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Identification Of Molecular Regulators Of Morphogenesis During Early Development Of Xenopus

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IDENTIFICATION OF MOLECULAR REGULATORS OF MORPHOGENESIS DURING EARLY DEVELOPMENT OF XENOPUS

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CELL BIOLOGY

ABSTRACT

During embryonic development of all animal species, dramatic changes in morphology occur to transform a single-celled zygote into a well-organized adult body that contains different organs positioned in stereotypical ways for the species. The processes that shape tissues and organs are called morphogenesis, and gastrulation is the first morphogenetic process in embryogenesis. During gastrulation, three germ layers are specified and a set of regionally specific cell movements act in concert to position these germ layers for proper establishment of future body plan of the developing animal. Although factors regulating cell lineage specification have been studied extensively, the molecular machinery controlling region-specific behaviors is less understood. In this study, we performed RNA-sequencing experiments to identify genes differentially expressed among various tissues during gastrulation, using the animal *Xenopus laevis* as our model. *Xenopus* gastrulation, as in other species, utilizes distinct cell behaviors, including cell shape changes (e.g. apical constriction), cell migration, and cell intercalation in different embryonic regions. These cell behaviors are preserved when tissues are isolated from the whole embryos. We sought to identify genes that are associated with different regions at developmental time points when cells in these regions exhibit characteristic behaviors. We aim to link these differentially expressed genes to cell behaviors they may be responsible for promoting. Identification of regulators of cell

behaviors during gastrulation can help to provide paradigms for understanding analogous cell behavior regulation seen in other contexts, both during embryogenesis and in adult.

Our results highlight several regionally enriched genes potentially involved in modulation of cell adhesion, extracellular matrix assembly, and modification of the cytoskeleton, the roles for many of these genes in development are previously unknown. Particularly, we investigate a secreted protein kinase, *pkdcc1*, and show that it is an important component in pathways regulating both migration of anterior mesendoderm and convergent extension of the prospective trunk. In addition, we describe an essential role for the Rho guanine nucleotide exchange factor *plekhg5* in induction of apical constriction within the bottle cells, which define the site of involution cell movements during gastrulation. Our studies thus uncover crucial function of previously uncharacterized regulators of cell movements.

Keywords: *Xenopus laevis*, apical constriction, convergent extension, *pkdcc1*, *plekhg5*

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INTRODUCTION

Overview of Gastrulation

Gastrulation is defined by a set of movements required for conversion of an embryo that is essentially a spherical ball of cells at the end of blastula stages into a more complex arrangement of cell layers that set up the body plan of the developing animal. It involves segregation and organization of three germ layers: the ectoderm, which develops into the nervous system and the epidermis; the endoderm, which lines gastrointestinal and associated organs (Zorn and Wells, 2009); and the mesoderm, which forms bone, muscle, blood, kidney, and other mesenchymal cells (Chal and Pourquié, 2017). Massive, coordinated cell movements occur during gastrulation, including the inward migration of endodermal and mesodermal cell populations to generate a rudimentary gut tube (Keller, 1975) and position the notochord and the somites underneath the ectoderm for induction of neural cell fates. The mesodermal tissues that are patterned along the dorsal-ventral (back-belly) and anterior-posterior (head-tail) axes move sequentially inside, with the dorsoanterior notochordal (embryonic backbone) cells migrating early, and the somites and the lateral plate mesoderm for heart, musculature, and skeleton following next, and the ventroposterior mesoderm for kidney and circulatory system moving in later. The ectodermal layer, which is initially restricted to the animal hemisphere and the upper

level of the equatorial region, expands to cover the entirety of the exterior of the embryo in a process termed epiboly (Solnica-Krezel and Sepich, 2012). Epiboly is driven by cell division and radial cell intercalation in the animal region and is accompanied by concerted actions of several morphogenetic behaviors exhibited by mesodermal and endodermal cells throughout the other parts of the embryo. Formation of bottle cells along the circumference of the vegetal hemisphere is apparent from the dark appearance of the cells, and this is driven by active shrinking of the apical surfaces of these cells. The bottle cells pull the surrounding epithelial cells to generate a furrow through which mesodermal and endodermal cells involute. Below the surface, deep cells encompassing anterior mesendoderm precursors migrate along the roof of an interior fluid-filled space called the blastocoel cavity toward the animal region. Concurrently, cells trailing anterior mesendoderm in the marginal zone extend vegetally and turn inward in a process termed involution (Keller, 1976). The cells around the marginal zone then undergo planar rearrangements which extend the tissue toward the vegetal pole while simultaneously driving constriction of the circular opening that will become the posterior end of the gut tube (Keller and Danilchik, 1988). Meanwhile, intercalation within the deep layer of ectodermal cells in the animal hemisphere results in thinning and expansion of the surface area of the ectoderm to enable coverage of the embryo exterior (Wilson and Keller, 1991). The diverse modes of cell movements in different tissues at gastrulation are regulated by distinct signals. Both intrinsic property of a cell and extrinsic signaling (biochemical and biomechanical) the cell receives can affect its behaviors and alter its adhesive strength and membrane protrusive dynamics. These will be summarized in the following.

Bottle cell formation

The onset of gastrulation is heralded externally by the formation of a darkly pigmented line of cells forming a crevice at the vegetal edge of the dorsal marginal zone. This dark crescent proceeds to spread laterally to encircle the entire vegetal side of the embryo, forming an invagination referred to as the blastopore. Comprising the floor of the blastopore are the bottle cells, so named for their elongated and expanded basolateral surfaces and tapered apical surface, which resemble a flask (Hardin and Keller, 1988) (Fig. 1C). The process of gastrulation is often referenced in terms of the blastopore; the transition from blastula stage into gastrula stage is first observed by the generation of the dorsal lip of the blastopore. Similarly, the transition from gastrula stage into neurula stage follows closure of the blastopore. In light of its central role in visualizing the progression of gastrulation, it is ironic that the bottle cells exert relatively little influence on the outcome of said process. Indeed, closure of the blastopore is completed even in embryos in which the bottle cell population has been micro-surgically removed, though these embryos did display defects in induction of anterior neural structures (Keller, 1981). Therefore, the primary function of the bottle cells seems to center on the formation of a crease to allow the superficial layer to buckle inward so that the forces driving involution can generate consistent and reproducible outcomes in terms of blastopore closure and neural induction. Though bottle cells are not generally essential for completion of gastrulation, loss of precision in cell movements in their absence can be catastrophic for development of the animal.

The cell behavior typified by bottle cells is apical constriction (Hardin and Keller, 1988). Apical constriction entails assembly of a network of filamentous actin arranged either as a circumferential belt of oriented filaments below the apical surface and perpendicular to what will be the axis of elongation (Baker and Schroeder, 1967), or in a somewhat more loosely connect meshwork directly beneath the medioapical plasma membrane (Martin et. al, 2009). Crosslinking these actin fibers are bipolar filaments of non-muscle myosin proteins (Franke et. al, 2005), which walk along actin fibers in a polarized fashion from the pointed end toward the barbed end of actin, such that two antiparallel actin filaments can be made to slide relative to one another to promote contraction of the actomyosin network (Murrell et. al, 2015) (Fig. 1B), and thereby exert tensile force on the cell surface through linkages anchoring the actin cytoskeleton to the plasma membrane via cell adhesion complexes (Mason et. al, 2013). Non-muscle myosin is a hexamer, comprising two heavy chains with ATPase domains responsible for motor activity and capable of binding to filamentous actin, two "essential" light chains, and two regulatory light chains (Ricketson et. al, 2010). Phosphorylation of the regulatory light chain promotes multimerization of myosin into bipolar filaments, and increases affinity of myosin heavy chain for actin, consequently increasing the ATPase activity of the heavy chain (Vicente-Manzanares and Horwitz, 2010) (Fig. 1A). Threonine 18 and Serine 19 of myosin regulatory light chain are targets of the Calmodulin-regulated kinase MLCK (Watanabe, 2007), the Cdc42 effector kinase MRCK (Marston et. al, 2016), as well as the RhoA effector kinases Citron (Yamashiro, 2003) and ROCK1/2, the latter of which can also inhibit the phosphatase responsible for dephosphorylation of Ser19 (Amano, 1996). Phosphorylation-mediated activation of myosin leads to increased

contractility of the actomyosin network, resulting in active reduction of apical cell area and bottle cell morphology.

While the dominant-negative RhoA T19N has been demonstrated to block bottle cell formation (Ossipova et. al, 2015), ROCK inhibitor treatment was ineffective in preventing apical constriction (Lee and Harland, 2007), perhaps pointing to a role for citron kinase, MRCK, or MLCK. In support of a role for the latter component, bottle cell formation is regulated by cytosolic calcium influx in other species (Nakajima and Burke, 1996). It is also possible that the requirement for RhoA might indicate a role for some other effector in this process, most notably the Diaphanous family of formins (Rose et. al, 2005), which promote assembly of unbranched actin filaments (Kovar et. al, 2006). Additionally, Diaphanous proteins, and perhaps formins generally, are capable of recruiting microtubules into non-centrosomal arrays (Ishizaki et. al, 2001), which are often seen in columnar epithelial cells (Akhmanova and Hoogenraad, 2015), and are present in bottle cells at the time of blastopore formation. Microtubule is shown to regulate bottle cell morphology. Furthermore, endocytosis of the apical membrane is observed in *Xenopus* bottle cells, and inhibition of endocytosis with dominant-negative Dynamin results in impairment of apical constriction (Lee and Harland, 2010). This is similar to what is seen upon nocodazole treatment to disrupt microtubules (Lee and Harland, 2010), perhaps indicative of a requirement for transport of cell membrane from the shrinking apical surface toward the expanding basolateral surface by way of traffic of vesicles along apicobasally oriented microtubules.

At the level of transcriptional regulation, the appearance of bottle cells can be blocked by treatment of embryos with the nodal pathway inhibitor SB-431542, as well as

by injection of antisense morpholino targeting the Smad 2/3 associated transcription factor Foxh1, (Chiu et. al, 2014) or the Smad/Foxh1 cofactor Tcf3 (Yoon et. al, 2011). Conversely, injection of TGF-beta family ligands in the animal hemisphere can induce formation of ectopic bottle cells, occurring as a consequence of induction of mesendodermal cell fate program in the animal cells (Kurth and Hausen, 2000). It has generally proven difficult to uncouple the process of bottle cell formation from the pathways required for establishment of mesendodermal cell fate, rendering identification of the molecular mediators of this cellular behavior challenging. To truly know the role of bottle cells in development would require identification of a gene specific to bottle cells and central to their function.

Head Mesendoderm Migration

Even before the bottle cells take shape on the exterior of the embryo, the internal cells of the prospective head mesendoderm have already mobilized to move toward the animal pole in a process called vegetal rotation. As the blastopore lip spreads circumferentially from the dorsal pole to encircle the endoderm, the population of dorsal mesendodermal cells migrates collectively along the blastocoel roof, eventually converging with the ventral mesendodermal cells to form an archenteron, which is the gastrointestinal cavity (Fig. 2A,B,C). These early migrating dorsal mesendoderm cells then differentiate into head mesendoderm in the tadpoles. Migration of these internal cells has a big impact on closure of the mesodermal mantle (Keller, 1976).

Explants of dorsal mesendodermal tissue behave similarly as in vivo and will adhere to and migrate on blastocoel roof (BCR) or on BCR conditioned substrate.

