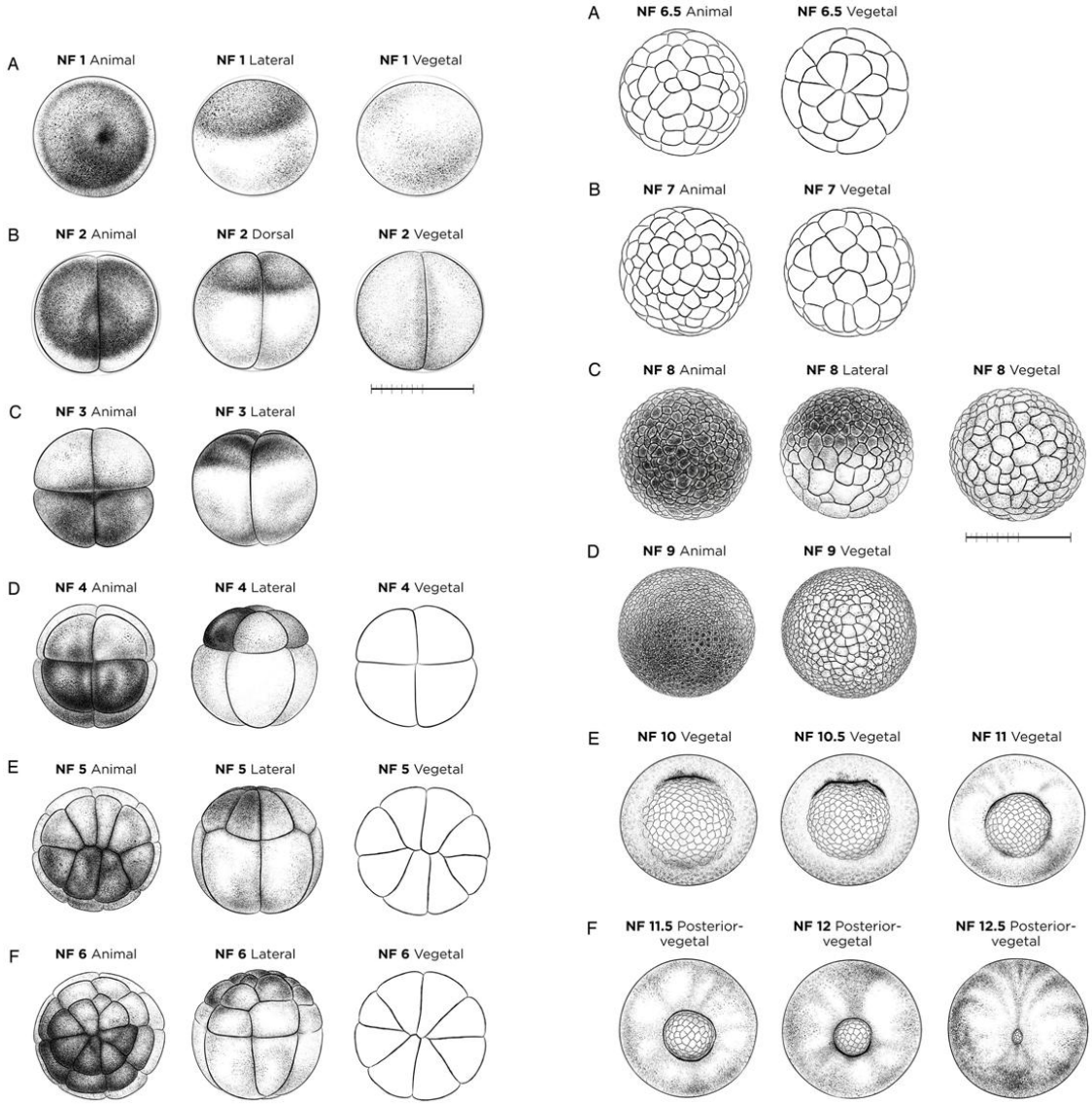


Figure 1. Developmental stages in *Xenopus laevis* embryos. Stages of *Xenopus laevis* development based on NF staging. NF stages 1-6: early cleavage, NF stages 7-9: blastula, NF stages 10-12: gastrula (Zahn et al., 2022).

Gastrulation in *Xenopus laevis*

Gastrulation is a critical phase in embryonic development and fundamental to the establishment of the basic body plan of an organism. This process leads to the formation of the three germ layers, which include the ectoderm, endoderm, and mesoderm, as well as the organization of these tissues. The ectoderm spreads over the embryo as mesoderm and endoderm internalize, with the mesoderm becoming positioned between ectoderm and endoderm. Cells from the inner layer of the vegetal pole give rise to the endoderm and deep marginal layers give rise to the mesoderm (Figure 2A).

At stage 10, gastrulation starts with the formation of bottle cells through apical constriction. These cells reduce their apical surface area, forming the dorsal lip of the blastopore (Shook et al., 2018). The formation of the dorsal lip of the blastopore in the dorsal marginal zone is followed by involution of the prospective head endoderm and mesoderm. The prechordal mesoderm migrates inward at the dorsal lip of the blastopore and travels towards the animal pole. The chordamesoderm follows through the movements of convergent extension (Figure 2A).

These coordinated movements collectively function to decrease the circumference of the blastopore. As the dorsal mesoderm cells move inward, the blastopore closes eccentrically, internalizing all endodermal precursors, and the ectoderm spreads to encircle the entire embryo, a process known as epiboly.

At the end of gastrulation, the ectoderm will give rise to the nervous system and epidermis; the mesoderm develops into connective tissues, blood, heart, kidneys, muscles, bones; and the endoderm will form the lining of the digestive tract, pancreas, and liver.

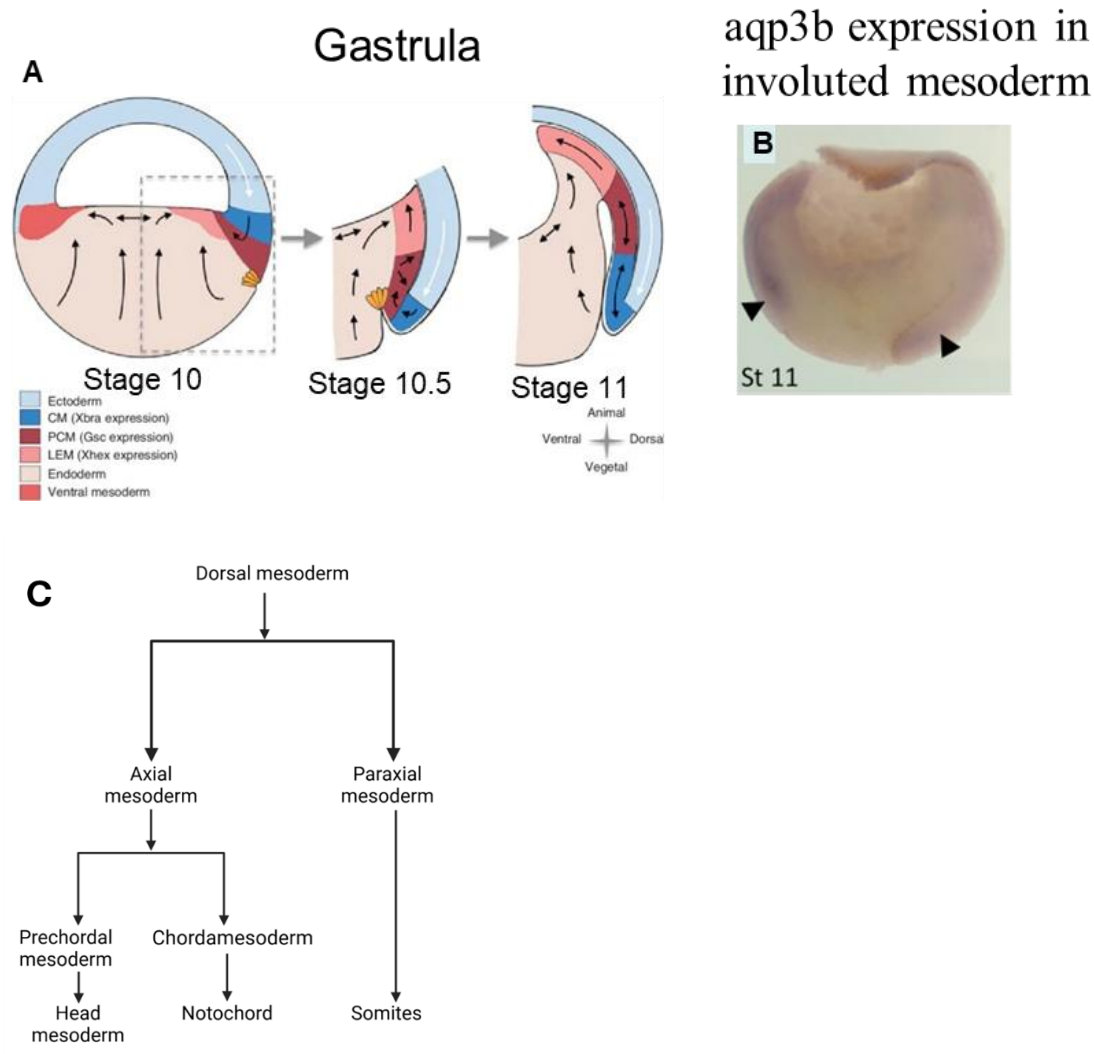


Figure 2. *aqp3b* is expressed in the involuting dorsal mesoderm. (A) Onset of gastrulation and initial formation of the dorsal lip of the blastopore (DLB; stage 10). The dorsal marginal zone cells involute and migrate (mid-gastrula, stage 10.5), giving rise to the dorsal mesoderm (dark red and dark blue). Anterior prechordal (head) mesoderm migrates actively, while more posterior chordamesoderm undergo convergent extension to form the notochord (dark blue; stage 11). Ectoderm (outermost layer of the gastrula) spreads and covers the surface of the embryo through epiboly. CM (chordamesoderm), PCM (prechordal mesoderm), LEM (leading edge mesendoderm) (Huang and Winklbauer 2018). (B) *aqp3b* is expressed in the involuting dorsal (right arrowhead) and ventral (left arrowhead) mesoderm (Forecki et al., 2018). (C) The dorsal mesoderm is specified into axial and paraxial mesoderm. Axial mesoderm consists of the prechordal and chordamesoderm giving rise to head mesoderm (by cell migration) and notochord (by convergent extension), respectively. Paraxial mesoderm gives rise to the somites.

Convergent extension

Convergent extension (CE) is a fundamental morphogenetic process that drives the movement and rearrangement of cells at several stages of embryonic development. During gastrulation, this process involves the mediolateral intercalation of adjacent cells in the axial (dorsal) mesoderm (Figure 2C), resulting in elongation and narrowing of the tissue. During CE, adjacent cells polarize and elongate along their mediolateral axis, forming a spindle-shape. These polarized cells then intercalate perpendicularly to the anterior-posterior (AP) axis, which narrows and lengthens the tissue (Figure 3) (Sutherland et al., 2020). CE occurs primarily in the dorsal mesoderm (Figure 2A), driving the narrowing and lengthening of the dorsal involuting marginal zone (Figure 3). CE drives mediolateral intercalation of dorsal mesoderm cells to form the presumptive notochord (Figure 2C).

The dorsal mesoderm consists of the axial and paraxial mesoderm (Figure 2C). The prospective head mesoderm (prechordal mesoderm) leads involution forming the anterior axial mesoderm, with these cells migrating actively towards the animal pole by crawling on the inner surface of the blastocoel roof without mediolateral intercalation. This anterior mesoderm does not undergo CE (Figure 2A). It undergoes directed migration guided by cues associated with the blastocoel roof, including extracellular matrix components such as fibronectin, as well as signaling molecules like PDGF-A (Platelet-Derived growth factor-A). The posterior axial mesoderm (chordamesoderm) follows by showing active CE, driven by mediolateral intercalation (Figure 2A) (Keller, 1976). The paraxial mesoderm later forms the somite (Figure 2C).