Moreover, the mesendodermal explant displays directional migration toward the region of the BCR that would have represented the animal pole, but migrates randomly upon reaching the animal pole (Winklbauer and Nagel, 1991). This would indicate that the BCR encodes a polarized substrate which provides directional cue(s) that guides mesendodermal migration. The polarity of the BCR was found to depend on FGF and nodal signaling and manifests itself in the assembly of the ECM component fibronectin into organized fibrils, a process which requires FGF signaling and is inhibited by high levels of nodal signaling (Nagel and Winklbauer, 1999). These fibronectin fibrils immobilize dimers of PDGF-alpha, which is expressed in a gradient centered about the animal pole, and present this ligand to PDGF receptor (PDGFR)-alpha, which is expressed in the cells of the prospective anterior mesendoderm (Ataliotis et. al, 1995). The distinct tissue distribution of the ligand and the receptor leads to chemotactic movements of the dorsal (head) mesendoderm.

At the cellular level, the anterior mesendoderm has been described as resembling shingles on a roof, with multiple rows of cells underlapping each other and extending monopolar protrusions uniformly oriented toward the animal pole (Winklbauer and Nagel, 1991) (Fig. 2D). Adhesion of mesendodermal cells to the blastocoel roof is mediated by binding of integrin receptors present on the mesendodermal cells to a layer of extracellular matrix predominantly composed of fibronectin deposited by the BCR cells on the inner surface of the blastocoel cavity. RGD peptide and fibronectin antibody blocks pseudopod protrusion of mesendodermal cells. While this changes the timeline of epiboly and head mesendoderm migration, it does not block blastopore closure (Winklbauer and Keller, 1996). Similarly, targeting PDGF-alpha with antisense

morpholino retards directional migration of mesendodermal cells, resulting in defective induction of anterior neural structures, but does not prevent closure of the blastopore (Nagel et. al, 2004).

At the molecular level, the PDGFR receptor tyrosine kinases dimerize upon binding to their ligand, triggering activation of the kinase domain and transphosphorylation of the receptors (Van Stry et. al, 2005). Three principle downstream effectors of PDGFR-alpha participating in mesendoderm migration are activated via binding to different docking sites created by PDGFR tyrosine phosphorylation. The first is Src non-receptor tyrosine kinase, which can phosphorylate the Abl family non-receptor tyrosine kinases (Plattner et. al, 1999) and the RhoGEF Tiam1 (Servitja et. al, 2003). The second is Phosphatidylinositol 3'-Kinase, which converts PtdIns(4,5)P2 to PtdIns(3,4,5)P3 (Rankin et. al, 1996) and phosphorylates many non-lipid substrates. The third is Phospholipase-C gamma, which can also activate Abl family kinases and hydrolyze PtdIns(4,5)P2 to liberate inositol-1,4,5-trisphosphate, resulting in calcium efflux from the endoplasmic reticulum into the cytosol (Plattner et. al, 2003).

All three of these effector pathways converge on the Wave family of actin nucleation promoting factors (Oikawa et. al, 2004) to influence mesendodermal cell migration. The Wave proteins reside within a heteropentameric complex which holds them inactive (Chen et. al, 2010). A combination of stimuli can activate Wave proteins. The first is tyrosine phosphorylation of key residues within the Wave proteins themselves, which is performed by the Src and Abl kinases. The second is PtdIns(3,4,5)P3, which Wave proteins bind through a conserved patch of basic amino acids, allowing for their translocation to the plasma membrane. The final input is binding

of the Wave protein complex to the Rac family of monomeric small GTPases (Lebensohn and Kirschner, 2009). These small GTPases require RhoGEF proteins such as Tiam1 to promote exchange of GDP for GTP, thereby converting Rac into an active form (Vetter and Wittinghofer, 2001). Integration of all of these signaling inputs releases the catalytic domain of Wave family proteins from repression by its regulatory complex, allowing Waves to nucleate branching of actin off existing actin filaments to generate lamellipodial protrusions (Machesky et. al, 1999).

Protrusion formation at the leading edge imparts tension on cadherin mediated adhesions at the trailing edge of migrating cells, which recruits Plakoglobin, facilitating assembly and engagement of the keratin intermediate filament network (Weber et. al, 2012). Application of tensile force to the keratin network activates Arhgef40, stimulating nucleotide exchange of the monomeric small GTPase RhoA, which in turn promotes assembly of stress fibers (Fujiwara et. al, 2016). Engagement of nascent focal adhesions by these stress fibers allows the cell to exert traction force on the underlying ECM, resulting in clustering of integrins, maturation of focal adhesions, and activation of FAK, which in turn stimulates disassembly of mature focal adhesions, allowing the cell to migrate (Moore et. al, 2010). Injection of morpholino targeting either plakoglobin, xCK1(8), or FAK disrupts directionality of protrusions formed by migrating cells of the mesendoderm, resulting in an embryonic phenotype closely resembling that of PDGF-alpha or Fibronectin knockdown (Bjerke et. al, 2014; Weber et. al, 2012).

Intercalation and Convergent Extension

Whereas the preceding examples have dealt with changes in tissue morphology that involve folding of a sheet of cells, or translocation of a sheet of cells along a surface, intercalation movements serve to change the dimensions of a cell sheet. Blastopore closure in amphibian gastrulation is dependent upon the formation and extension of a folded sheet of tissue originating from the field of cells around the equatorial margin of the blastula. This region is referred to as the marginal zone. It is delimited on the vegetal side by the site of bottle cell formation and extends, along with the gradient of nodal signaling, toward the animal pole (Keller, 2005). This region can be subdivided into the vegetal and the animal halves. The vegetal half is referred to as the involuting marginal zone (IMZ), the superficial layer of which will involute to line the archenteron while the deep layer will contribute to the mesodermal tissues. The animal half, which is ectodermal facing the exterior of the embryo and will generate posterior epidermal and neuroectodermal lineages, is termed the non-involuting marginal zone (NIMZ) (Fig. 3A). At the junction of these two regions is formed the lip of the blastopore (Keller, 1975). Explants taken from the dorsal marginal zone display convergence and extension movements, and are useful in illustration of the simultaneous execution of two morphological processes, one regulating constriction of the blastopore lip, and the other regulating extension of the IMZ and NIMZ tissue, which becomes more pronounced with increasing proximity to the blastopore lip from either the animal or vegetal directions (Keller and Danilchik, 1988). These processes are meant to work in concert in the context of the intact embryo to affect blastopore closure. An explant, which is initially a square piece of DMZ, will over time adopt more of an hour-glass shape, as the cells in the

middle of the explant rearrange to narrow the tissue mediolaterally and extend it along the anteroposterior axis. The narrowest point mediolaterally in the middle of that explant represents the blastopore lip (Fig. 3B). If in this example the IMZ and NIMZ are folded over on themselves, as they would be in the whole embryo, the net effect of this rearrangement would be to extend the mass of tissue toward the posterior of the embryo, and simultaneously reduce the diameter of the blastopore until the vegetal cells are completely enveloped (Fig. 3C).

At the cellular level, the process of convergent extension is driven by intercalation of the deep cell layer, while rearrangements within the superficial cell layer are thought to occur passively (Keller et. al, 1985). This is reflected in differences in the manifestation of convergent extension in epithelial cells versus mesenchymal cell populations, with the former exhibiting neighbor exchange and rosette formation (Nishimura et. al, 2012), while the latter adopt a bipolar cell morphology and actively intercalate between one another (Wilson and Keller, 1991) (Fig. 3D). This characteristic cell morphology is a product of the reduction in overall protrusion formation frequency prior to conspicuous cell elongation and intercalation, followed by mediolateral alignment and bipolar lengthening of cells in the marginal zone (Shih and Keller, 1992).

What makes convergent extension at the tissue scale possible is coordination of oriented protrusion formation across individual cells throughout the entire field of the tissue. Central to this coordination is the non-canonical arm of the Wnt signaling pathway. Initially characterized in *Drosophila*, the core of this pathway comprises the transmembrane proteins Frizzled, Van Gogh, and Flamingo, along with the cytosolic proteins Prickle and Dishevelled, which are recruited to the plasma membrane upon

pathway activation (Seifert and Mlodzik, 2007). In cells of the fly wing epithelia, these proteins form complexes on opposite faces of the cell, with one complex composed of Frizzled and Dishevelled and its counterparty containing Van Gogh and Prickle, while Flamingo is a component of both complexes, residing on both surfaces and is essential for communicating polarization information between cells (Tree et. al, 2002; Feiguin et. al, 2001; Das et. al, 2004; Olofsson et. al, 2014). Orthologs of these core components are present in vertebrates, where they also function in a similar fashion to coordinate polarization among a field of cells within the plane of the tissue (Ossipova et. al, 2015b; Butler and Wallingford, 2015; Devenport and Fuchs, 2008; Aw et. al, 2016). While examples of developmental processes that invoke this pathway are numerous and growing (Tada and Kai, 2012), the most prominent example in the context of *Xenopus* gastrulation are the convergence and extension movements of the marginal zone, which were determined to be the primary engine driving blastopore closure (Keller and Jansa, 1992). Unlike in flies, the vertebrate orthologs of core planar polarity proteins are manifold. For example, multiple Frizzled and Dishevelled genes are expressed during gastrulation with overlapping expression patterns. As a consequence, morpholino-mediated depletion of a particular protein may not result in the expected phenotype owing to compensatory action of the remaining homologs (Zhang et. al, 2011). Nevertheless, several key mediators of the non-canonical Wnt signaling pathway have been tied to the process of convergence and extension in gastrulation (Hikasa et. al, 2002; Darken et. al, 2002; Shibata et. al, 2005; Habas et. al, 2001). While it is important to explore whether non-canonical Wnt components are distributed asymmetrically as in drosophila, studies from different groups have provided somewhat conflicting reports on

this issue. One of the possible reasons is that unlike epithelial cells which are largely static in nature, mesenchymal cells undergoing convergent extension movements are motile and change their positions constantly. The localization of planar polarity components may thus be dynamic. The mechanisms for generation and maintenance of planar polarity are therefore likely to differ between different model organisms and also between different cell types.

While the exact manner in which dorsal mesodermal cells interpret asymmetric cues of planar polarization to generate mediolateral intercalation behavior is not entirely clear at present, some general themes have emerged. First, non-canonical Wnt signal activates Phospholipase-C, leading to production of diacylglycerol which then recruits Dishevelled proteins, the primary mediators of Wnt signaling, to the plasma membrane in complex with PKC-delta (Kinoshita et. al, 2003). Second, the small GTPase RhoA is activated by Dishevelled proteins through the formin family member Daam1 and some unidentified and potentially variable RhoA exchange factor (Habas et. al, 2001; Nishimura et. al, 2012). The small GTPase Rac is also activated through unspecified mechanism(s) (Habas et. al, 2003). The result of these sequential activation steps appears to be modulation of the strength of C-cadherin mediated cell-cell adhesions as well as maturation and turnover of focal adhesions. While C-cadherin function is vital to gastrulation movements (Brieher and Gumbiner, 1994), a reduction in C-cadherin adhesive strength is required for execution of mediolateral intercalation behavior (Zhong et. al, 1999). This can be achieved either through tyrosine phosphorylation of Ctnnb1 by Src family kinases (Vassilev et. al, 2017), direct competition for Ctnnb1-binding sites on cadherins by Vangl proteins (Nagaoka et. al, 2014), or induction of Pcdh8 transcription

by the Ror family of Wnt co-receptors (Brinkmann et. al, 2016), which inhibits C-cadherin-mediated adhesion by an unknown mechanism (Chen and Gumbiner, 2006). Similarly, clustering of integrin receptors to form focal adhesions and turnover of those adhesions appears to be crucial for both extension of mediolateral protrusions (Iioka et. al, 2007) and coordination of the orientation of these protrusions between cells (Davidson et. al, 2006; Muñoz et. al, 2006). Control of both adhesion-related processes seems to rely on cross-talk with the non-canonical Wnt signaling pathway. Activation of RhoA by non-canonical Wnt signaling is the common thread linking both of these cell adhesion pathways through the regulation of actomyosin filament assembly (Schlessinger et. al, 2009). RhoA-dependent stimulation of Myo2b contractility applies tensile forces (Skoglund et. al, 2008) that link cadherins to the actin cytoskeleton (Buckley et. al, 2014) and promote maturation of focal adhesions (Moore et. al, 2010). In contrast to the head mesendoderm where PDGFR receptor tyrosine kinase signal promotes formation of protrusions and stimulates expansion and maturation of focal adhesions via Src and FAK, it would seem that cadherin-based and integrin-based adhesions in the trunk are regulated mainly by application of tensile force downstream of Wnt signaling. Consistent with this, it has been reported that mechanical strain or tissue deformation can help alignment of planar polarity, highlighting the interplay between mechanical and biochemical signals in regulation of planar cell polarity (Aw et. al, 2016). Finally, it should be emphasized that the above list of mediators of convergent extension is by no means exhaustive, and novel signaling intermediates in this pathway as well as the mechanisms that these signaling molecules employ to control convergent extension await further discovery.

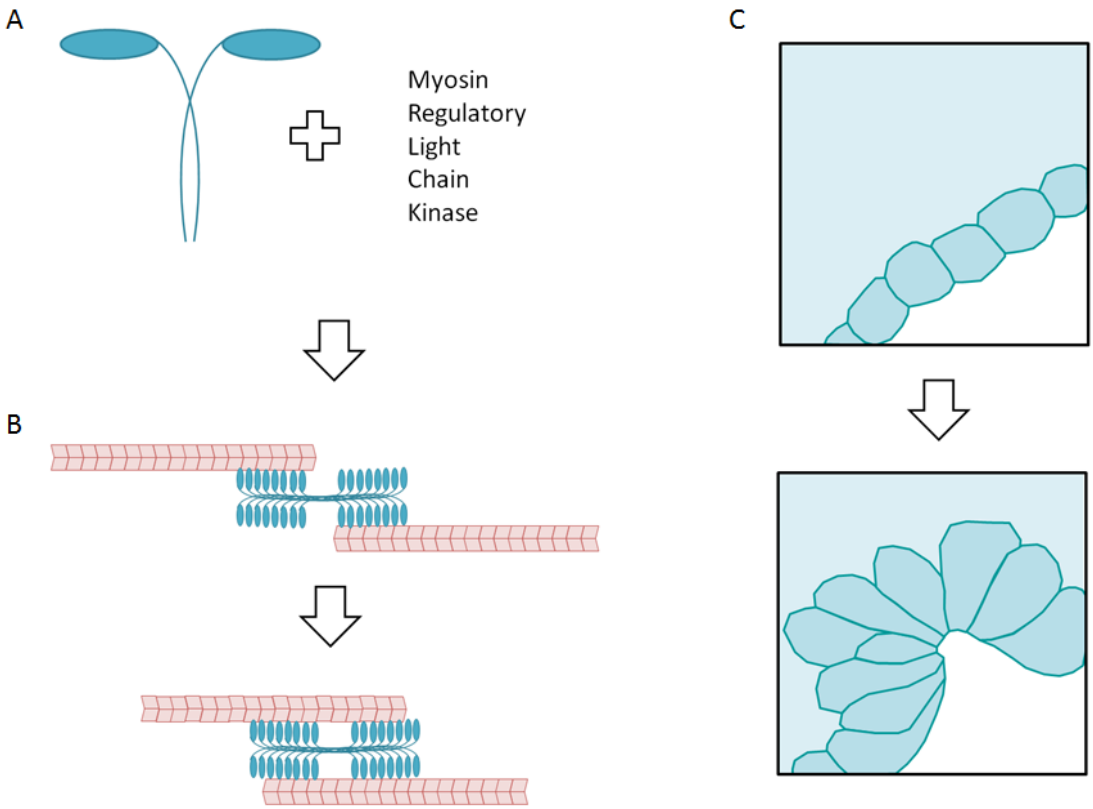

Figure 1. Myosin2b activation induces apical constriction. A) Phosphorylation of myosin regulatory light chain by ROCK, Citron, MRCK, or MLCK promotes activation and multimerization of myosin. B) Myosin translocation along actin filaments brings them toward each other. C) Contraction of the apical cytoskeleton reduces the apical surface area of epithelial cells (outlined), and results in bending of the epithelial sheet.

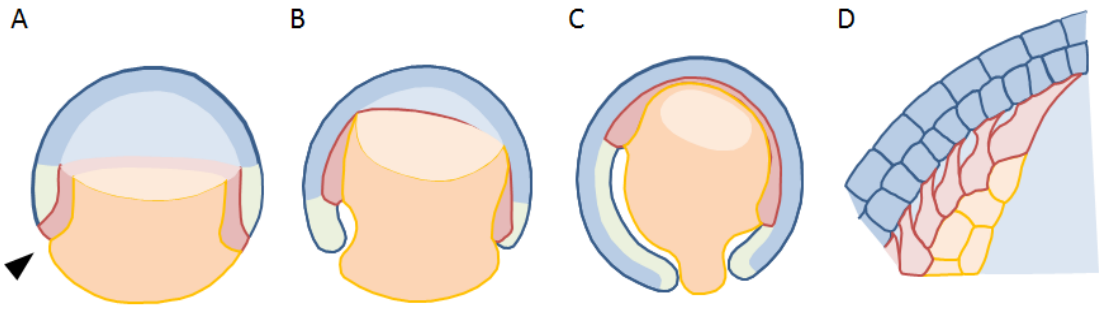

Figure 2. Mesendodermal tissue migrates toward the animal pole. A) *Xenopus* embryo shortly after stage 10 (site of dorsal blastopore lip formation indicated by pointer). B) Head mesendoderm (colored red and yellow) migrates along the inner circumference of the blastocoel roof (lighter coloring indicates blastocoel cavity) toward the animal pole. C) Migrating mesendoderm converges at the animal pole resulting in closure of the mesodermal mantle. D) Cartoon depicting migrating cells (red) extending protrusions along the surface of the blastocoel roof.

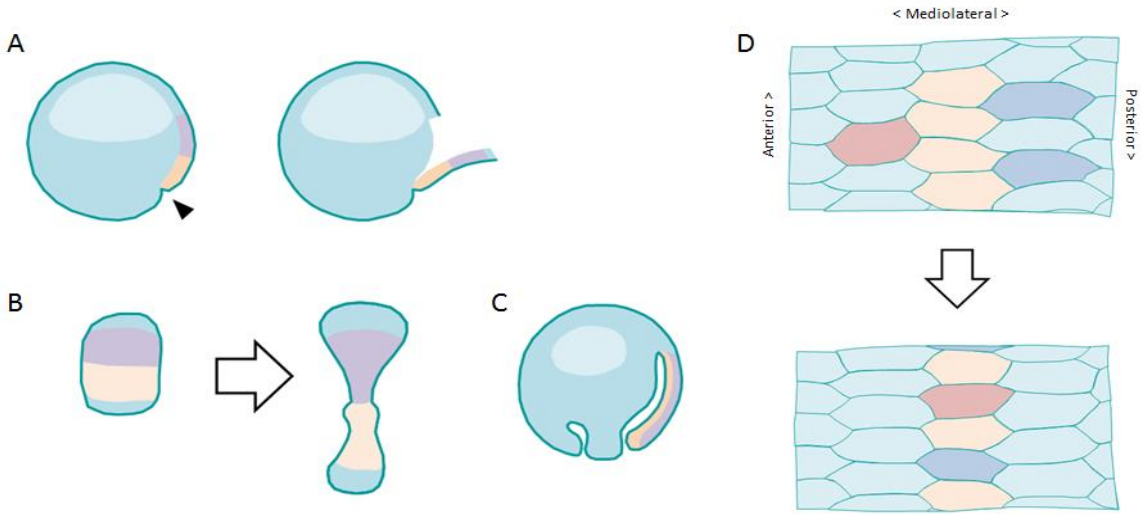

Figure 3. Convergent extension elongates the marginal zone. A) *Xenopus* embryo shortly after stage 10 (site of dorsal blastopore lip formation indicated by pointer, the involuting marginal zone (IMZ) is yellow, while the non-involuting marginal zone (NIMZ) is purple). B) An explant taken of the dorsal marginal zone elongates along its anteroposterior axis and narrows along its mediolateral axis as blastopore closure progresses. C) Position of the IMZ and NIMZ around stage 12, the IMZ forms the dorsal side of the archenteron, the junction between the IMZ and NIMZ represents the blastopore lip. D) Convergent extension is achieved by intercalation of cells along the mediolateral axis to generate lengthening of the anteroposterior axis.

IDENTIFICATION OF NEW REGULATORS OF EMBRYONIC PATTERNING AND MORPHOGENESIS IN XENOPUS GASTRULAE BY RNA SEQUENCING

by

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ABSTRACT

During early vertebrate embryogenesis, cell fate specification is often coupled with cell acquisition of specific adhesive, polar and/or motile behaviors. In *Xenopus* gastrulae, tissues fated to form different axial structures display distinct motility. The cells in the early organizer move collectively and directionally toward the animal pole and contribute to anterior mesendoderm, whereas the dorsal and the ventral-posterior trunk tissues surrounding the blastopore of mid-gastrula embryos undergo convergent extension and convergent thickening movements, respectively. While factors regulating cell lineage specification have been described in some detail, the molecular machinery that controls cell motility is not understood in depth. To gain insight into the gene battery that regulates both cell fates and motility in particular embryonic tissues, we performed RNA sequencing (RNA-seq) to investigate differentially expressed genes in the early organizer, the dorsal and the ventral marginal zone of *Xenopus* gastrulae. We uncovered many known signaling and transcription factors that have been reported to play roles in embryonic patterning during gastrulation. We also identified many uncharacterized genes as well as genes that encoded extracellular matrix (ECM) proteins or potential regulators of actin cytoskeleton. Co-expression of a selected subset of the differentially expressed genes with activin in animal caps revealed that they had distinct ability to block activin-induced animal cap elongation. Most of these factors did not interfere with mesodermal induction by activin, but an ECM protein, *efemp2*, inhibited activin signaling and acted downstream of the activated type I receptor. By focusing on a secreted protein kinase *pkdcc1*, we showed with overexpression and knockdown experiments that *pkdcc1* regulated gastrulation movements as well as anterior neural patterning during early

Xenopus development. Overall, our studies identify many differentially expressed signaling and cytoskeleton regulators in different embryonic regions of *Xenopus* gastrulae and imply their functions in regulating cell fates and/or behaviors during gastrulation.

INTRODUCTION

Allocation of embryonic cells to distinct germ layers is one of the earliest events in vertebrate development. Cells in each germ layer also distinguish from each other according to their locations within the embryos, as cell positions influence their exposures to different maternal and zygotic signaling molecules and transcription factors. This patterning process endows cells not only distinct fates, but also different behaviors that are intimately linked to their fates. Hence, cells fated to become anterior mesoderm and endoderm migrate long distances to reach the head region, whereas cells that contribute to the trunk structures undertake polarized cell intercalation to alter the morphology of the tissues. Coordination of cell fate specification and cell movements in different embryonic regions is critical for proper vertebrate development.