To change shape, chordamesoderm cells exhibit rapid and randomly directed protrusive activity as they progressively involute following the head mesoderm. The cells' ability to protrude slows down on its anterior and posterior ends (Figure 3B, 4), during the mid-gastrula stage of gastrulation, limiting it to the medial and lateral ends (Figure 3C, 4) (Shih and Keller et al, 1992). The chordamesoderm cells adopt a bipolar morphology, promoting the mediolateral intercalation of adjacent cells and thereby facilitating convergent extension (Figures 3C, D and 4), (Shih and Keller, 1992, Keller and Danilchik, 1988). Failure of CE can result from disruptions in either the stability or polarity of these protrusions (Wallingford and Harland, 2001).

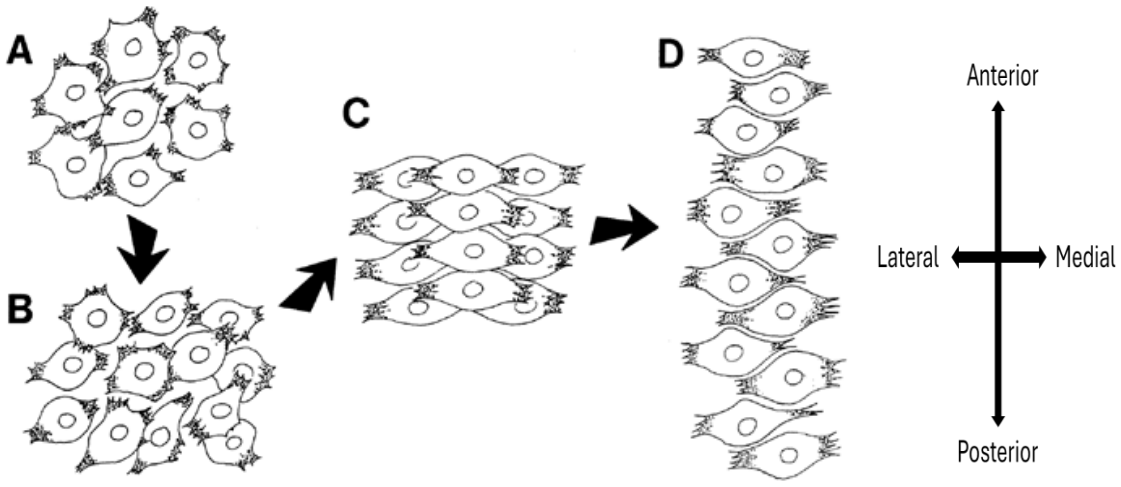


Figure 3. Convergent extension movements in *Xenopus laevis.* (A) At the beginning of gastrulation (stage 10), dorsal mesodermal cells exhibit rapid, randomly protrusive activity. (B, C) In the early midgastrula (stage 10.25-10.5), chordamesoderm cells undergo a progressive transition, limiting protrusive activity to the medial and lateral sides, elongating mediolaterally, and aligning parallel to each other. (C) Cells stabilize and polarize, exhibiting a reduction in protrusive activity. (D) When mesodermal cells intercalate along the mediolateral axis, they exert traction on adjacent cells producing convergence and extension movements (Shih and Keller, 1992).

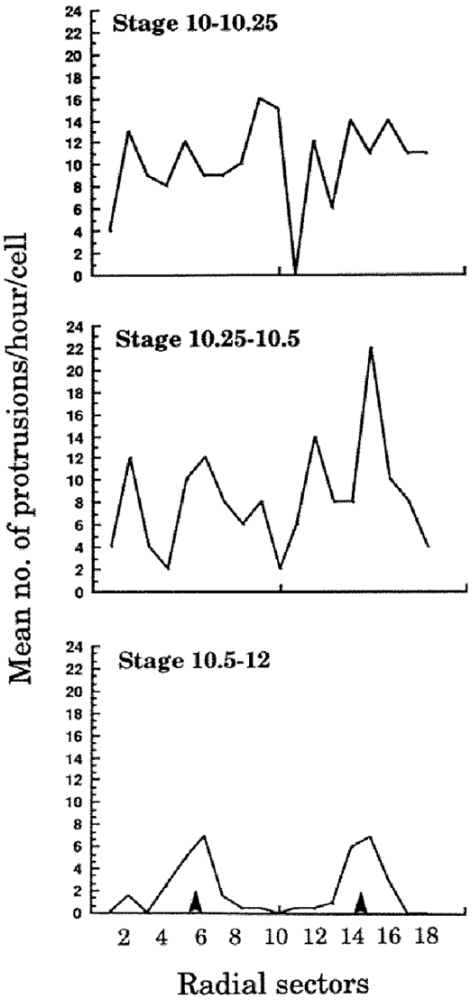


Figure 4. Protrusive activity during *Xenopus* gastrulation. At stage 10-10.25, cells show random and unstable protrusive activity. At stage 10.25-10.5, protrusions show initial bias towards medial and lateral ends (sectors 5.5 and 14.5). At the onset of convergent extension (stage 10.5), protrusive activity is only found at the medial and lateral ends of the cells (Shih and Keller, 1992).

Polarized protein localization during convergent extension

Polarized protein localization during convergent extension is an essential process that involves planar cell polarity (PCP) signaling and the actin cytoskeleton. During convergent extension, cells establish polarity in response to signals from neighboring cells (Irvine and Wieschaus, 1994).

PCP is a geometric property of multicellular tissues that enables the coordination of cellular behaviors within the plane of a sheet of cells, perpendicular to the apical-basal axis of these cells. Core PCP genes were initially identified in gain- and loss-of-function analyses in the genetic regulation of wing hair and body bristle orientation in Drosophila (Gubb and Garcia-Bellido, 1982). In vertebrates, the core PCP proteins include transmembrane proteins such as Vangl, Celsr and Frizzled, and cytoplasmic proteins including Disheveled and Prickle (Figure 5). Downstream of these proteins are the Rho family of GTPases, JNK, Daam1, and Rho kinase.

Prickle and Vangl proteins localize to the anterior or proximal side of the cell, while Disheveled and Frizzled localize to the posterior or distal side, forming antagonistic complexes that establish and maintain cellular polarity (Tree et al., 2002; Bastock et al., 2003) (Figure 5). Deletion of any of the core PCP components results in a loss of asymmetry of the others. Disruption of *prickle* and *vangl* led to shorter body axis of the Zebrafish and *Xenopus* embryo (Tada and Kai, 2009). Disheveled was reported to lack asymmetric localization during early stages of convergent extension in *Xenopus*, but it becomes mediolaterally localized at later stages (Jiang et al., 2005). In *Xenopus*, Wnt11(Tada and smith, 2000) and Wnt5 (Moon et al., 1993), ligands of Frizzled, are required for convergent extension. Dominant interfering versions of these ligands inhibit convergent extension and are rescued by Disheveled.

Mediolateral intercalation of mesodermal cells during *Xenopus* convergent extension is driven by mediolaterally polarized protrusive activity. This behavior progresses from anterior to posterior, with cells at tissue boundaries transitioning from bipolar to monopolar protrusive activity (Shih and Keller, 1992). Asymmetric protein localization plays an important role in regulating these cell shape changes and motility patterns. For instance, fibronectin is deposited at the tissue boundaries during convergent extension, forming an extracellular matrix relevant for mediolateral intercalation and stabilization (Wallingford, 2005; Davidson et al., 2006).

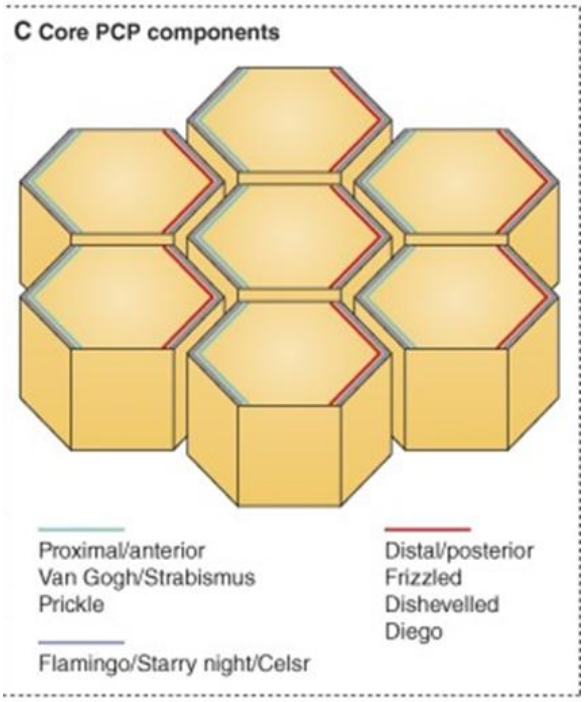


Figure 5. Core planar cell polarity (PCP) components. Core PCP components are asymmetrically localized to the plasma membrane. Frizzled, Dishevelled and Diego are positioned on one edge of the cell (distal/posterior) and on the opposite side (proximal/anterior) lie Van Gogh/Strabismus, Prickle and Flamingo/Starry night/Celsr (Devenport, 2014).

Non-canonical Wnt signaling pathway

Non-canonical Wnt signaling pathway (Figure 6) is a β-catenin-independent pathway that regulates cell polarity, migration and calcium signaling. It comprises the Wnt/PCP pathway and the Wnt/Ca2+ pathway. The Wnt/PCP pathway is activated when Wnt ligands (such as Wnt 5 or Wnt 11) bind to Frizzled receptors, recruiting Disheveled to the plasma membrane. Disheveled contains different domains, of which the PDZ and DEP domains are required for non-canonical Wnt signaling (Sheldahl et al., 2003). Disheveled interacts with Daam1, activating RhoA but not Rac1, which then activates JNK to regulate cytoskeleton rearrangement and cell polarity (Yamanaka et al., 2002). In the Wnt/Ca2+ pathway, Frizzled (Frz) activation by Wnt5 or Wnt11 mediates the activation of heterotrimeric G-proteins, a common feature of all G-protein-coupled receptors (GPCR) (Ahumada et al., 2002). However, what distinguishes the Wnt/Ca2+ pathway is that activated G-proteins specifically lead to an increase in intracellular calcium levels. This increase in calcium ions activates Protein Kinase C (PKC) (Shedahl et al., 2003), which regulates the small GTPase Cdc42 independent of Disheveled (Choi and Han, 2002). (Figure 5).

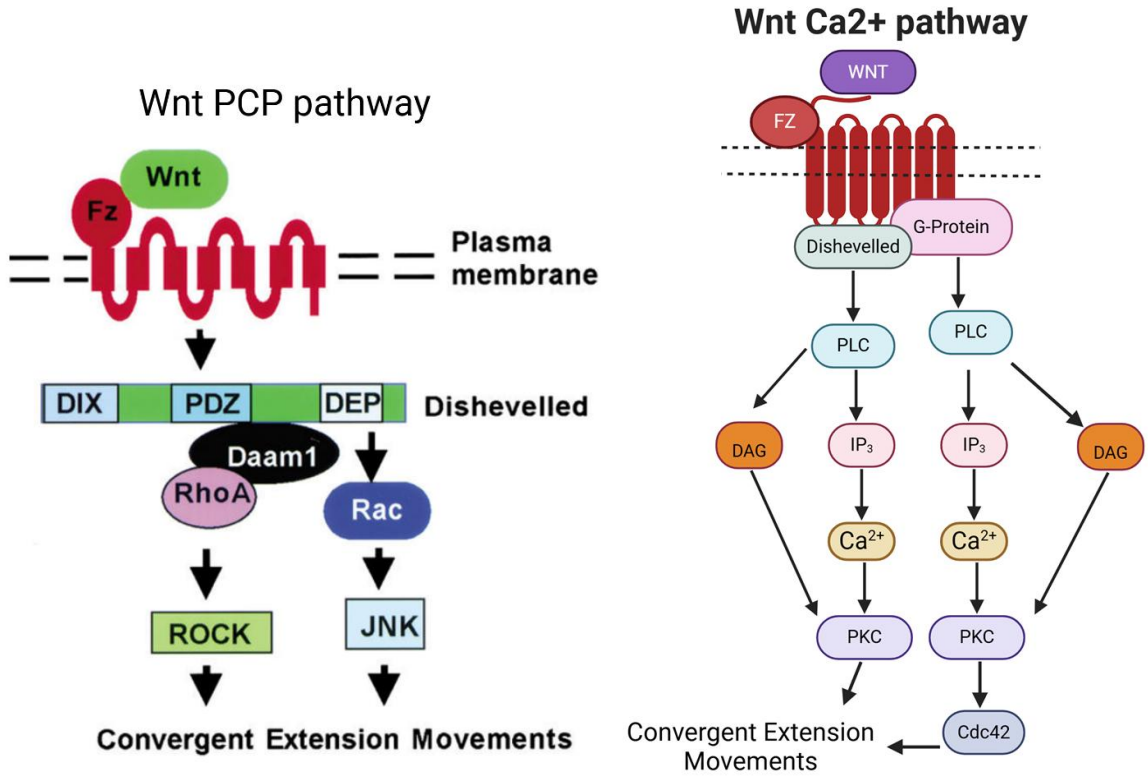


Figure 6. Non-canonical Wnt signaling pathways regulate convergent extension. Wnt ligands bind to the Frizzled receptor, which activates non-canonical Wnt pathways. The Wnt/PCP pathway is mediated by the activation of RhoA and Rac1, downstream of Disheveled (middle two pathways). In the Wnt/Ca2+ pathway, Disheveled activates PKC and Cdc42 activation is independent of Disheveled activity (right pathway) (Habas et al., 2003).

The role of noncanonical Wnt signaling in convergent extension

Overexpression of Wnt5 (Moon et al., 1993) or dominant negative Wnt11 blocks convergent extension movements in *Xenopus* (Tada and Smith, 2000). This led to the discovery that Wnt signaling is involved in regulating convergent extension movements. WNT signaling consists of canonical and non-canonical Wnt pathways (Figure 6). Convergent extension defects could not be rescued by canonical Wnt pathway components, such as β-catenin. However, non-canonical

Wnt signaling plays an important role in regulating the complex cellular behaviors required for convergent extension (Wallingford et al., 2000). It regulates convergent extension through Wnt /PCP (Habas et al., 2003) and Wnt/Ca^{2+} (Kühl et al., 2001) signaling pathways (Figure 5). This pathway uses components such as Wnt11, Wnt5, Frizzled, Disheveled, JNK, PKC, and various small GTPases. Disruption of these components inhibits convergent extension. Disheveled and Frizzled 7 are core components of the PCP pathway (Figure 5) that regulate the adhesion properties and polarization of tissues during gastrulation by activating the non-canonical Wnt signaling pathway (Wallingford et al., 2000, Tada et al., 2002). Disruption of Xdsh (Disheveled) impairs neural tube closure and it is also known to control convergent extension in dorsal mesoderm and posterior neural ectoderm of *Xenopus* (Wallingford and Harland, 2001, 2002). Xdsh mutants disrupt small GTPases regulation, impairing convergent extension (Habas et al., 2003).

Role of small GTPases in convergent extension

In serum-starved Swiss 3T3 fibroblasts, RhoA, Rac1 and Cdc42, members of the Rho family of small GTPases, were originally identified to induce stress fibers, lamellipodia and filopodia respectively (Hall and Nobes, 2000). Stress fibers, which consist of long filamentous actin, regulate cell contractility. RhoA induction of stress fiber involves the activation of ROCK and myosin in stabilizing protrusive activity during convergent extension. Migrating cells form lamellipodia induced by Rac1 at the leading edge, controls directional movements necessary for active migration. Cdc42 triggers filopodia formation, a finger-like extension that explores extracellular environment by detecting chemical and mechanical cues (Settleman, 2001).

Members of the small GTPase family alternate between a GTP-bound active form and a GDP-bound inactive form. In their inactive state, Rho family proteins are sequestered in the cytoplasm in complex by guanine nucleotide dissociation inhibitors (GDIs), which prevent their activation and membrane translocation. Upon stimulation by extracellular signals, guanine nucleotide exchange factors (GEFs) promote the exchange of GDP for GTP, activating the GTPases. Active Rho GTPases translocate to the plasma membrane and initiate signaling cascades that regulate cytoskeletal dynamics. The regulated cycling between the active GTP-bound and inactive GDP-bound states of small GTPases allows them to control processes such as convergent extension, migration, and adhesion.

In Drosophila and *Xenopus*, the small GTPases Rac1, RhoA act downstream of Wnt/Frz/Dsh signaling, which ultimately regulates cytoskeletal rearrangement (Fanto et al., 2000, Muñoz-Descalzo et al., 2007; Habas et al., 2003). Both RhoA and Rac1 are activated through different branches of the non-canonical Wnt pathway downstream of Disheveled (Wallingford et al., 2002, Habas et al., 2003). Disheveled interacts with Damm1 which is important for RhoA activation but not Rac1 (Hayat et al., 2022). RhoA and Rac1 have distinct roles in regulating convergent extension. Rac1 induces both lamellipodia at the leading edge of migrating mesodermal cells, controlling directional movement. RhoA regulates the lifespan of transient protrusions and cell shape changes necessary for mediolateral intercalation during convergent extension. These distinct pathways ensure precise regulation of cytoskeletal dynamics during *Xenopus* convergent extension.

Cdc42 does not act downstream of Disheveled in *Xenopus* gastrulation. Instead, it is activated by PKC via the Wnt/Ca2+ signaling pathway. The activation of Cdc42 controls cell

adhesion, balancing plasticity, and tissue integrity during convergent extension (Choi and Han, 2002).

Aquaporins

Aquaporins (Aqps) are a group of integral membrane channel proteins that facilitate the passive passage of water across cell membranes and, in some cases, other small solutes (Figure 7) (Heymann and Engel, 2000). Aqps play a crucial role in osmoregulation, kidney function, cell migration, skin hydration, cell proliferation, neural signaling, epithelial fluid transport (Kortenoeven and Fenton; Kitchen et al., 2015). They are classified based on their selectivity. The pores of aquaglyceporins are lined with relatively hydrophobic residues, contributing to their ability to allow the passive passage of small solutes besides water molecules. Their pore diameter is generally greater than that of water-selective aquaporins. The channel of water-selective aquaporins is lined with polar residues that enhance their water selectivity (Kitchen et al., 2015). Aquaporins that function as water-selective channels include Aqp1, Aqp2, Aqp4, Aqp5 and Aqp8. The aquaglyceporins are Aqp3, Aqp7 and Aqp9. Aquaglyceroporins also facilitate the passive passage of water and small solutes including glycerol, H202, urea and other metabolites.

Studies have shown that expression of Aqps enhances cell migration with Aqps localizing to the leading edge of migrating cells and increasing lamellipodial dynamics (Hara-Chikuma and Verkman, 2006, Hara-Chikuma and Verkman, 2008; Kitchen et al., 2015). AQP1-null mice show slower endothelial cell migration, resulting in impaired tumor angiogenesis as compared to wild-type cell culture (Saadoun et al., 2005, Papadopoulos et al., 2008). Aqp3-deficient keratinocytes revealed the impairment of p38 MAP kinase signaling activation (Hara-Chikuma and Verkman,

2008). Silencing the aqp3 gene inhibited FGF2-induced cell migration in human breast cancer cell lines. Different varieties of cancer, such as skin and pancreatic cancer, have been reported to exhibit high expression of aqp3, which has been shown to promote cell migration and cell-cell adhesion (Cao et al., 2013, Silva et al., 2022, Silva et al., 2024).

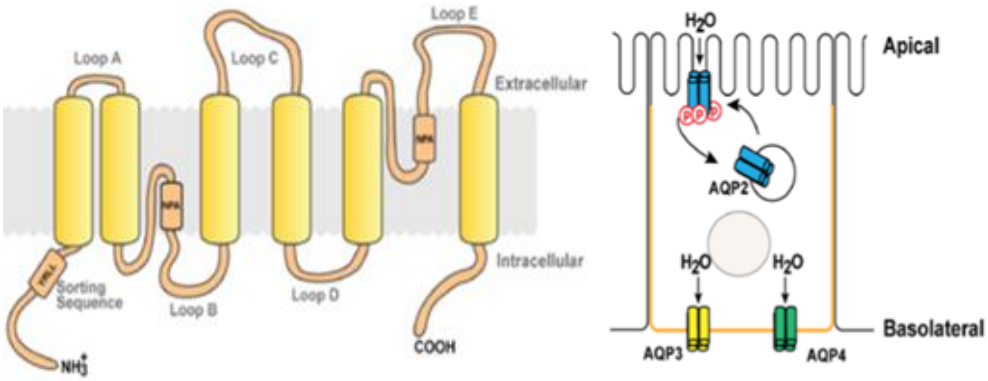


Figure 7. Aquaporins. Aquaporins are water channels in cell membranes. Aquaglyceroporins, such as Aqp3, allow the passage of small polar molecules in addition to water. Aqp3 is expressed in various tissues and found in multiple organs. It is basally localized on the cell membrane in polarized epithelial cells. Aquaporins function as homo-tetramers with each monomer functioning as an independent water or solute channel. The tetrameric organization is important for proper folding and stability.

Role of the aquaporin gene, *aqp3b* (=*aqp3L*) in *Xenopus laevis* embryonic development

The *aqp3b* gene was identified as a direct target of the Zic1 transcription factor, highlighting its role in *Xenopus laevis* embryonic development (Cornish et al., 2009). *Xenopus laevis* is a pseudo-tetraploid organism with two subgenomes, long (L) and short (S), resulting in the presence of two aqp3 genes: *aqp3.S* (*aqp3*) and *aqp3.L* (*aqp3b*). While both genes are expressed in adult tissues, their developmental roles differ (Figure 8B). Specifically, *aqp3b* is

expressed throughout early development, whereas aqp3 is not expressed during these stages (Figure 8 A) (Cornish et al., 2009).

The expression pattern of *aqp3b* during the early developmental stages (Figure 8A) revealed its role in gastrulation (stage 10-12). In early gastrula, *aqp3b* is expressed in the inner cell layers of the blastocoel roof and marginal zones (Figure 2B). As gastrulation progresses, *aqp3b* becomes localized to the marginal zone and involuted mesoderm. These expression patterns were observed using *in situ* hybridization, highlighting the spatial distribution of *aqp3b* transcripts (Forecki et al., 2018).

Functional studies revealed the role of *aqp3b* in convergent extension movements during gastrulation. Knockdown of *aqp3b* using morpholino oligonucleotides disrupts convergent extension movements in the dorsal axial mesoderm, as shown by the wider expression of chordin (Figure 9O) (Forecki et al., 2018). This disruption suggests that *aqp3b* is required for proper mediolateral intercalation during *Xenopus* convergent extension.

Fibronectin, an extracellular matrix glycoprotein, is relevant in guiding cell movements by mediating cell polarity and adhesion at tissue boundaries (Davidson et al., 2006). Interference with *aqp3b* expression disrupts fibronectin matrix assembly at the dorsal marginal zone but not on the free surface on the blastocoel. This loss of fibronectin correlates with compromised border integrity between involuted mesodermal cells and non-involuted ectoderm, further impairing convergent extension (Forecki et al., 2018).