In the frog *Xenopus laevis*, anterior mesendoderm is first manifested at the morphological level by the appearance of a small pigmented line in the vegetal region of early gastrula embryos. Cells surrounding this dorsal lip, the organizer, have three basic properties: they self-differentiate into the head mesoderm and the anterior endoderm; they migrate collectively as a sheet toward the animal, the future anterior, region; and they emit signaling molecules to induce adjacent tissues to adopt dorsal cell fates (Winklbauer, 1990; Winklbauer and Nagel, 1991; Winklbauer et al., 1996; Vodicka and

Gerhart, 1995; Harland and Gerhard, 1997; De Robertis et al., 2001; Heasman, 2006). Cell trailing behind the organizer in involuting gastrula embryos do not spread efficiently on extracellular matrix (ECM) for migration. Instead, these cells actively modify cell-cell contact for directional cell intercalation, resulting in tissue convergence toward the midline and simultaneous extension along the anterior-posterior axis (convergent extension, or CE, cell movements), resulting in elongation of the trunk tissues (Shih and Keller, 1992a, 1992b; Smith and Howard, 1992; Symes et a., 1994; Vodicka and Gerhart, 1995; Keller and Shook, 2004, 2008). Cells located opposite to the organizer will contribute to the ventral and posterior structures. These cells also intercalate among themselves, but preferentially do so to produce multiple cell layers instead of following planar cell intercalation. This results in tissue thickening (convergent thickening, or CT, movements) at the tail end of the embryos (Wilson and Keller, 1991; Keller and Shook, 2008; Keller et al., 2008). Thus, gastrulating *Xenopus* embryos display region-specific cell behaviors corresponding to the distinct differentiation paths of these cells.

The molecular signatures of specific tissues in *Xenopus* gastrulae have been explored for almost three decades, and the functional relevance of tissue-specific molecules in embryonic patterning has also been scrutinized. These studies employ a variety of approaches, including differential gene expression analysis, functional expression library screening, individual candidate gene interrogation, and transcriptional target investigation for effectors of growth factor signals and nuclear proteins. Several prominent signaling pathways have emerged as crucial regulators of the formation and the inducing activity of the organizer (reviewed in Harland and Gerhard, 1997; Heasman,

2006). For example, maternal Wnt and zygotic nodal signals are shown to cooperate to induce the organizer, whereas the ventralizing and the caudalizing activities of bone morphogenetic proteins (BMPs) and zygotic Wnts are modulated by organizer-secreted BMP and Wnt antagonists. Fibroblast growth factors (FGFs) also participate in early embryonic induction and patterning. A plethora of transcription factors act downstream of these signals to control germ layer specification and dorsal-ventral patterning. Although these studies greatly advance our knowledge on cell fate determination, relatively limited molecules have been uncovered that can control cell movements without affecting cell fates. The most investigated signaling is the planar cell polarity (PCP) pathway, which regulates mediolateral cell intercalation during CE (reviewed in Wallingford et al., 2002; Roszko et al., 2009; Skoglund and Keller, 2010; Wallingford, 2012). Different tyrosine kinase signals have also been shown to affect gastrulation morphogenesis (Ataliotis et al., 1995; Conlon and Smith, 1999; Nutt et al., 2001; Nagel et al., 2004; Sivak et al., 2005; Nie and Chang, 2007a, 2007b; Damm and Winklbauer, 2011; Park et al., 2011; Evren et al., 2014). The PCP and the tyrosine kinase pathways may converge to control the activities of Rho family small GTPases to influence cell behaviors (Habas et al., 2001, 2003; Choi and Han, 2002; Pendo-Mendez et al., 2003; Tahinci and Symes, 2003; Ren et al., 2006). However, it is unclear whether other signals are involved, what common and divergent signal transducers are employed, and how factors involved in embryonic patterning may influence the expression and/or the function of the molecules involved in cell movements.

To fully understand molecular integration of cell fate and cell motility, it is imperative that we survey all the expressed genes in embryonic tissues with distinct fates and behaviors and investigate the function of the genes that show differential expression patterns. Several previous studies have been conducted to use non-biased approaches, such as differential library screening or microarray-based strategies, to address roles of differential gene functions in embryonic patterning (Sasai et al., 1994; Altmann et al., 2001; Munoz-Sanjuan et al., 2002; Smith and Harland, 1992; Wessely et al., 2004; Baldessari et al., 2005; Peiffer et al., 2005; Taverner et al., 2005; Hufton et al., 2006). Though productive, these studies tend to favor the discovery of genes with abundant expression due to technical limitations. Recent advances in genomic level analysis of gene expression promise to overcome the previous limitations and open the door for whole transcriptome study of each tissue without any bias. In this work, we employed the RNA sequencing (RNA-seq)-based strategy to analyze differentially expressed genes in the organizer, the dorsal trunk tissue, and the ventral-posterior region. We uncovered many known patterning molecules as well as scores of uncharacterized genes that might potentially regulate embryonic patterning and/or morphogenesis. Using activin-induced animal cap assay, we showed that several uncharacterized, differentially expressed genes had distinct ability to modulate elongation of the animal caps without affecting mesodermal cell fates. However, one ECM protein, *efemp2*, inhibited mesodermal induction by activin. Using both gain- and loss-of-function assays, we further demonstrated that the secreted protein kinase, *pkdcc1*, regulated gastrulation movements and anterior neural patterning during early *Xenopus* development.

RESULTS

RNA-sequencing analysis of differentially expressed genes in the organizer, the dorsal trunk, and the ventral-posterior tissues

To understand how cells in different embryonic regions acquire distinct cell fates and migratory behaviors, we performed an RNA sequencing experiment using tissues dissected from three regions of gastrulating *Xenopus* embryos. The early organizer, which forms anterior mesendoderm and migrates collectively on the ECM, was dissected from stage 10 embryos as the tissue surrounding, and including, the dorsal lip. The dorsal and ventral marginal zones (DMZ and VMZ) that encompass the dorsal trunk and the ventral-posterior tissues that display CE or CT behaviors, respectively, were dissected from stages 11 to 11.5 embryos from the regions above the blastopore (Fig. 1). Total RNA was extracted and subjected to sequencing on Illumina HiSeq2000 platform. Two independent dissections and RNA-seq experiments were performed, and the results were analyzed based on *Xenopus laevis* genome version 7.1 (Suppl. Table 1). Subsequent analysis based on newly released genome version 9.1 validated the genes we identified (Suppl. Tables 1 and 2; also see Materials and Methods).

Pairwise comparison of the transcriptomes as well as ANOVA-like analysis for all three sample sets revealed differentially expressed genes in each region (Suppl. Table 1 and data not shown). These included the known organizer-specific genes, such as Nodal-related 3 (Xnr3) and Siamois, the dorsal trunk genes, such as Sonic Hedgehog (Shh) and Follistatin (Fst), and the ventral genes, such as Sizzled (Szl) and Vent

transcription factors (Hemmati-Brivanlou et al., 1994; Ekker et al., 1995; Lemaire et al., 1995; Smith et al., 1995; Onichtchouk et al., 1996; Salic et al., 1997). In addition, hundreds of previously uncharacterized genes were uncovered from the study. A greater number of genes showed significant enrichment in the DMZ than either the organizer or the VMZ (320 and 98 genes were expressed at higher levels in the DMZ and the organizer, respectively; and 177 and 85 genes were enriched in the DMZ and VMZ, respectively). Among the genes with higher expression in the organizer, several seemed to encode proteins involved in the endodermal development, such as GATA4, Mix1, nodal-related 6, and KLF4 (Rosa, 1989; Lemaire et al., 1998; Takahashi et al., 2000; Weber et al., 2000; Cao et al., 2012). These genes might play a general role in germ layer specification rather than dorsal-ventral (DV) patterning, though several have also been implicated in cell spreading and migration of the leading edge mesendoderm (e.g. Mix1 and GATA4, Wacker et al., 1998; Fletcher et al., 2006). Other genes in this group included many transcription factors and signaling molecules, as well as a number of genes involved in metabolism, stress responses to DNA-damage or unfolded proteins in the ER, and ion and solute transporters (Suppl. Table 1). Interestingly, several genes encoded factors that might regulate Rho family signaling or actin organization. These included *plekhg5*, a Rho guanine nucleotide exchange factor (GEF) family member, *cdc42ep2*, an effector protein of Cdc42, *cass4*, a member of the Cas scaffolding protein family that is often involved in integrin signaling, and *filip1*, a Filamin A interacting protein. The preferential expression of these genes in the organizer implied that they might regulate anterior mesendoderm cell migration on the ECM. Of the genes with significant enrichment in the DMZ, 84 of them were expressed at higher levels in the

DMZ than that in both the organizer and the VMZ, whereas the rest showed differential expression over only one tissue (Suppl. Table 1 and data not shown). There was a high representation of transcription factors and signaling molecules in this group. Additionally, there were many ECM proteins and ECM receptors, such as laminin β1, different collagens (e.g. type V α1, XI α2, and XIII α1), and integrins (e.g. integrin α2 and α3). Regulators and effectors of Rho family GTPases were also present, such as *arhgef3* and *fgd5*, both are RhoGEFs, and *cdc42ep3*. These ECM proteins and cytoskeleton regulators might participate in modulation of directional intercalation cell behaviors in the DMZ. The genes enriched in the VMZ comprised a large number of transcription factors that had been associated with ventral cell type development, including the Vent, Msx, Dlx, AP2, Grainyhead, GATA, and p63 family members. Signaling molecules and cytokeratin genes were also found in this group, but genes that might control dynamic cytoskeleton organization were conspicuously missing when compared with those expressed in the DMZ (Suppl. Table 1). Taken together, the data suggest that region-specific transcriptomes in *Xenopus* gastrulae contain specific members of ECM proteins and actin regulators in addition to signaling molecules and transcription factors. These ECM and actin regulatory genes may be co-modulated with patterning factors that determine cell lineages in each region, so that cells with particular fates can adopt appropriate migratory behaviors for correct morphogenesis.

Differential expression of region-specific genes during early *Xenopus* development

To confirm the differential expression patterns of the genes identified from our RNA-seq experiments, we dissected the organizer, the DMZ and the VMZ explants as

above and performed RT-PCR to examine the expression of a subset of the genes. We focused our attention on potential regulators of embryonic patterning and movements and thus selected a group of ECM proteins, cytoskeleton regulators, signaling molecules and transcription factors. Assay for known markers expressed in the dissected regions attested the identity of these tissues (Fig. 2A). Examination of the other genes showed that they could be divided into five groups based on their RT-PCR patterns (Fig. 2 and Suppl. Fig. 1, summarized in Suppl. Table 3). Group I genes were expressed at the highest levels in the organizer and included the genes *plekhg5*, *cdc42ep2*, *cass4* and *filip1*. Group II genes showed higher expression in the DMZ than both the organizer and the VMZ, and included the ECM proteins *sparc*, *efemp2*, the signaling molecules *gprc5c*, *pskh2*, the transcription factors *atf3*, *zc4h2*, and the RhoGEF *arhgef3*, among others. Group III genes were expressed at higher levels in both the organizer and the DMZ, whereas group IV genes showed high level expression in both the DMZ and the VMZ versus the organizer. Group V genes were enriched in the VMZ (Fig. 2, Suppl. Fig. 1, and Suppl. Table 3). In each group, some genes were probably abundantly expressed and showed strong signals, such as *cdc42ep2* and *gprc5c*, but some others showed low levels of expression and weak PCR signals, such as *lpar5* and *pskh2*. The results indicate that RNA-seq not only uncovers abundant genes, but also identifies genes with weak, but nonetheless differential, expression profiles. The patterns of these genes in specific regions imply that they may play roles in tissue specification and/or morphogenesis in distinct embryonic regions.

Distinct regulation of activin-induced animal cap elongation by differentially expressed genes

Molecules with the ability to pattern embryonic tissues during *Xenopus* gastrulation have been identified and described in some details for many genes, but factors that can modulate cell motility without affecting cell fates are relatively less studied. To uncover additional regulators of cell movements, we employed the animal cap assay system. Animal caps excised from the blastula ectoderm normally develop into atypical epidermis and assume a round morphology. However, when activin is included in the system, it induces dorsal mesoderm which undergoes CE movements, resulting in elongation of the animal caps (Chang, 2016). Factors that modulate CE movements can block activin-induced animal cap elongation when ectopically expressed. Using this assay system, we surveyed several molecules uncovered from our RNA-seq analysis for their ability to interfere with animal cap elongation. We chose to analyze two signaling molecules, *gprc5c* and *cxcr7*, three RhoGEF members, *arhgef3*, *plekhg5* and *fgd5*, three CDC42 effector proteins, *cdc42ep2*, *cdc42ep3* and *cdc42se2*, three transcription factors, *atf3*, *fos* and *klf10*, and two ventrally-enriched Ras family proteins, *diras2* and *rasl11b*. Both *gprc5c* and *cxcr7* are seven transmembrane domain-containing G-protein coupled receptors (GPCRs) and showed higher expression in the DMZ. The regulators and the effectors of Rho family GTPases displayed differential expression in the organizer and the DMZ, whereas the three transcription factors showed higher expression in the DMZ over the organizer (Fig.2, Suppl. Fig. 1; also see *in situ* patterns of *arhgef3* and *cxcr7* in Hufton et al., 2006 and Mishra et al., 2013). We co-injected RNAs encoding these molecules with activin RNA into the animal region of two-cell stage embryos, dissected

the animal caps at the blastula stages, cultured them to late neurula stages, observed the morphology of the caps, and examined the mesodermal marker expression in these caps (Fig. 3). In addition, we also used activin protein rather than activin RNA in these assays and obtained similar results (Suppl. Fig. 2).

As shown in Fig. 3, while none of these genes affected dorsal mesodermal induction by activin, they displayed different capacity in reducing animal cap elongation. Both the transmembrane GPCRs, *gprc5c* and *cxcr7*, reduced the elongation of activin-induced animal caps, and *gprc5c* also seemed to influence cell adhesion, as increased cell shedding was observed (Fig. 3A, Suppl. Fig. 2B, and data not shown). In contrast, among the three RhoGEF proteins, *arhgef3* alone displayed high efficiency in blocking animal cap elongation (Fig. 3B and Suppl. Fig. 2D). Similarly, CDC42 effector proteins showed differential ability to interfere with activin-induced animal cap elongation, with *cdc42ep3* as the most effective inhibitor. At higher doses, *cdc42ep2* and *cdc42se2* also blocked animal cap elongation, and *cdc42se2* tended to cause dissociation of the animal caps, implying a role of this protein in regulation of cell adhesion (Fig. 3C, Suppl. Fig. 2D and data not shown). The transcription factors *atf3* and *fos* also reduced animal cap elongation when ectopically expressed with activin, but *klf10* did not have much effect (Fig. 3D and Suppl. Fig. 2C). The two ventrally-enriched Ras family members could not block activin-induced animal cap elongation (Fig. 3E). Our results reveal that differentially expressed proteins belonging to the same gene family may have differential activities in modulating cell behaviors during gastrulation.

Efemp2, an ECM protein, regulates activin signaling downstream of the activin receptors

Several ECM genes were differentially expressed according to our RNA-seq analysis (Fig. 2, Suppl. Fig. 1, and Suppl. Tables 1 and 3). As ECM proteins often modulate cell adhesion and signaling to influence cell motility, we examined the effect of ectopic expression of two ECM genes, *sparc* and *efemp2*, on activin-induced animal cap elongation. Co-expression of *efemp2* with activin efficiently blocked animal cap elongation. However, unlike the other genes we assayed, *efemp2* inhibited dorsal mesodermal induction by activin, suggesting that it regulated mesodermal cell fate to affect cell movements indirectly (Fig. 4A). To see whether *efemp2* may block activin from interacting with its receptors to prevent downstream signaling, we co-expressed *efemp2* with a constitutively active type I receptor CA-ALK4, which stimulated activin signaling independent of the activin ligand (Chang et al., 1997). Interestingly, *efemp2* inhibited dorsal mesodermal induction by CA-ALK4 as well (Fig. 4B). However, co-expression of *efemp2* with Smad2, the cytoplasmic signal transducer of activin/nodal signaling, did not interfere with mesodermal induction or animal cap elongation (Fig. 4B). The data suggest that *efemp2* blocks activin signaling downstream of the activated receptors but upstream of Smad2. Examination of BMP-dependent ventral mesodermal induction in animal caps indicated that *efemp2* did not inhibit BMP signaling (data not shown), demonstrating that *efemp2* preferentially regulates the activin/nodal branch of the TGF-β signaling.

Pkdcc1, a secreted protein kinase, regulates gastrulation and anterior neural patterning during early *Xenopus* development

To confirm the function of differentially expressed genes in early *Xenopus* development, we need to perform loss-of-function studies. For this purpose, we focused our attention on one gene that we identified that had higher expression in the organizer and the DMZ over the VMZ (Fig. 2). The gene, *pkdcc1* (also known as VLK, or vertebrate lonesome kinase), encoded a novel secreted protein kinase that likely exerted its function in the extracellular space (Bordoli et al., 2014). RT-PCR assay demonstrated that *pkdcc1* was first expressed during early gastrulation (stage10), and its expression persisted until at least late tailbud stage (Fig. 5A). In situ hybridization revealed that *pkdcc1* transcripts were localized around the organizer at stage 10, but were subsequently up-regulated in the anterior neural domain at the mid- to late gastrula stages. The anterior neural expression remained throughout the neurula stages. In tailbud embryos, *pkdcc1* was detected in the eyes, the lateral plate mesoderm, and the heart primordium (Fig. 5B). Co-expression of RNAs encoding *pkdcc1* and activin resulted in reduction of activin-induced animal cap elongation without defects in dorsal mesodermal cell fates (Fig. 5C). Overexpression of *pkdcc1* RNA in early frog embryos led to severe gastrulation defects. The affected embryos showed shortened and bent body axis, reduced head, and split dorsal axis (Fig. 5D). These defects occurred in the absence of inhibition of the pan-mesodermal marker Brachyury (Bra, Smith et al., 1991) at the gastrula stages, though *in situ* hybridization indicated that the cells expressing Chordin (Chd, Sasai et al., 1994) and Goosecoid (Gsc, Cho et al., 1991) showed delayed involution inside the embryos (Fig.

5E). These results indicated that ectopic *pkdcc1* likely interfered with gastrulation morphogenesis without affecting mesodermal cell fates.

To further examine the role of *pkdcc1* in early *Xenopus* embryogenesis, we designed a translational blocking antisense morpholino oligo (*pkdcc1*-MO) that hybridized to the 5'-untranslated region (5'-UTR) of the *pkdcc1* transcript (Fig. 6A). Injection of *pkdcc1*-MO into the dorsal marginal zone region of 4-cell stage embryos resulted in dose-dependent reduction of the head structure as well as the body axis, and the defects were largely rescued by a construct that contained only the coding region, but not 5'-UTR, of *pkdcc1* (Fig. 6B and data not shown). The reduction in body axis elongation was not accompanied by ventralization of the embryos, as in situ hybridization of marker expression demonstrated the presence of the somatic mesodermal marker MyoD in these embryos (not shown). In addition, at the gastrula stages, the expression levels of Bra, Chd and Gsc appeared normal, but the Chd- and Gsc-expressing cells showed delayed internalization inside the embryos and remained around the blastopore when cells expressing these markers had moved away from the blastopore in control embryos (Fig. 6C). The results demonstrated that knockdown of Pkdcc1 affected *Xenopus* gastrulation without changing mesodermal cell fates.

Since both gain- and loss-of-function of *pkdcc1* resulted in body axis defects, we inquired whether *pkdcc1* modulated gastrulation movements. We thus analyzed tissue morphogenesis in explanted dorsal mesodermal tissues in culture. Both overexpression and knockdown of Pkdcc1 led to impaired CE movements, as the DMZ explants

dissected from the RNA- or MO-injected embryos displayed decreased length to width ratio when cultured in vitro (Fig. 7A). We also assayed for migration of anterior mesendoderm by dissecting the organizers from the injected embryos at stage 10+ and plating them on fibronectin-coated tissue culture dishes. Examination of the explants showed that there were no discernable defects in collective cell sheet migration when the anterior mesendodermal explants were cultured for about 6 hours. However, prolonged incubation for over 8 hours resulted in dissociation of some peripheral cells from the core explants, and the effect was more pronounced in explants from the morphant embryos (Fig. 7B). The data implied that *pkdcc1* might play a role in cell adhesion during collective migration of the anterior mesendoderm.

Since we observed distinct microcephaly in morphant embryos, we analyzed whether this was due to impairment of dorsal tissue formation, or it was caused by defects in anterior neural development. We thus examined dorsal mesodermal and anterior-posterior neural markers by *in situ* hybridization (Fig. 8). The notochord marker Chordin (Sasai et al., 1994) and the paraxial mesodermal marker MyoD (Hopwood et al., 1989) were expressed normally in the morphants, and the pan-neural marker Sox2 (Mizuseki et al., 1998) was not affected either. However, the expression domain of the forebrain/midbrain marker Otx2 (Blitz and Cho, 1995; Pannese et al., 1995) was reduced, and there was a concurrent anterior shift of the midbrain marker Engrailed (En, Brivanlou and Harland, 1989) in the morphants (Fig. 8). The results suggested that anterior neural patterning, but not dorsal-ventral axis specification, was defective in *pkdcc1* morphants.

DISCUSSION

Since the discovery of the organizer by Mangold and Spemann about a century ago (see a translated version of Spemann and Mangold, 2001), there has been an enduring fascination of the molecules synthesized by the organizer that can influence the surrounding tissues to adopt axial and paraxial cell fates (Harland and Gerhard, 1997; Gerhart, 2001; De Robertis, 2001). Multiple growth factor signals, including activin/nodal, BMP, Wnt, and fibroblast growth factor (FGF), have been shown to modulate organizer formation and/or activities. The downstream targets of these signals have also been investigated, including at the genomic scale (Wessely et al., 2004; Perffer et al., 2005; Hufton et al., 2006; Chiu et al., 2014; Gupta et al., 2014). However, most of the functional studies are focused on genes with the ability to induce or pattern embryonic tissues, and some large scale studies also employ techniques that are biased toward abundant genes. Organizer molecules that may control cell polarity, adhesion or motility have not been explored with the same rigor. Similarly, while it is known that temporal changes in tissues surrounding the dorsal blastopore is associated with anterior-posterior (AP) patterning of the body axis (Slack and Tannahill, 1992; Durston and Zhu, 2015), factors that may link AP cell fates and distinct motile cell behaviors are not well scrutinized. To acquire a more detailed understanding of how cells destined to differentiate into specific lineages also adopt particular motile behaviors, we performed RNA-seq analyses in this study to gain insight into distinct transcriptomes in tissues with defined cell fates and migratory patterns.