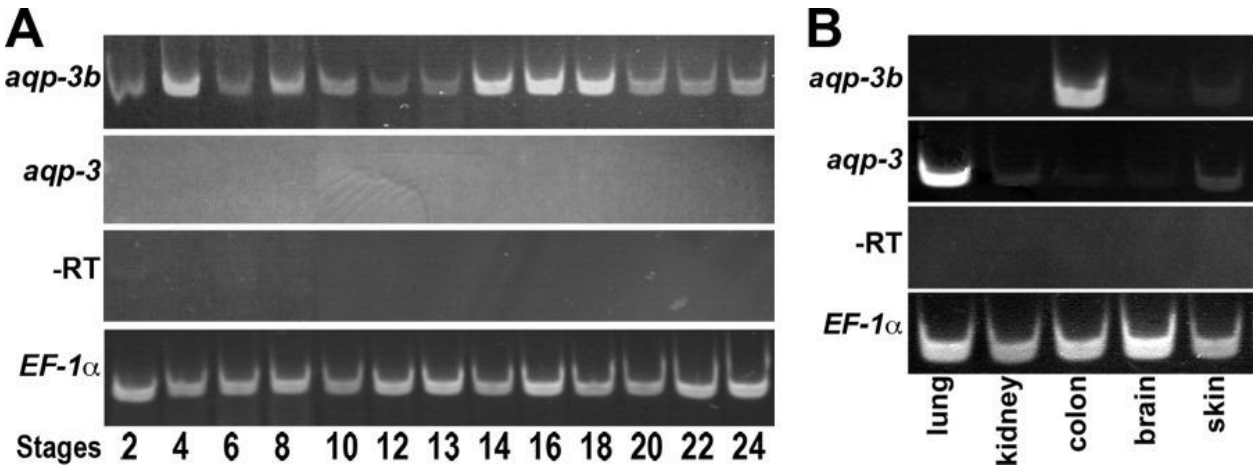


Figure 8. Expression of *aqp3b* and *aqp3*. RT-PCR analysis revealed the developmental expression profile of *aqp3b* (*aqp3L*) and *aqp3* (*aqp3S*). *aqp3b* is expressed maternally and throughout early development. *aqp3* is not expressed in any of the embryonic stages. (Cornish et al., 2009). (B) The expression pattern of *aqp3b* and *aqp3* in the various adult tissues. *aqp3b* was expressed in the colon and slightly in the skin, while *aqp3* was expressed in the lung, kidney, colon, brain, and skin (Cornish et al., 2009).

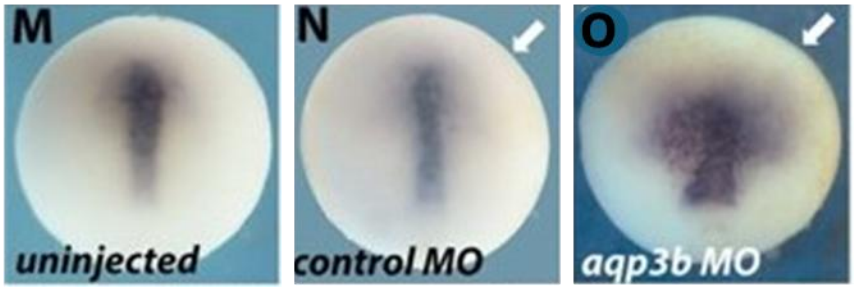


Figure 9. Inhibition of *aqp3b* causes defective convergent extension. (M, N) Uninjected and control MO-injected embryos (late gastrula stage 13) show normal convergent extension of the dorsal mesoderm, which is stained by *in situ* hybridization with chordin probe. (O) *aqp3b* MO-injected embryos show defects in convergent extension and do not form a notochord (Forecki et al., 2018).

CHAPTER TWO

MATERIALS AND METHODS

Xenopus husbandry

Female adult *Xenopus laevis* were induced to lay by injection with 150 units Pregnant Mare Serum Gonadotrophin (PMSG). After 48–72-hours, females were bathed in 200mM progesterone to induce ovulation. Testes were obtained from adult male *Xenopus laevis* after euthanasia with an overdose of MS-222. Testes were kept in Modified Barth's Solution (1X MBS; 10mM Herpes [pH 7.6], 88mM NaCl, 1mM KCl, 2.4mM NaHCO3, 1mM MgSO4.7H2O, 0.1M CaCl2) supplemented with 10% lamb serum.

Embryo culture

Xenopus laevis eggs were fertilized with a small amount of macerated testis and cultured in 0.1X MBS. Embryos were dejellied using 2% cysteine (pH 8.0) to remove the protective coat surrounding them. Embryos were staged according to Nieuwkoop and Faber (1994).

Morpholino oligonucleotides, plasmid construct, sense RNA preparation and microinjections

Aqp3b MO were designed to target the translation start site (ATG) or the 5'UTR. The sequences were as follows: ATG MO, 5'-CAAAATCCTTCTGGCGACCCATGTT-3' and 5'UTR MO, 5'-TTGGCTGGATCTCAGTTTGTCCTTA-3' (Gene Tools, Philomath, OR). To ensure specificity, 5-base mismatch control morpholinos were designed for each *aqp3b* MO: ATG control MO, 5'- CAAATTACTTCTCGCCACCCATCTT and 5'UTR control MO, 5'-

TTGCCTCGATGTCCACTTTCTCCTTA. The pCMVSPORT-*aqp3b* plasmid used for sense RNA synthesis was previously described (Forecki et al., 2018). The plasmids pCS2+ Dvl1, pCS2+ Dvl2 (Gentzel et al., 2014), pCS2+ PKC GFP, pCS2+ Frz7 (Sertz et al., 2014) were a kind gift from Alexandra Schambony. All sense RNAs were synthesized from linearized plasmids using mMessage mMachine kit (Invitrogen). Plasmid DNA was used directly for RhoA, Rac1 and Cdc42. They were constitutively active (Unterseher et al., 2004) and were also provided by Alexandra Schambony.

Embryos were injected into both blastomeres at the 2-cell stage for animal cap assays and into both dorsal blastomeres at the 4-cell stage for Keller explants. For single MO experiments, 8ng of either 5'UTR or ATG or control MO plus an emerald dextran tracer were co-injected into each cell, totally 16ng per embryo. For synergism experiments, 4ng of each MO was injected per cell (totaling 8ng per cell and 16ng per embryo) along with a tracer. Synergism injections targeted the dorsal marginal zone for animal cap assay, RT-PCR analysis and immunofluorescence analysis.

For rescue of convergent extension defects, embryos were co-injected with either 16ng of 5'UTR or ATG MO and mRNAs per embryo: 200pg Dvl1 or Dvl2 mRNA, 200pg PKC-GFP and 500pg Frz7 mRNA or plasmid DNA: constitutive-active (ca) RhoA 5pg, Rac1 10pg, Cdc42 10pg (Unterseher et al., 2004). For RT-PCR analysis, 300pg of aqp3b mRNA was injected into dorsal blastomere of each embryo at the 4-cell stage.

Keller explant and animal cap dissection

Keller explants are derived from *Xenopus* dorsal marginal zone of early gastrula embryos. These explants consist of a rectangle of dorsal mesendoderm and ectoderm, extending from just above the early dorsal lip to the blastopore to the animal pole (Figure 8A). To ensure proper selection of injected embryos, emerald dextran tracer was used for visualization.

For convergent extension assays, Keller explants were dissected between stages 10-10.25 and cultured flat under a coverslip resting on silicone vacuum grease. They were cultured in 1X MBS coated with 0.1% Bovine Serum Albumin (BSA). During overnight culture, explants initially roll up into a sphere which then undergoes convergent extension movements. To prevent these protrusions from curling under the explants, Keller explants are cultured beneath a coverslip resting on spacers of silicone vacuum grease (Sive et al., 2000).

Keller explants injected with either control MO or synergy MO were cultured in either 1X MBS or 200nM PMA in 1X MBS or 0.1% DMSO in 1X MBS. They were cultured overnight to allow convergent extension movements.

Explants were scored as described by (Kuehl et al, 2001). Elongation was quantified by normalizing to uninjected control embryos (Figure 8B). Deep mesodermal cells are what generate the force needed for convergent extension movement (Keller and Danilchik, 1988).

Animal caps are excised from the animal pole region of mid-blastula *Xenopus* embryos. In normal *Xenopus* development, animal caps are destined to form ectoderm, which, when isolated, differentiates into epidermal tissues. However, when exposed to inducers or growth factors, these cells can be directed to differentiate into tissues from all three germ layers.

Animal caps were excised from uninjected and injected embryos at stage 9 (Figure 9A) and cultured in Marc's Modified Ringer's Solution (0.5XMMR; 50mM Nacl, 1mM KCl, 0.5 mM MgCl2, 1mM CaCl2, 0.05mM EDTA, 2.5mM HEPES [pH 7.6]), supplemented with or without 5ng/ml of Activin. Animal cap assay allows for the study of cell movements and early patterning events in mesoderm and ectoderm formation (Green, 1999).

Reverse transcription polymerase chain reaction (RT-PCR)

For RT-PCR assays, Keller explants were dissected at stage 10 and cultured to the equivalent of stages 11 and 12.5. Total RNA was extracted from animal caps, Keller explants, and whole embryos (Merzdorf and Goodenough, 1997). cDNA was prepared using SuperScript II reverse transcriptase (Invitrogen). RT- PCR was performed using 30 PCR cycles for all target primers and 23 cycles for loading control primer. Find forward and reverse primers used from Table 1. Ef1α primers were used as a loading control to normalize the cDNAs.

Table1. Forward and reverse primers used.

Gene Name	Forward Primer	Reverse Primer	Reference
Noggin	AGTTGCAGATGTGGCTCT	AGTCCAAGAGTCTCAGCA	Aguis et al., 2000
Chordin	TTTCGCAACAGGAGCACAGACAC	TACCGCACCCACTCAAAATACCAT	New
Otx2	GAGGCCAAAACAAAGTGAGA	ACAGTCCATACCCCCAAAGT	New
Goosecoid	ACAACTGGAAGCACTGGA	TCTTATTCCAGAGGAACC	Wallingford et al., 1997
Xbra	GTTTGACTTTGCTAAAAGAGACAGG	TTCTGAAGGTGAGCATGTCG	Imberman et al., 2015
HoxB9	GCCCCTGCGCAATCTGAAC	CAGCAGCGGCTCAGACTTTGAG	New
FGF2	CTGCTGAGGCACTGTAGGAG	GCTTGTAGCTGTAATTTTATGTGCG	New
eFGF	CGGGTTTCATATCCAGGTTTTAC	GCGTTATAGTTGTTGGGCAGAAG	Sun et al., 1999
Ef1α	TCTTGACTGCATTCCACCAC	GACCAACTGGTACAGTACCAATACC	Gammill and Sive, 1997

Immunofluorescence and microscopy

Animal caps were fixed in MEMFA for 1 hr at room temperature, washed with 1X Phosphate-Buffered Saline (PBS) and blocked in 1X PBS/0.1% saponin/2% BSA/1%NaAzide. The primary antibody used for immunofluorescence was chicken anti-EGFP polyclonal antibody at 1:500 dilution (Abcam #ab13970) and goat anti-chicken IgG-Alexa-488 (Life Technologies, Carlsbad, CA) for secondary antibody at 1:500 dilution. After staining, animal caps were dehydrated in methanol followed by clearing using Murray's Clear (Dodt et al., 2007). The stained animal caps were examined using an Axiophot microscope.

Keller explants fixed in MEMFA and blocked with 1X PBS/0.1% Saponin/ 1% BSA were incubated in 10 units/ml Phalloidin-alexa-488 (Life Technologies). Stained Keller exlants were dehydrated in isopropanol and examined using confocal microscope.

CHAPTER THREE

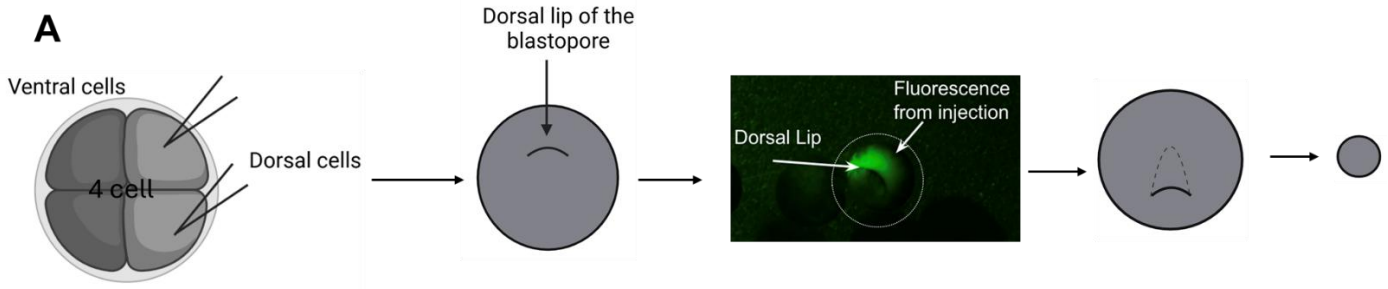
RESULTS

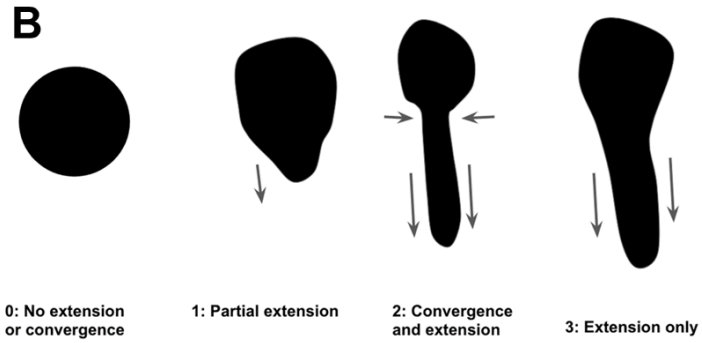
Aqp3b is required for convergent extension movements during gastrulation.

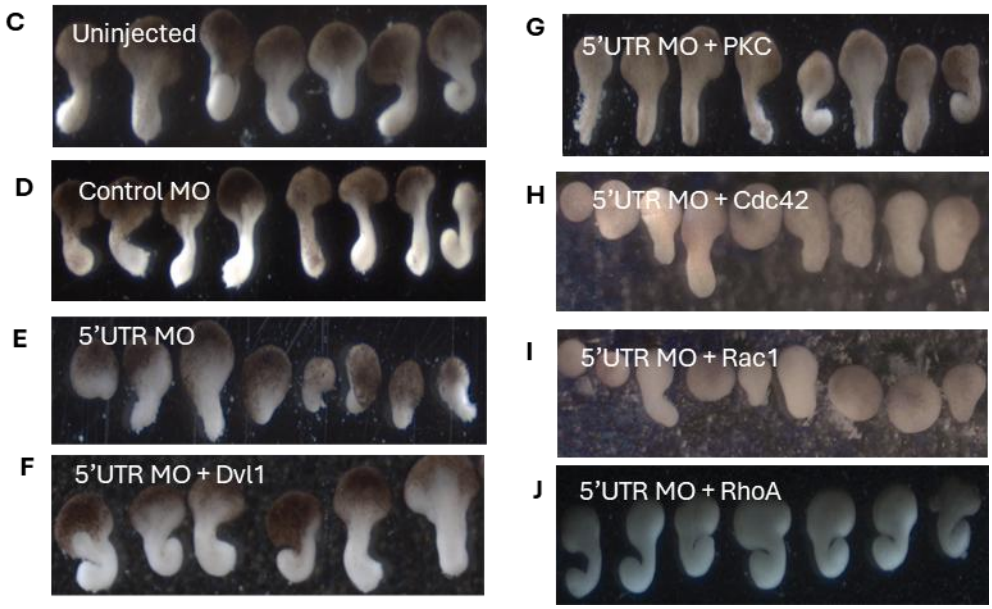
Inhibition of Aqp3b translation with morpholino oligonucleotides (MOs) disrupted convergent extension (CE) movements in the dorsal mesoderm, as indicated by a wider chordin probe expression pattern (Figure 9) (Forecki et al., 2018). To further investigate the role of *aqp3b* in convergent extension during gastrulation, we conducted a loss-of-function analysis using Keller explants. Injection of 16ng of 5'UTR *aqp3b* MO led to greatly reduced CE in these explants (Figure 10E). In contrast, explants injected with control MO showed nearly normal rates of CE, similar to uninjected Keller explants (Figure 10C, D). These results suggested that *aqp3b* was required for CE movements within the dorsal mesoderm.

The non-canonical Wnt pathway, consisting of Wnt/PCP and Wnt/Ca2+ signaling pathways, regulate convergent extension movements, including in *Xenopus* (Kühl et al., 2001, Habas et al., 2003). We investigated whether pathway components of the non-canaonical Wnt pathway could rescue convergent extension defects caused by *aqp3b* MO. Inhibition of *Xenopus* Disheveled disrupts CE movements of dorsal mesodermal cells during gastrulation (Wallingford et al., 2000). PKC, a downstream effector of the Wnt/Ca2+ pathway, has been implicated in regulating CE (Kühl et al., 2001). In addition, the small GTPases, RhoA, Rac1 and Cdc42 downstream of the non-canonical pathway, regulate convergent extension by reorganization of the actin cytoskeleton and cell adhesion (Tahinci et al., 2003, Choi and Han, 2002). We tested this

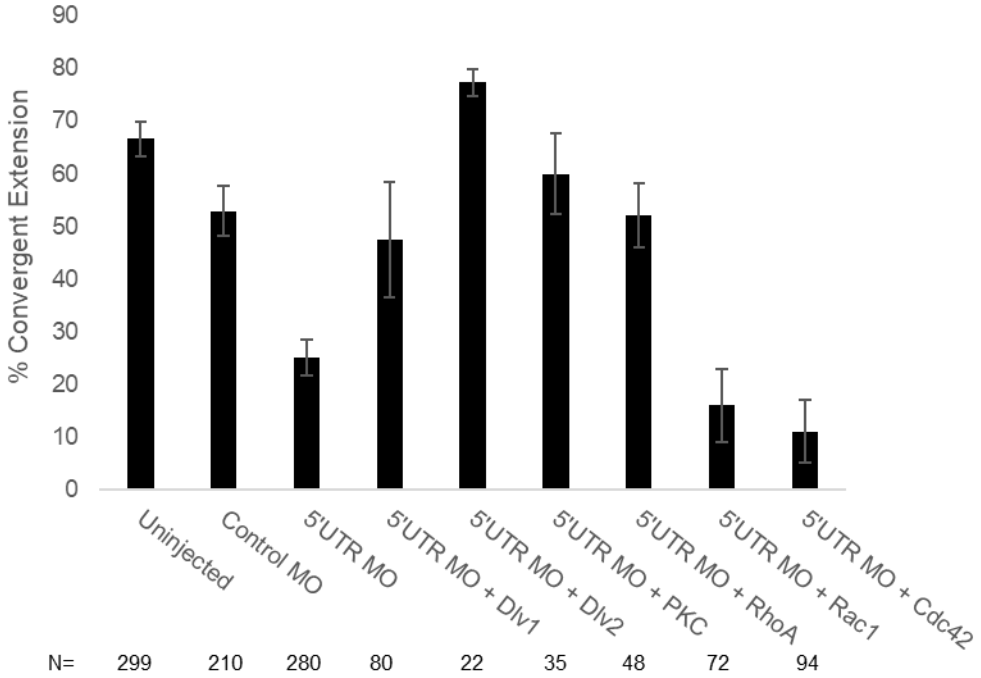
possibility by rescuing with Disheveled first, since it is at the apex of all these pathways. When that succeeded (Figure 10F), we hypothesized that the other components of the individual pathways should rescue convergent extension defects caused by aqp3b MO. PKC (Figure 10G) rescued *aqp3b* MO CE defects when co-injected with 5'UTR *aqp3b* MO, which was confirmed using the PKC agonist PMA (Figure 10M). Next, we investigated the potential of Cdc42 in rescue, given its involvement in regulating CE through the Wnt/Ca2+ pathway. Surprisingly, it did not rescue either 5'UTR or ATG aqp3b MO-injected embryos (Figure 10H). Rac1 also did not rescue either 5'UTR or ATG *aqp3b* MO-injected embryos (Figure 10I), but RhoA did (Figure 10J). This was intriguing because the Wnt/PCP pathway activates both RhoA and Rac1, both of which have distinct roles in regulating CE (Tahinci and Symes, 2003). In summary, *aqp3b* appeared to be involved in the regulation of CE through non-canonical Wnt signaling, but specifically not through Cdc42 and Rac1.



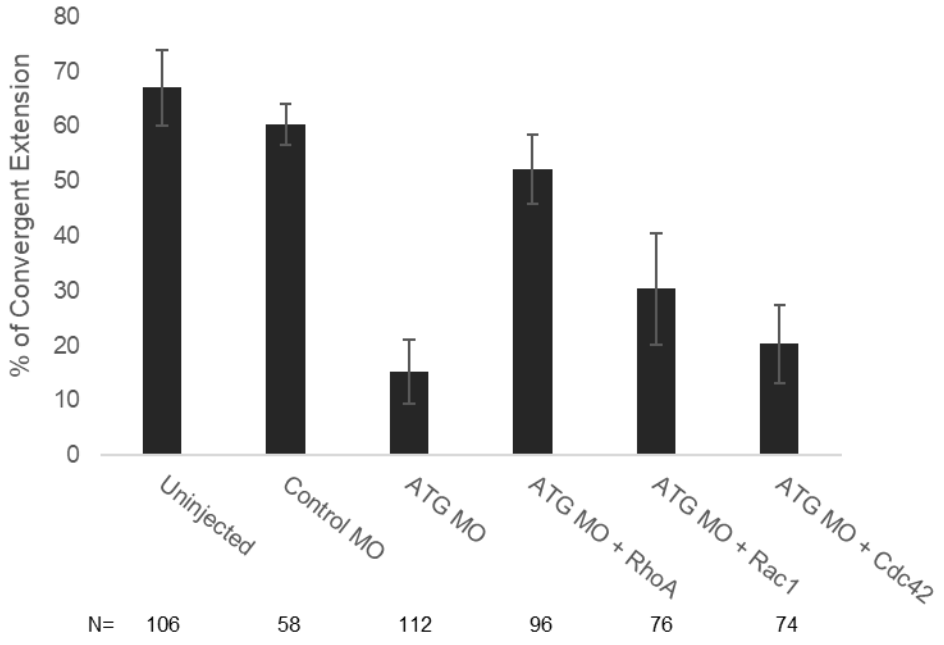

B
0: No extension or convergence
1: Partial extension
2: Convergence and extension
3: Extension only


C
Uninjected
D
Control MO
E
5'UTR MO
F
5'UTR MO + Dvl1
G
5'UTR MO + PKC
H
5'UTR MO + Cdc42
I
5'UTR MO + Rac1
J
5'UTR MO + RhoA

K


% Convergent Extension
90
80
70
60
50
40
30
20
10
0
Uninjected
Control MO
5'UTR MO
5'UTR MO + Dlv1
5'UTR MO + Dlv2
5'UTR MO + PKC
5'UTR MO + RhoA
5'UTR MO + Rac1
5'UTR MO + Cdc42
N= 299 210 280 80 22 35 48 72 94

L


% of Convergent Extension
80
70
60
50
40
30
20
10
0
Uninjected
Control MO
ATG MO
ATG MO + RhoA
ATG MO + Rac1
ATG MO + Cdc42
N= 106 58 112 96 76 74