Our studies uncover a plethora of transcription factors and multitude of signaling molecules differentially enriched in the organizer, the DMZ or the VMZ. Many of these genes have been linked to cell fate determination events in previous reports. The VMZ-enriched factors are especially conspicuous in their abundance in signaling and transcription factors, with only a minor portion encoding metabolic and adhesion molecules (Suppl. Table 1). In contrast, both the organizer and the DMZ contain many distinctive potential regulators of cytoskeleton and/or cell motility, such as the ECM proteins and the actin regulators. In comparing the differentially expressed genes, we reason that those enriched in the DMZ over the organizer, but not the DMZ over the VMZ (group IV), may potentially play roles in AP patterning and/or general cell intercalation behaviors, regardless whether it is CT or CE. The observed lack of VMZ-specific enrichment of actin regulators may indicate that the type of the cell movements in the VMZ, convergent thickening, is probably the default cell behaviors in the trunk. Acquisition of new actin regulators and ECM proteins in the DMZ over the VMZ (group II) may then help to endow the DMZ cells to respond to specific dorsal or ECM signals to undergo CE. Thus, group II genes may be important for dorsal cell fates as well as CE, whereas group IV genes may be crucial for caudal cell specification and CT movements. The genes that are differentially expressed in the organizer and the DMZ (group I and II), in addition to having an effect on embryonic patterning of the head and the trunk, may also differentially influence cell-ECM and cell-cell interactions to modulate cell migration on ECM or over other cells. It is interesting to note that several genes in our dataset, such as *arhgef3*, *irg1* and *rasl11b*, overlap with those with altered expression when an ectopic organizer was induced in the ventral side by simultaneous inhibition of

BMP and Wnt signals (Hufton et al., 2006). This suggests that the organizer signals can influence the expression of genes with roles in cell movements, such as *arhgef3*, to coordinate cell fate and motility.

Our initial analyses of a group of signaling, transcription and cytoskeleton regulatory factors show that members of the same gene family often have distinct functions in regulating CE in activin-induced animal caps. The difference in functionality of the genes is often associated with different expression patterns of these genes. For example, the organizer-enriched RhoGEF *plekhg5* is not effective in blocking activin-induced animal cap elongation, but the DMZ-enriched RhoGEF *arhgef3* interferes with CE in dorsalized animal caps efficiently. Similarly, among the Cdc42 effectors, *cdc42ep3*, which is expressed at high levels in both the organizer and the DMZ, displays the greatest activity in preventing activin-induced animal cap elongation, whereas *cdc42ep2*, which is enriched in the organizer only, has a weaker activity in this assay. The results suggest that distinct members of RhoGEF and Cdc42EP families may detect and respond to different signals in the head and the trunk regions to modulate actin organization and cell behaviors. The sequences outside the conserved GEF or Cdc42 binding domains are therefore crucial to determine the potency and/or the specificity of gene functions. Recognition of the sequence motif(s) that confers specific activities to particular family members is thus important in the future for us to understand how genes belonging to the same family possess unique functions in regulating different migratory cell behaviors during *Xenopus* gastrulation.

In addition to genes that regulate cell movements without affecting cell fates, we have also identified an ECM protein as a new regulator of the activin signaling. The gene, *efemp2*, contains multiple EGF and calcium-binding EGF domains and is also known as fibulin-4. Two other fibulin family genes have been reported to regulate TGF-β family signaling. Fibulin-1 regulates BMP signaling through direct binding to BMP-2 (Cooley et al., 2014), whereas Fibulin-3 inhibits TGF-β1 signaling by interacting with TβRI/ALK-5 to prevent formation of a functional ligand-receptor complex (Tian et al., 2015). Fibulin-4 mutations are associated with patients with aortic aneurysms, arterial tortuosity and stenosis. TGF-β signaling is enhanced in these patients, though the underlying molecular mechanism has not been shown (Renard et al., 2010). In mice, Fibulin-4 deficiency results in increased expression of TGF-β ligands in isolated aortic smooth muscle cells, leading to enhanced TGF-β signaling (Ramnath et al., 2015). In our study, we find that *efemp2* inhibits activin signaling downstream of the activated receptor ALK-4, suggesting that it may regulate other growth factor signals to stimulate a distinct pathway to block activation of the Smad2/3 signal transducers by activated ALK receptors. Further experiments are required to understand the detailed mechanisms underlying *efemp2* function.

To investigate the roles of endogenous genes that show differential expression during gastrulation, we focused on *pkdcc1* in this study. The protein, also known as VLK, is shown to be a novel secreted tyrosine kinase that can phosphorylate a broad range of extracellular molecules and ectodomains of transmembrane proteins. These include many ECM proteins, such as collagens, and matrix metalloproteinases (MMPs),

such as MMP1 and MMP14. Phosphorylation of these proteins can potentially control their activities and impact on cell adhesion and migration (Bordoli et al., 2014). Targeted disruption of Pkdcc/Vlk gene in mice results in multiple developmental defects, such as short limb, cleft palate, and lung hypoplasia (Imuta et al., 2009; Kinoshita et al., 2009; Probst et al., 2013). In *Xenopus*, *pkdcc1* and 2 have been reported to regulate JNK-dependent planar cell polarity pathway to control blastopore and neural tube closure (Vitorino et al., 2015). In our studies, we show that *pkdcc1* regulates gastrulation movements and anterior neural patterning. *pkdcc1* modulates CE of the trunk mesoderm and the sustained cohesion of the anterior mesendoderm. These phenotypes partially mimic those associated with altered expression of Arg/Abl2 (Bonacci et al., 2012), implying that Pkdcc1 may influence or converge with Arg/Abl2-dependent signaling pathway. As the transcripts of *pkdcc1* are expressed at high levels in the organizer and the anterior neural tissues, but not abundantly in the trunk mesoderm, it implies that the secreted protein product may diffuse a long distance to influence cell behaviors in the trunk mesoderm. *pkdcc1* also regulates anterior neural patterning, with the forebrain most sensitive to the reduction of Pkdcc1 levels. The AP neural patterning is controlled by multiple growth factor signals, including Wnt, FGF, BMP, and retinoid acid (Slack and Tannahill, 1992; Chang and Hemmati-Brivanlou, 1998). Pkdcc1 may phosphorylate one or several extracellular components of these signals to influence the development of the anterior neural tissues. It is likely that the targets of Pkdcc1 in the mesoderm and the neural tissues may differ. For example, one of the known targets of *pkdcc1* is *mmp14*, which has been shown to modulate CE in zebrafish (Coyle et al., 2008; Williams et al., 2012). In contrast, potential regulation of the canonical Wnt signaling may contribute to

the neural patterning activity of *pkdcc1* (Bordoli et al., 2014). Modification of distinct substrates in different tissues will allow Pkdcc1 to regulate cell movements and cell fates in two different developmental contexts.

In summary, we have uncovered in this work many differentially expressed genes in different embryonic tissues during gastrulation. These not only include factors that can control embryonic patterning, but also proteins that may potentially regulate cell adhesion, polarity and motility to influence cell movements on ECM (migration) or over other cells (intercalation). Further studies employing loss-of-function approaches are required to interrogate the endogenous roles of these genes in particular cell movements during gastrulation, and how expression and/or activities of these proteins are modulated by signals that impart cell fates. These studies promise to provide more in depth understanding on how cell fate specification and cell behaviors are coordinated during vertebrate embryogenesis.

MATERIALS AND METHODS

Embryo culture and explant dissection

The *Xenopus* embryos were obtained and maintained using standard protocols as described (Sive et al., 2000). The early organizer was dissected from stage 10 to 10.25 embryos by removing the tissues surrounding, and including, the dorsal lip. The DMZ and the VMZ explants were dissected from stage 11 to 11.5 embryos by removing tissues from the dorsal and the ventral sides of the embryos above the blastopore, respectively.

Total about 150 explants were pooled together for each sample. RNA extraction was performed using Qiagen RNeasy mini-spin columns. Two different sets of the samples were prepared independently. The second set of the samples were made at somewhat later stages, which were reflected by stronger expression of certain genes and weaker expression of the others when compared with the first set of the samples. The sample comparison was thus performed independently for each set and combined together. There were variations in calls of differentially expressed genes in the two sets, which might be due to combined effects of natural variations of gastrula embryos (Vodicka and Gerhart, 1995; Ewald et al., 2004) and variations in dissections of pooled samples. RT-PCR was thus performed to confirm and validate the differentially expressed genes, as marker expression in these samples could be easily tested.

RNA sequencing and data analyses

RNA sequencing was performed on Illumina HiSeq2000 platform with 2x50bp paired-end sequencing configuration. Briefly, the quality of the total RNA was assessed using the Agilent 2100 Bioanalyzer followed by 2 rounds of poly A+ selection and conversion to cDNA. The TruSeq library generation kit was used to construct the cDNA library as per the manufacturer's instructions (Illumina, San Diego, CA). The cDNA library was quantitated using qPCR in a Roche LightCycler 480 with the Kapa Biosystems kit for library quantitation (Kapa Biosystems, Woburn, MA) prior to sequencing. Paired-end reads were mapped to the reference *Xenopus laevis* transcriptome 'MayBall' annotation (Chung et al., 2014), based on *Xenopus laevis* genome (version 7.1), using Bowtie (version 1.0.0). After the latest release of *Xenopus*

laevis genome (version 9.1), we re-analyzed our RNA-seq data with it using BWA (version 0.7.12) against primary transcripts (based on JGI version 1.8 annotation) to quantify the expression of L and S homeologs separately (Supplemental Table 2). Differentially expressed genes were identified using edgeR packages (version 3.12.0, Robinson et al., 2010), with both classic pairwise comparison and ANOVA-like multiple comparison. However, we noticed that several genes on our original differentially expressed gene list based on 7.1 genome, such as *arhgef3* and *cxcr7*, were lost in new annotation (although there are two *arhgef3* candidates, Xelaev18003042m and Xelaev18040996m, according to sequence similarity, they were not annotated as *arhgef3* in JGI version 1.8 annotation). Therefore, we kept using the original analysis for differential gene expression as listed in Supplemental Table 1 (only ANOVA-like multiple sample comparison was included in this table).

Animal cap assay

RNAs encoding 2pg activin and 0.25-1ng of RNA-seq clones were injected into the animal region of two-cell stage embryos. Ectodermal explants were excised from blastula stage embryos and cultured in 0.5X MMR solution until late neurula stages. The morphology of the explants was observed and photographed, and the animal caps were subjected to RNA extraction and RT-PCR as described (Chang, 2016). The primers used for RT-PCR are listed in the Suppl. Table 4. Alternatively, animal caps from the embryos injected with RNA-seq clones were dissected at blastula stages and incubated with 8-10ng/ml Activin B protein (R&D Systems) until late neurula stages before they were imaged.

Assays for dorsal mesoderm convergent extension and anterior mesendoderm migration

The DMZ explants were dissected from stage 10.5 to 11 embryos and cultured in vitro in 0.5X MMR solution. The elongation of the explants was examined and photographed at late neurula stages, and the length to width ratio was measured using the NIH ImageJ program (Bonacci et al., 2012). For anterior mesendoderm migration, the organizer region was dissected from stage 10+ embryos and plated on fibronectin-coated dishes. The explants were cultured in DFA solution at the room temperature for about 6 hours before being imaged (Nie et al., 2007a). Some of the explants were allowed for longer incubation for over 8 hours to examine the effect of altered *pkdcc1* on tissue integrity and migration.