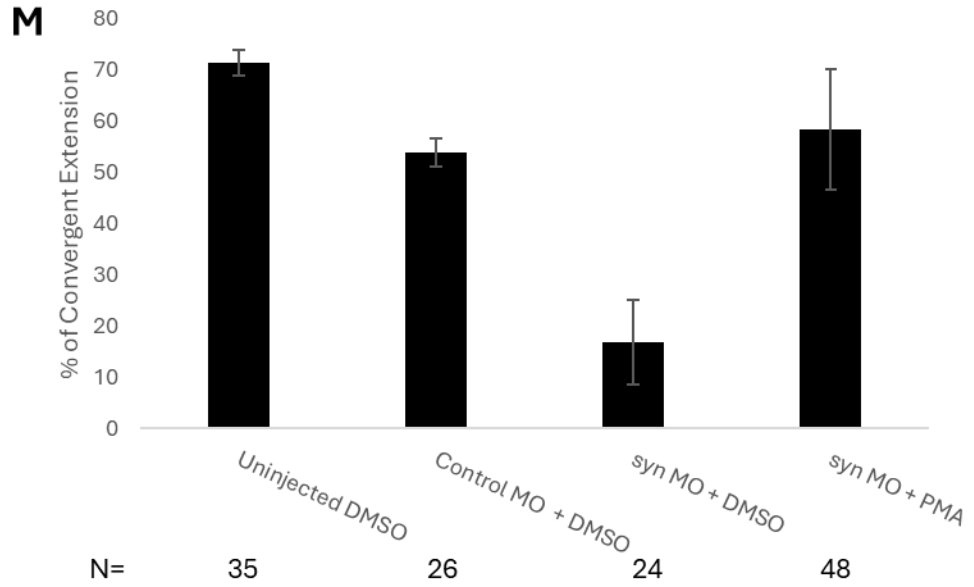


Figure 10. Aqp3b is required for convergent extension movements during gastrulation (A) Schematic showing the isolation and scoring of Keller explants. 4-cell embryos were injected into both dorsal blastomeres. A tracer (emerald dextran) was co-injected to ascertain that the dorsal marginal zone was injected. This region was dissected at stage 10. Explants were scored as 0 when no extension or convergence ensued, 1 when partial extension occurred, 2 when extension showed convergence and 3 when only extension occurred without convergence. (B, C) Keller explants from uninjected and control MO (16ng) injected embryos showed normal convergent extension. (D) Keller explants injected with 5'UTR *aqp3b* MO (16ng) suppressed convergent extension, which was rescued by co-injection of (E) 200pg Dvl1 mRNA, (F) 200pg PKC mRNA and (I) 5pg caRhoA plasmid but not (G) 10pg caCdc42 plasmid or (H) 10pg caRac1 plasmid. (K) Quantification of data from A-H. 5'UTR *aqp3b* MO caused convergent extension defects, which were rescued by Dvl2, Dvl1, PKC and caRhoA, but not caRac1 and caCdc42. (L) Keller explants injected with ATG *aqp3b* MO show convergent extension defects, which were rescued by co-injection with caRhoA but not caRac1 and caCdc42, thus confirming results in (K). (M) Keller explants injected with synergy MO and treated with PMA rescued convergent extension. These data suggest that Aqp3b may act upstream of Dsh-dependent PKC activation and Wnt/PCP RhoA activation. N represents the number of explants that were scored. The Y axis indicates the percentage of convergent extension. Error bars are standard error of the mean.

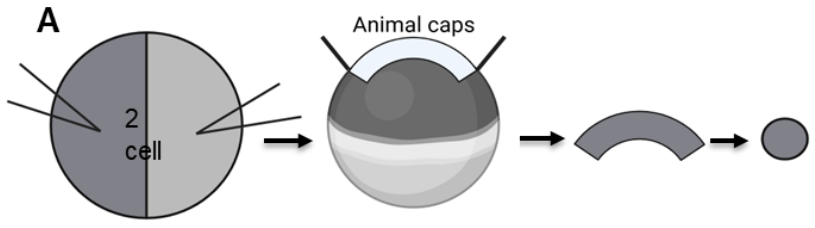
Aqp3b modulates anterior-posterior patterning in the dorsal mesoderm.

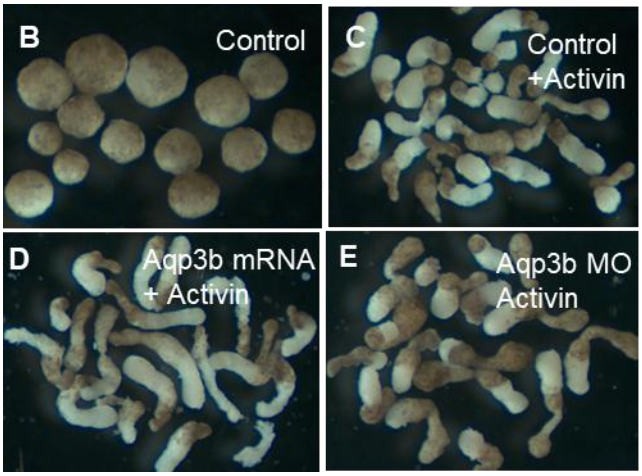
The *aqp3b* gene has been shown to be expressed in the dorsal mesoderm during gastrulation (Figure 2B). Animal caps, which are presumptive ectodermal explants, can be induced to form mesoderm (Jones and Woodland, 1987). Treatment of these explants with the mesoderm inducer Activin leads to a dose-dependent induction of mesoderm cell fates, which results in elongation of the animal caps (Green et al., 1992). Elongation of animal caps misexpressing *aqp3b* treated with activin was increased compared to uninjected animal caps and reduced in animal caps injected with *aqp3b* MOs (Figure 11D, E). These results suggested that *aqp3b* promotes elongation in activin treated animal caps.

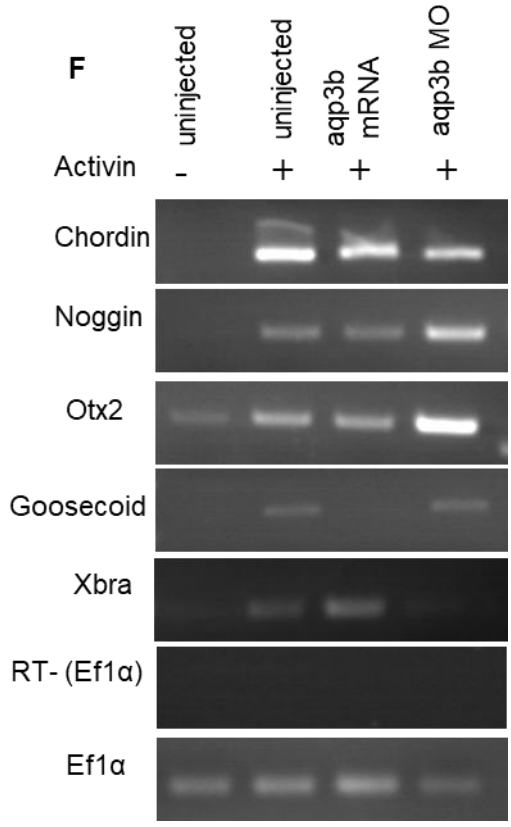
To test the hypothesis that *aqp3b* misexpression or inhibition disrupts mesodermal patterning or affects mesodermal cell fate decisions, we analyzed the expression of mesodermal markers. *goosecoid*, *otx2*, *noggin*, *chordin*, and *Xbra* are mesodermally expressed genes. RT-PCR analysis of the activin-treated animal caps revealed downregulation of *Xbra* and upregulation of *goosecoid*, *otx2*, and *noggin* when *aqp3b* was inhibited and vice versa when *aqp3b* was misexpressed (Figure 11F). The gene *chordin* expression did not change (Figure 11F). The gene expression of *otx2*, *goosecoid* and *noggin* are involved in specifying anterior mesoderm and neurectoderm (Ira and Cho, 1995; Steinbeisser et al., 1995; Lamb et al., 1993). Xbra plays an important role in posterior patterning during *Xenopus* development (Tada et al., 1997). Aqp3b has been shown to indirectly regulate gene expression through its passive passage functions and modulation of signaling pathways (Bollag et al., 2020). Collectively, these results suggest that Aqp3b in modulating anterior-posterior patterning in dorsal mesodermal cells, specifically inducing genes characteristic of posterior mesoderm. As shown in Figure 10, *aqp3b* inhibition

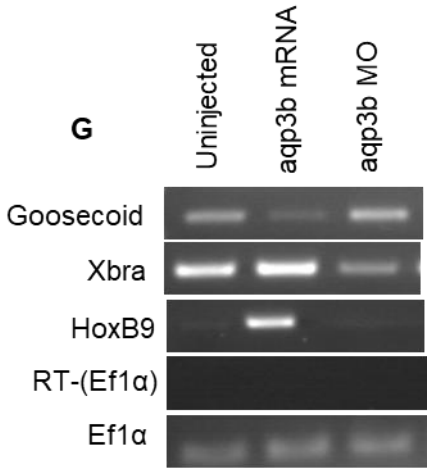
caused CE defects. Failure of mesodermal patterning can lead to defective CE movements (Keller and Danilchik, 1988). We confirmed this by performing RT-PCR analysis on Keller explants. Posterior genes (*Xbra, hoxB9*) were downregulated in *aqp3b* inhibition and upregulated in *aqp3b* misexpression and vice versa for the anterior gene, *goosecoid* (Figure 11G). This confirms that the *aqp3b* inhibition defect in convergent extension could be a result of failure in patterning of the posterior mesoderm.

FGF2 and eFGF have been found to upregulate *Xbra* expression during *Xenopus* gastrulation. Without FGF signaling, *Xbra* may not be expressed at all (Latinkić et al., 1997; Isaacs et al., 1994). The suppression of anterior genes and activation of posterior genes, when FGF is overexpressed (Hongo and Okamoto, 2022) was similar to *aqp3b* misexpression. We determined if *Xbra* modulation by *aqp3b* is via FGF signaling. *aqp3b* misexpression in activin-treated animal caps induced FGF and eFGF (Figure 11H). This suggests the modulation of FGF/Xbra by *aqp3b* in promoting convergent extension during *Xenopus* gastrulation.




B
Control
C
Control
+Activin
D
Aqp3b mRNA
+ Activin
E
Aqp3b MO
Activin


F
uninjected
uninjected
aqp3b mRNA
aqp3b MO
Activin
-
+
+
+
Chordin
Noggin
Otx2
Goosecoid
Xbra
RT- (Ef1α)
Ef1α


G
Uninjected
aqp3b mRNA
aqp3b MO
Goosecoid
Xbra
HoxB9
RT-(Ef1α)
Ef1α

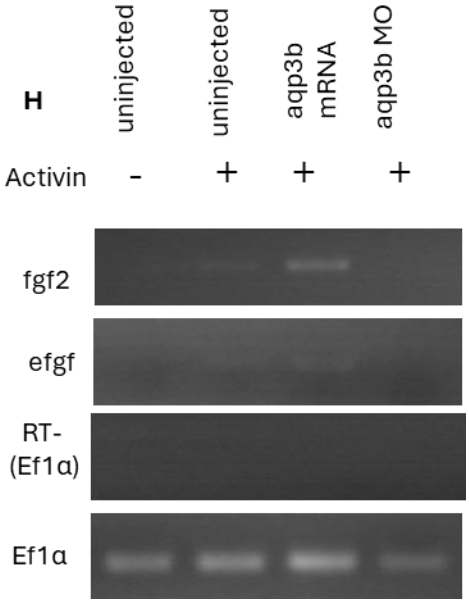

H
uninjected
uninjected
aqp3b mRNA
aqp3b MO
Activin
-
+
+
+
fgf2
efgf
RT-
(Ef1α)
Ef1α

Figure 11. Aqp3b modulates anterior-posterior patterning during gastrulation. Embryo patterning is a crucial process that requires signals to coordinate morphogenetic movements. This assay investigated the role of *aqp3b* in anterior-posterior patterning using animal caps and Keller explants. (A) Schematic showing the isolation of animal caps. 2-cell embryos were injected into both cells and dissected at stage 8. Animal caps were cultured without or with 5 ng/ml activin in 0.5X MMR. (B) Uninjected, untreated animal caps showed no elongation. (C) Uninjected animal caps treated with activin showed normal elongation. (D) Injection of 300pg *aqp3b* mRNA in activin treated animal caps resulted in increased elongation. (E) *aqp3b* MO (synergy) injected animal caps treated with activin showed reduced elongation. (F, H) animal caps from B-E were analyzed using RT-PCR. (F) *chordin* expression (along entire anterior/posterior axis) was not affected. *aqp3b* misexpression in activin-treated animal caps caused decreased *noggin, otx2*, and *goosecoid* expression (genes expressed in anterior mesoderm), while *aqp3b* MO caused increased *Xbra* expression (gene expressed in posterior mesoderm). This suggested that *aqp3b* modulates anterior-posterior patterning by downregulating anterior genes and upregulating posterior genes. (G) Keller explants excised and cultured until stage 12 were analyzed using RT-PCR: *aqp3b* misexpression reduced *goosecoid* expression and increased *Xbra* and *hoxB9*, confirming that *aqp3b* indirectly induced posterior fates. (H) Animal caps injected with *aqp3b* MO and treated with activin, showed inhibition of *fgf2* and *efgf* expression (both activate posterior genes). Based on these results, we hypothesize that *aqp3b* modulates fgf signaling to promote upregulation of posterior genes in order to promote convergent extension, which has been shown for *Xbra*. Each experiment was repeated a minimum of three times in independent experiments.

Inhibition of Aqp3b prevents PKC membrane localization and results in abnormal cell shape.

Accumulation of calcium in cells regulates convergent extension in *Xenopus*. Calcium levels are related to PKC activation (Shindo eta l., 2010, Sheldahl et al., 1999). Cdc42, a downstream effector of the Wnt/Ca2+ signaling pathway, is activated by PKC and has been shown to regulate convergent extension (Choi and Han, 2002). However, Cdc42 did not rescue convergent extension defects caused by *aqp3b* inhibition, but PKC did (Figure 10), raising the question whether *aqp3b* may regulate convergent extension via a different pathway than Wnt/Ca2+ signaling. To address this question, we examined the role of *aqp3b* in PKC membrane

translocation. Overexpression of Frz7 translocate PKC from the cytoplasm to the plasma membrane in *Xenopus* embryos (Medina et al., 2000). When injected alone, PKC-GFP did not translocate to the membrane (Figure 12A). Co-injection of Frz7 and PKC-GFP in animal caps translocated PKC-GFP to the membrane (Figure 12B). Control *aqp3b* MO co-injected with Frz7 and PKC-GFP also translocated PKC-GFP to the membrane (Figure 12C). However, the inhibition of *aqp3b* in animal caps expressing Frz7 and PKC-GFP resulted in impaired membrane localization of PKC-GFP (Figure 12D). Together, these results and the inability of Cdc42 to rescue *aqp3b* morphants suggest that *aqp3b* regulates convergent extension via the Wnt/Ca2+ pathway but not Cdc42.

During convergent extension cells acquire an elongated shape, cellular motility, and bipolar morphology, which drive tissue elongation and narrowing. Mediolateral intercalation is essential for convergent extension to occur. It relies on polarization which ensures directional movements, necessary for CE (Shindo, 2017). To determine if the morphology of the dorsal marginal zone cells was affected by *aqp3b* inhibition, we stained Keller explants with phalloidin. Keller explants of the dorsal marginal zone cells injected with *aqp3b* MO showed a wider cell shape as compared to the uninjected cells (Figure 12 E, F). However, disruption in cell shape by *aqp3b* inhibition may cause defects in polarization, which ultimately contribute to convergent extension failure.

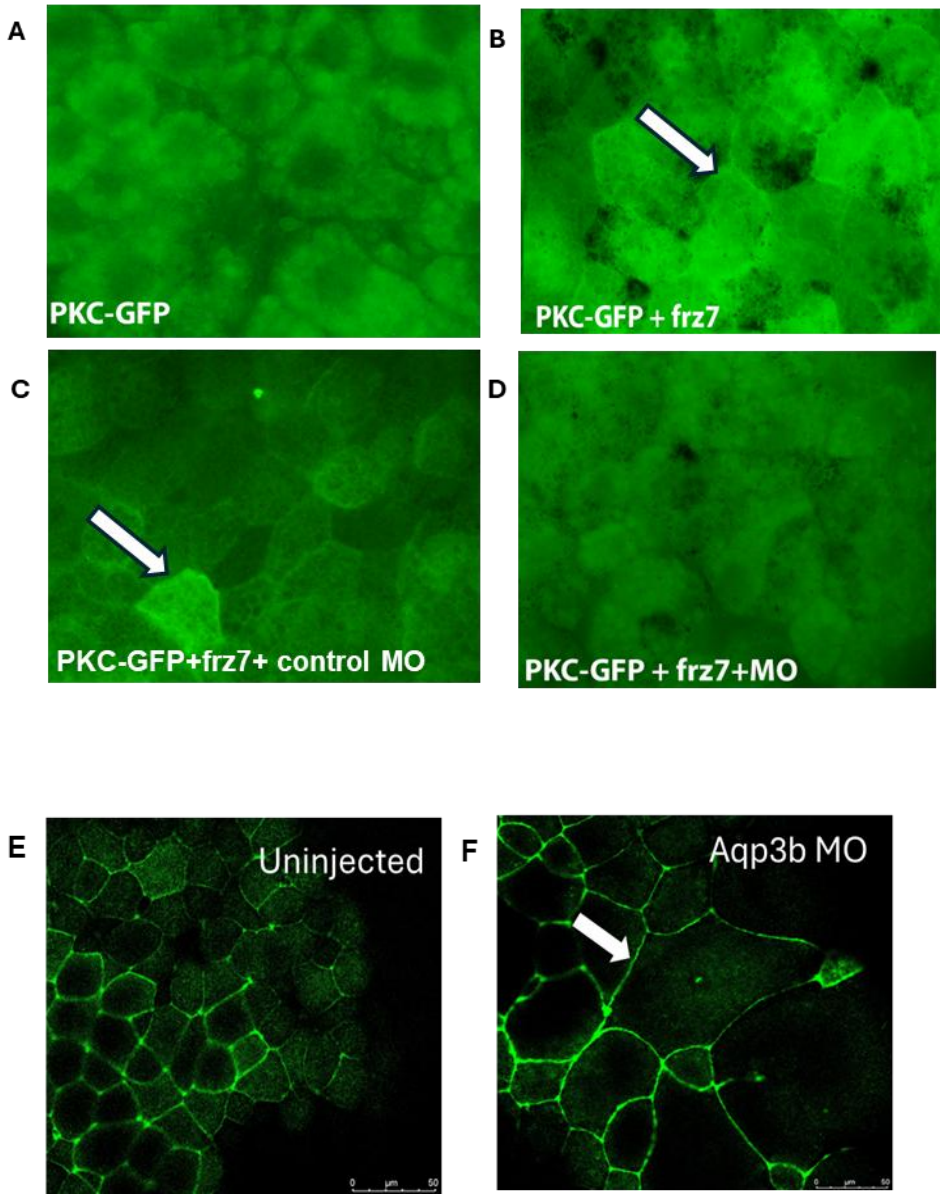


Figure 12. *aqp3b* MO impaired PKC membrane localization in animal caps and caused changes in cell shape in Keller explants. (A-D) Determination if *aqp3b* MO affects PKC activation. (A) PKC-GFP was cytoplasmically localized in animal caps. (B) Co-injection of 200pg Frz7 mRNA with 300pg PKC-GFP mRNA caused localization of PKC-GFP to the cellular membrane. (C) Co-injection with a control MO did not affect the membrane-localization of PKC-GFP. (D) Co-injection with *aqp3b* MO caused PKC-GFP to remain in the cytoplasm. Thus, Aqp3b may activate PKC through a Dsh-dependent pathway (Figure 10), rather than Cdc42 regulating convergent extension through PKC activation via the Wnt/Ca2+ pathway. (E-F) Keller explants at stage 10 where dissected and stained with phalloidin to determine cell shape. (E) Uninjected explants showed normal cell shape. (F) *aqp3b* MO injection caused changes in cell shape, which may cause defective convergent extension.

CHAPTER 5

DISCUSSION

During *Xenopus* gastrulation, anterior-posterior mesodermal patterning has been shown to be directly linked to convergent extension movements (Ninomiya et al., 2004). Cells of the anterior region undergo directed migration on the inner surface of the blastocoel, while posterior cells behind them undergo convergent extension, forming the notochord. The regulation and segregation of these two cell populations is based on differences in cell behavior and gene expression.

Aqp3b is an aquaglyceroporin expressed in the sensorial cell layers of the blastocoel roof and marginal zones. We previously demonstrated that embryos injected with *aqp3b* MO exhibit defects in convergent extension movements, shown by wider *chordin* expression, a marker of the dorsal axial mesoderm (Forecki et al., 2018). We demonstrate here that inhibition of *aqp3b* impaired convergent extension in Keller explants. This inhibition was rescued by Disheveled, PKC and RhoA, but not Rac1 or Cdc42 (Figure 10). This suggested that *aqp3b* can act through the non-canonical Wnt pathway.

At the beginning of gastrulation, mesoderm cells extend lamellipodia and filopodia in random orientations. However, these protrusions become polarized, stabilized and mediolaterally biased during convergent extension, guided by molecular cues such as the non-canonical Wnt pathway (Keller et al., 1992) (Figures 3,6). Disheveled, a key component of the non-canonical pathway, regulates cell polarity and protrusive stability during *Xenopus* convergent extension (Wallingford et al., 2000). Disheveled proteins have three conserved domains (Boutros and Mlodzik, 1999): the DIX domain, essential for canonical Wnt signaling but not for the non-

canonical Wnt pathway, and the PDZ and DEP domains, which are both required for non-canonical Wnt signaling (Tada and Smith 2000; Wallingford et al., 2000; Habas et al., 2001). Members of the Disheveled protein family exhibit both overlapping and distinct functions in the various Wnt signaling pathways (Shi, 2020). Dvl1 and Dvl2 are members of the *Xenopus* Disheveled protein family. They were constructed to lack the DIX domain, in order to only allow non-canonical Wnt signaling, and have been shown to regulate convergent extension. Dvl1 regulation of convergent extension is dependent on Protein Kinase C (PKC), whereas Dvl2 regulation does not rely on PKC (Genzel et al., 2015). This suggests a role for Dvl1 in Wnt/Ca2+ signaling and Dvl2 in the Wnt/PCP pathway. Disheveled (specific protein family not stated) has also been shown to activate the downstream effectors of the Wnt/Ca2+ pathway including Ca2+ flux and PKC. PKC plays a dual role in the Wnt/Ca2+ pathway by acting downstream of Disheveled and upstream of Cdc42 (Sheldahl et al., 2003; Choi and Han, 2002). Interestingly, we demonstrated rescue of convergent extension defects with both Dvl1 and Dvl2 in *aqp3b* MO morphants (Figure 10). This suggests that Aqp3b plays a role in regulating both the Wnt/Ca2+ and Wnt/PCP signaling pathways. The role of Aqp3b in Wnt/Ca2+ signaling was confirmed by rescue of *aqp3b* morphants by PKC.

PKC has also been shown to promote convergent extension by activating the small GTPase Cdc42 downstream of Wnt/Ca2+ signaling via a Disheveled-independent mechanism (Choi and Han, 2002). Interestingly, unlike PKC, Cdc42 did not rescue *aqp3b* morphants (Figure 10E, F). This suggest that regulation of convergent extension by Aqp3b relies specifically on PKC activation and excludes alternate pathways involving Cdc42. Further, inhibition of *aqp3b* impaired PKC-GFP localization to the cell membrane (Figure 12D), confirming an upstream role

of Aqp3b in PKC activation. Thus, Aqp3b may be upstream of Dvl-dependent PKC activation via the Wnt/Ca2+ pathway, but not of the Dvl-independent PKC/Cdc42 pathway.

Disheveled functions downstream of Wnt/Frz in the activation of the small GTPases RhoA and Rac1 via the Wnt/PCP pathway. RhoA and Rac1 are activated independently, each requiring different Disheveled protein domains. A Disheveled mutant lacking the PDZ domain blocks RhoA activation but allows Rac1activation. Thus, the DEP domain alone is sufficient for Rac1 activation, but both PDZ and DEP domains are required for RhoA activation (Habas et al., 2001).