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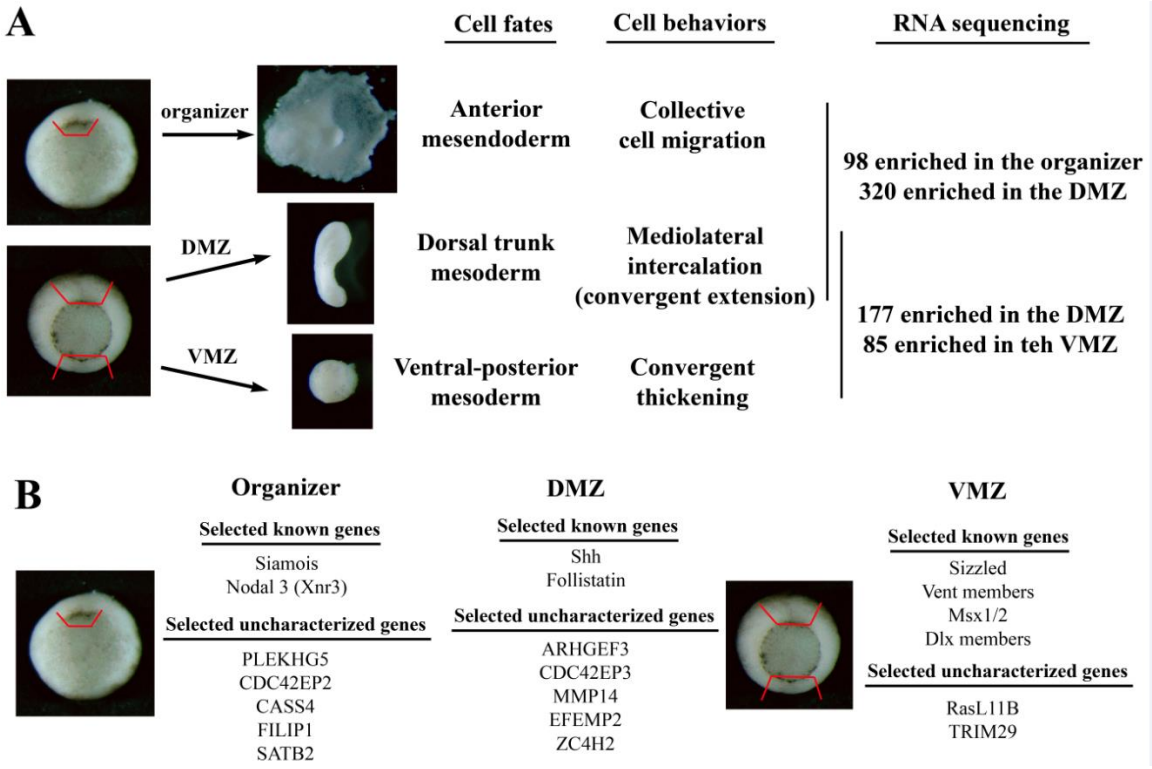


Figure 1. Schematic representation of the RNA-seq experiment. A) The early organizer from stage 10+ embryos and the dorsal and the ventral marginal zone (DMZ and VMZ) explants from mid-gastrula stage embryos were dissected and subjected to RNA sequencing. Cells in these regions have distinct fates and motile behaviors. Pairwise comparison of differential expressed genes was performed between the organizer and the DMZ, and the DMZ and the VMZ, samples. Genes with potential to regulate embryonic patterning and/or movements were especially scrutinized in more detail in this study. B) A list of the selected known and uncharacterized genes with differential expression patterns in different embryonic regions is shown.

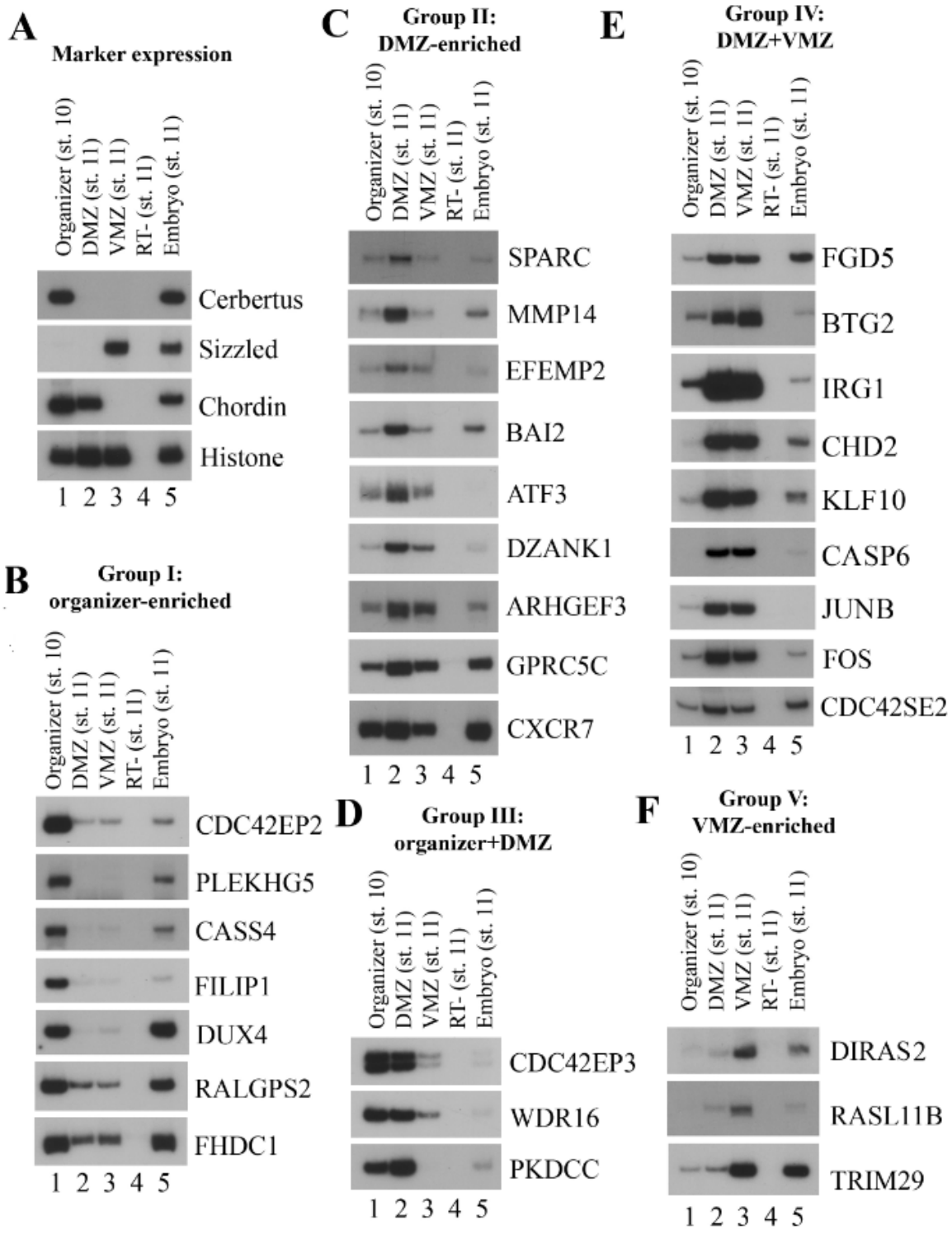


Figure 2. Differential gene expression in the organizer, the DMZ, and the VMZ.

RT-PCR was performed to assay for gene expression in different embryonic regions. A)

Marker analysis confirmed the expression of known region-specific genes in the dissected tissues. B) Group I genes were enriched in the organizer. C) Group II genes showed highest expression in the DMZ. D) Group III genes were expressed at high levels in both the organizer and the DMZ. E) Group IV genes were expressed at high levels in both the DMZ and the VMZ. F) Group V genes were enriched in the VMZ.

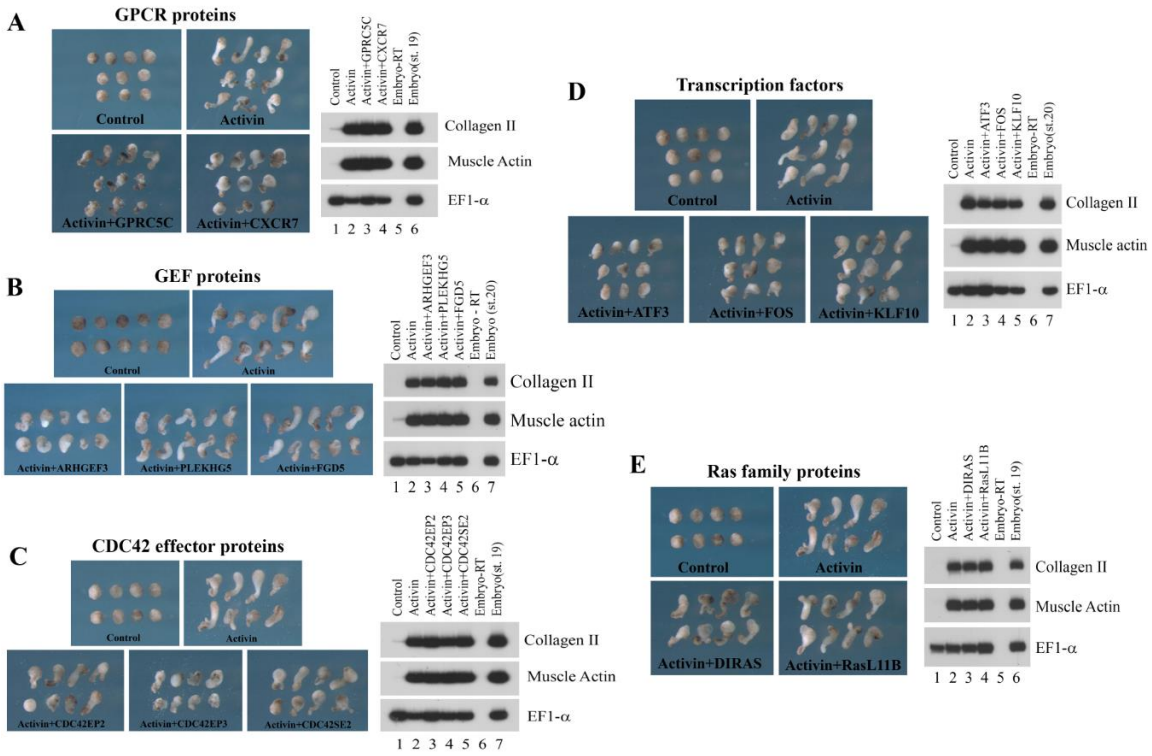
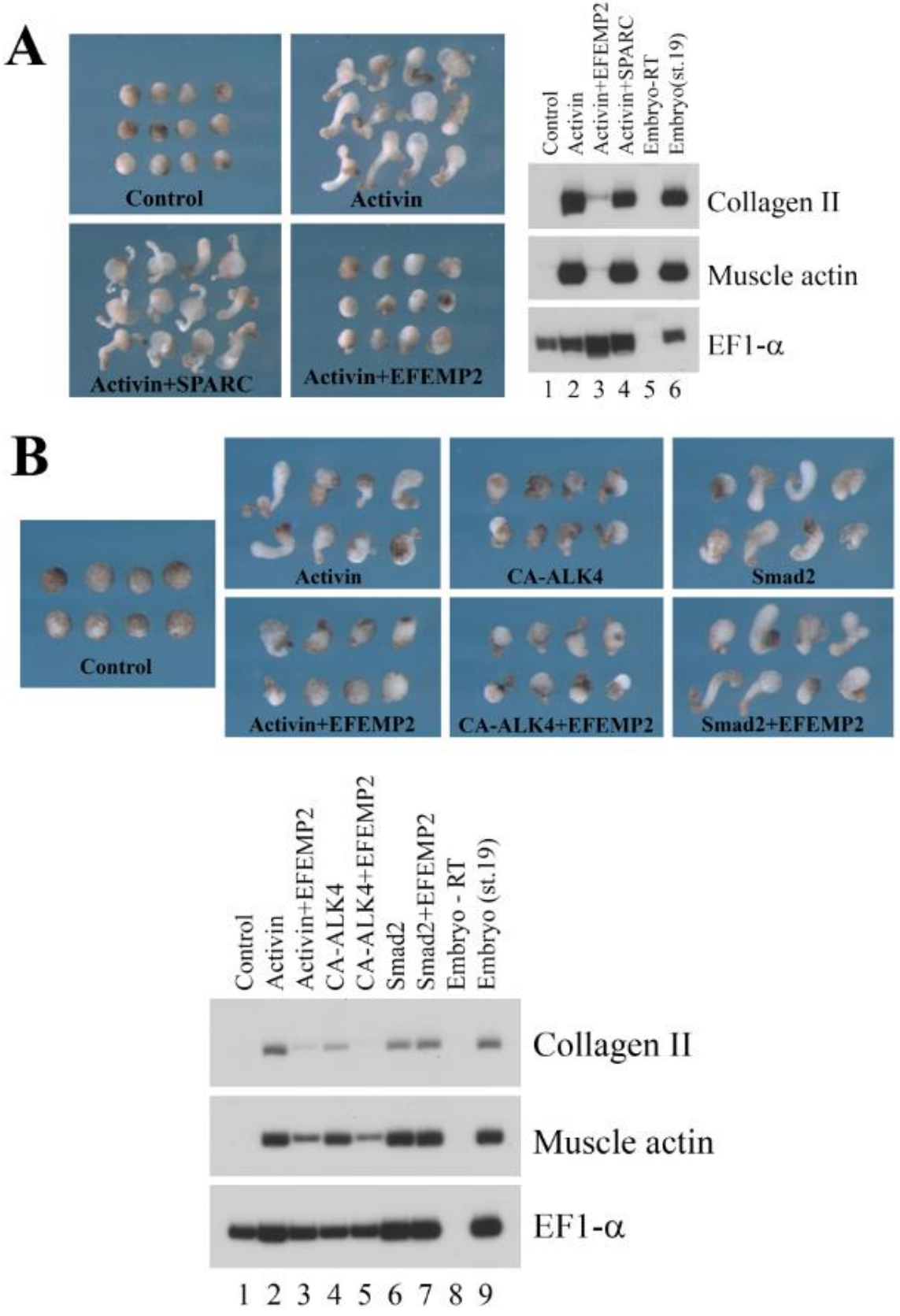