RhoA and Rac1 play distinct and antagonistic roles in regulating convergent extension. Experimental inhibition of both RhoA and Rac1 decreases the overall protrusive activity in dorsal mesoderm cells, but their individual roles are antagonistic. (Tahinci and Symes, 2003). RhoA is primarily involved in stabilizing protrusions through the activation of Rho-associated kinase (ROCK), a downstream effector, which increases actomyosin contractility to generate mediolateral tension. This stabilization of the cytoskeleton allows cells to establish protrusive polarity (Totsukawa et al., 2000; Katsumi et al., 2002; Tahinci and Symes, 2003). In contrast, Rac1 promotes protrusive actin dynamics, increasing lamellipodia formation. Excessive Rac1 activity prevents cells from achieving polarized protrusions, disrupting cell polarity and leading to convergent extension defects. (Katsumi et al., 2002; Tahinci and Symes, 2003). The combined action of RhoA and Rac1 results in suppression of lamellipodia and protrusive activity along the anterior and posterior sides of dorsal mesoderm cells, generating mediolateral tension, thereby enhancing cell polarity (Keller, 1984; Keller et al., 1992). This suppression ensures that protrusive activity is concentrated at the mediolateral ends of the cells, promoting effective

polarization and directional movement along the mediolateral axis, which can be inhibited by an increase in Rac1 activity (Katsumi et al., 2002). Since RhoA, but not Rac1, could rescue convergent extension defects in *aqp3b* morphants, we concluded that *aqp3b* may regulate the stabilization of polarized protrusive activity during convergent extension via the Dvl/RhoA pathway. Future experiments will determine if *aqp3b* is involved in establishing protrusive polarity in mediolateral convergent extension.

During gastrulation, dorsal mesoderm cells in *Xenopus* embryos exhibit two distinct cell behavior patterns: cell migration (anterior dorsal mesoderm) and convergent extension (posterior dorsal mesoderm). Cell migration is the directed movement of cells driven by molecular cues, while convergent extension is characterized by the anterior-posterior polarization of individual mesodermal cells and their mediolateral intercalation (Ninomiya et al., 2004). *In vitro*, activin acts as a mesoderm inducer of naïve animal cap tissue. Activin causes the elongation of animal caps by establishing anterior-posterior tissue specification, which produces both cell migration (anterior-type region) and convergent extension (posterior-type region) (Green et al., 1992). The induction of these cellular behaviors during activin-induced elongation results from its ability to establish graded differences in gene expression, with the induction of *Xbra*-expression equivalent to posterior chordamesoderm and *otx2*-expression equivalent to anterior prechordal mesoderm in different regions of the animal caps (Green et al., 1992; Wacker et al., 1998; Ninomiya et al., 2004; Sander et al., 2007). These data are consistent with the expression of the *Xbra* transcription factor in posterior *Xenopus* dorsal mesoderm, where it promotes convergent extension by inhibiting cell migration (Kwan and Kirschner, 2003).

Xbra directly activates *Xwnt11* gene expression and therefore regulates convergent extension via the noncanonical Wnt pathway (Tada and Smith, 2000; Yokota et al., 2003). Keller explants expressing either a dominant negative Wnt11 or a PDZ-deleted Disheveled inhibit convergent extension and increase the number of cells undergoing cell migration. Since PDZ-deleted Disheveled activates only Rac1, this deregulates Rac1 activity by overriding RhoA's stabilizing function. Excessive and unregulated Rac1 activity promotes active and disorganized cell migration which disrupts convergent extension. Xbra inhibits Rac1-mediated formation of lamellipodia protrusions, suggesting that it indirectly stabilizes Rac1 protrusions via the non-canonical pathway. This stabilization enables polarized mediolateral intercalation movements during convergent extension. (Kwan and Kirschner, 2003). Our data show that *Xbra* expression was upregulated in *aqp3b* mRNA injected animal caps treated with activin and in Keller explants, suggesting that Aqp3b may modulate the expression of *Xbra* to promote convergent extension. Consistent with Rac1 not rescuing *aqp3b* morphants, we can conclude that Aqp3b may promote protrusion stabilization through *Xbra* activation.

Since FGF signaling activates *Xbra* gene expression (Hongo and Okamato, 20022), we determined if *aqp3b* may modulate *Xbra* expression via FGF signaling. The misexpression of *aqp3b* in activin-treated animal caps induced *FGF2* and *eFGF* expression, which were reduced in *aqp3b*-depleted activin-treated animal caps (Figure 11H). The reduction in *Xbra* expression seen in *aqp3b*-depleted explants is thus likely due to diminished FGF signaling. We therefore conclude that *aqp3b* may modulate *Xbra* expression via FGF signaling.

In contrast to the role of *Xbra* in promoting convergent extension, misexpression of the anterior genes *goosecoid* and *otx2* in *Xenopus* embryos promotes anterior migration of dorsal

mesoderm cells while inhibiting convergent extension (Niehrs et al., 1993, Carron et al., 2005; Ulmer et al., 2017). The inhibition of *goosecoid* in activin-treated animal caps did not disrupt elongation but failed to induce anterior markers like *otx2* (Sander et al., 2007). Activin treated animal caps misexpressing *aqp3b* promoted convergent extension as demonstrated by increased elongation (Figure 11D), upregulation of *Xbra* and downregulation of *goosecoid* (Figure 11F). Conversely, inhibition of Aqp3b expression reduced elongation in activin-treated animal caps and allowed expression of *goosecoid* and promoted the expression of *otx2*, while inhibiting the expression of *Xbra*. These data suggest that Aqp3b inhibited *goosecoid* and *otx2* expression to prevent cell migration characteristic of anterior dorsal mesoderm, in addition to modulating *Xbra* expression to promote convergent extension as already discussed above.

The homeobox transcription factor gene goosecoid is expressed in the anterior dorsal mesoderm and required for the expression of Spemann's organizer genes (Niehrs et al., 1993). Noggin and Chordin, secreted by Spemann's organizer, antagonize bone morphogenetic proteins (BMPs) in order to promote neural tissue formation (Harland and Gerhart, 1997). The *noggin* and *chordin* genes have different modes of activation. The *noggin* gene cannot be directly activated by Goosecoid, while Goosecoid directly activates *chordin* transcription (Sasai et al., 1994). We determined if downregulation of goosecoid by *aqp3b*-MO could affect expression of these organizer genes. Noggin is expressed in the prechordal mesoderm (Lamb et al., 1993) and exhibited increased expression in *aqp3b*-depleted activin treated animal caps (Figure 11F). However, Chordin is expressed in both the prechordal mesoderm and chordamesoderm (Kurth et al., 2004). Its expression remained unchanged in animal caps during both *aqp3b* gain- or loss-of-function experiments (Figure 11F), consistent with its role in promoting elongation in the

notochord during activin treatment (Sander et al., 2007). Since noggin is expressed only in the prechordal mesoderm, we confirmed Aqp3b inhibition of anterior genes.

Cell shape plays an important role during mediolateral intercalation, which is part of convergent extension. *aqp3b* MO caused changes in cell shape in Keller explants (Figure 12F), but it is not yet clear whether this may affect convergent extension. Future experiments will investigate the role of *aqp3b* in the polarization of mesodermal cells, since a prerequisite for convergent extension is the ability of cells to change shape by adopting a bipolar morphology.

In summary, these findings provide evidence for the regulation of anterior-posterior patterning and convergent extension movements by *aqp3b*. Anterior-posterior patterning and convergent extension are necessary during *Xenopus* development for shaping the posterior body axis in order to form the notochord and ultimately the trunk. Both processes are influenced by Aqp3b through its interaction with noncanonical Wnt pathway components involved in establishing polarized protrusive activity and mediolateral intercalation necessary for convergent extension (Figure 13). Our results confirm that Goosecoid and Xbra act antagonistically in regulating cell behaviors and mesodermal patterning in the dorsal mesoderm. Aqp3b modulation of *Xbra* expression in activin-treated animal caps influenced elongation, while inhibiting *goosecoid* from activating cell migration. It will be interesting to know how *aqp3b* regulates the balance between cell polarity and protrusive activity during convergent extension. Understanding these mechanisms will provide deeper insight into the regulation of convergent extension and mesoderm patterning in *Xenopus* embryos.

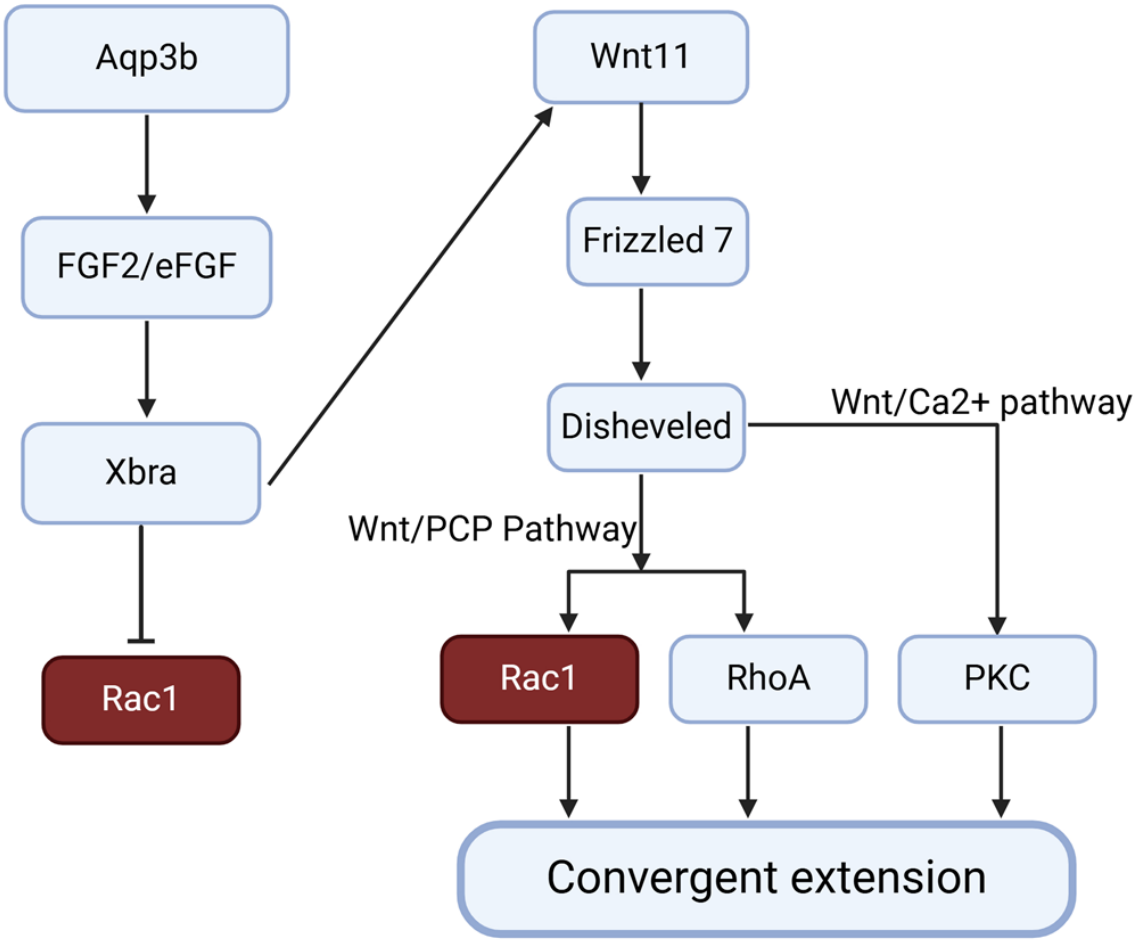


Figure 13. Gene modulation in posterior dorsal mesoderm that undergoes convergent extension. Aqp3b expression promotes FGF signaling to induce *Xbra*. Xbra directly activates Wnt11 expression, a key activator of non-canonical Wnt signaling essential for convergent extension. Xbra inhibits lamellipodia formation by inhibiting Rac1. RhoA and PKC are downstream of Aqp3b activity. Both promote protrusive stability through non-canonical signaling. Rac1 is not downstream of Aqp3b activity. This further highlights the potential role of Aqp3b in protrusive stability during convergent extension movements.

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