Figure 3. Distinct activities of the differentially expressed genes to block activin-induced animal cap elongation. RNAs encoding the RNA-seq clones and activin were co-injected into the animal region of 2-cell stage embryos. Animal caps were dissected at blastula stages and cultured to late neurula stages. A) Two GPRC proteins with the highest expression levels in the DMZ, *gprc5c* and *cxcr7*, reduced activin-induced animal cap elongation without interfering with the mesodermal induction by activin. B) The DMZ-enriched GEF, *arhgef3*, efficiently blocked animal cap elongation; but the organizer-enriched GEF, *plekhg5*, or RhoGEF expressed in the DMZ and VMZ, *fgd5*, could not do so. C) *cdc42ep3*, which was expressed at high levels in both the organizer and the DMZ, blocked activin-induced animal cap elongation more efficiently than *cdc42ep2*, a gene enriched in the organizer. D) The transcription factors *atf3* and *fos* both reduced the elongation of the animal caps, but *klf10* was ineffective in doing so. E) The

ventrally-enriched Ras family proteins, *diras* and *rasl11b*, did not block activin-induced animal cap elongation. The doses of RNA used: activin, 2pg; RNA-seq clones, 0.25-1ng.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Control
Activin
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Collagen II
Muscle actin
EF1-α
1 2 3 4 5 6
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Control
Activin
CA-ALK4
Smad2
Activin+EFEMP2
CA-ALK4+EFEMP2
Smad2+EFEMP2
Control
Activin
Activin+EFEMP2
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CA-ALK4+EFEMP2
Smad2
Smad2+EFEMP2
Embryo - RT
Embryo (st.19)
Collagen II
Muscle actin
EF1-α
1 2 3 4 5 6 7 8 9

Figure 4. The ECM protein Efemp2 inhibits activin signaling downstream of the activated receptor. A) The ECM protein Efemp2, but not SPARC, inhibited activin-induced mesodermal formation in animal caps. B) Efemp2 interfered with mesodermal induction by both activin and the activated type I activin receptor ALK4 (CA-ALK4), but did not inhibit mesodermal induction by Smad2 or Smad2-induced animal cap elongation. The doses of RNA used: activin, 2pg; CA-ALK4, 1ng; Smad2, 1ng; *efemp2*, 1ng.

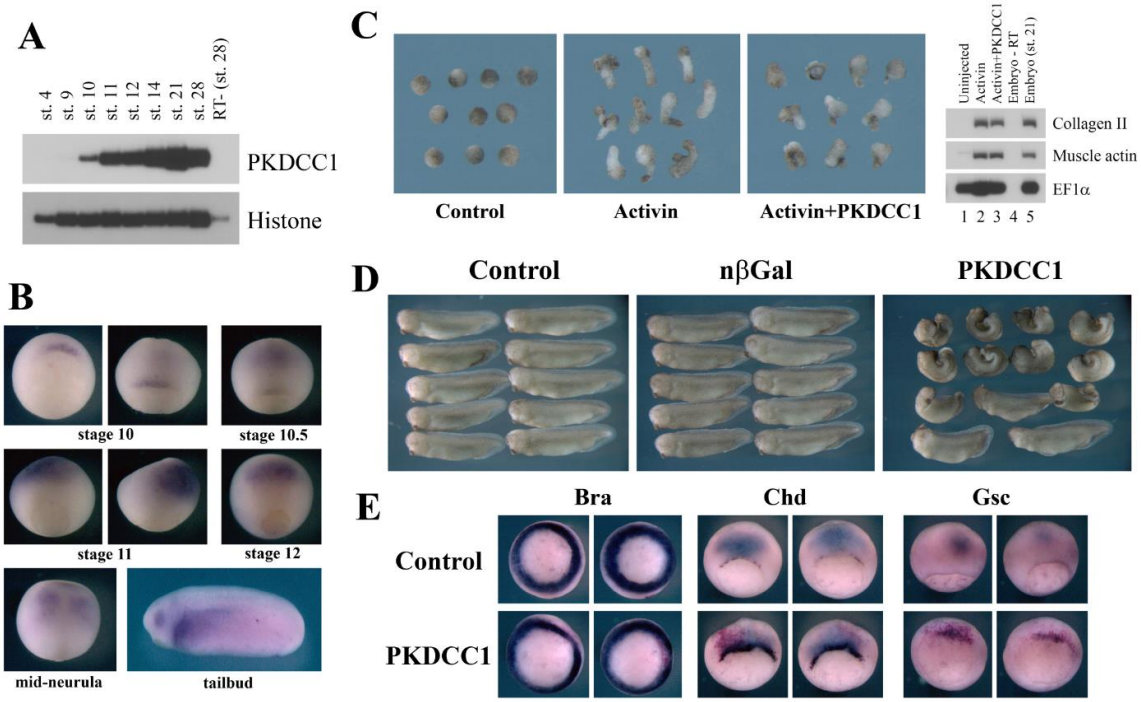

Figure 5. *pkdcc1*, a dorsally enriched gene, induced gastrulation defects when ectopically expressed. A) RT-PCR showed that *pkdcc1* was first expressed during early gastrulation, and its expression persisted until at least tailbud stages. B) *In situ* hybridization showed that *pkdcc1* was expressed in the organizer at the dorsal lip region in early gastrula embryos. Its expression was then up-regulated in the anterior neural tissues during mid-gastrula to neurula stages. At tailbud stages, *pkdcc1* transcripts were seen in the eyes, the lateral plate mesoderm, and the heart primordium. C) *pkdcc1* reduced activin-induced animal cap elongation without affecting mesodermal cell fates. D) Ectopic expression of *pkdcc1* in early *Xenopus* embryos induced gastrulation defects, with the resulting embryos displaying reduced body axis, smaller head, and failure in neural tube closure. E) Overexpression of *pkdcc1* did not inhibit mesodermal formation in early embryos, as indicated by normal expression of Brachyury (Bra), but delayed

involution and/or migration of dorsal mesodermal cells marked by Chordin (Chd) and Goosecoid (Gsc).

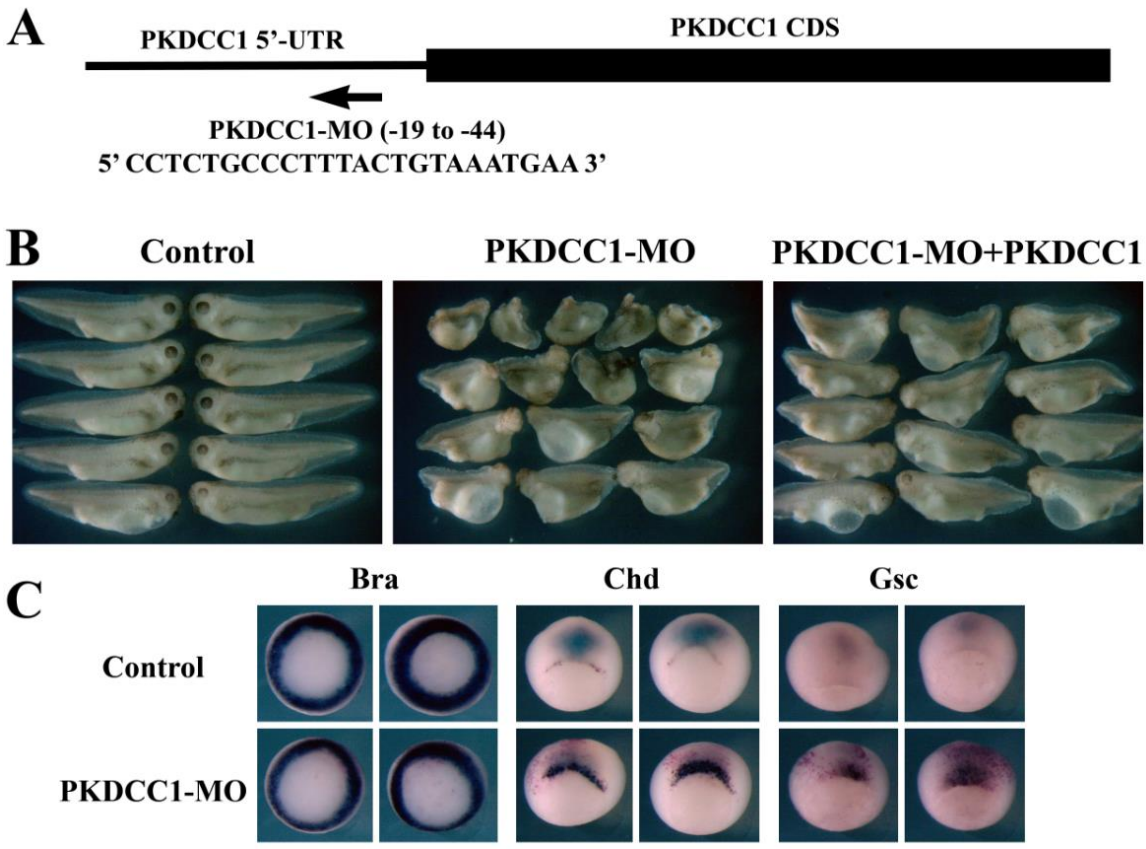

Figure 6. Knockdown of Pkdcc1 resulted in gastrulation defects. A) A translational blocking antisense *pkdcc1*-MO was designed to hybridize to the 5'-UTR sequence of the *pkdcc1* transcript. B) Expression of *pkdcc1*-MO led to embryos with reduced body axis and smaller head, which were greatly rescued by co-expression of low doses of *pkdcc1* RNA that did not contain the 5'-UTR MO-target sequence. C) Knockdown of Pkdcc1 resulted in delay in internalization of dorsal mesodermal cells marked by Chd and Gsc.

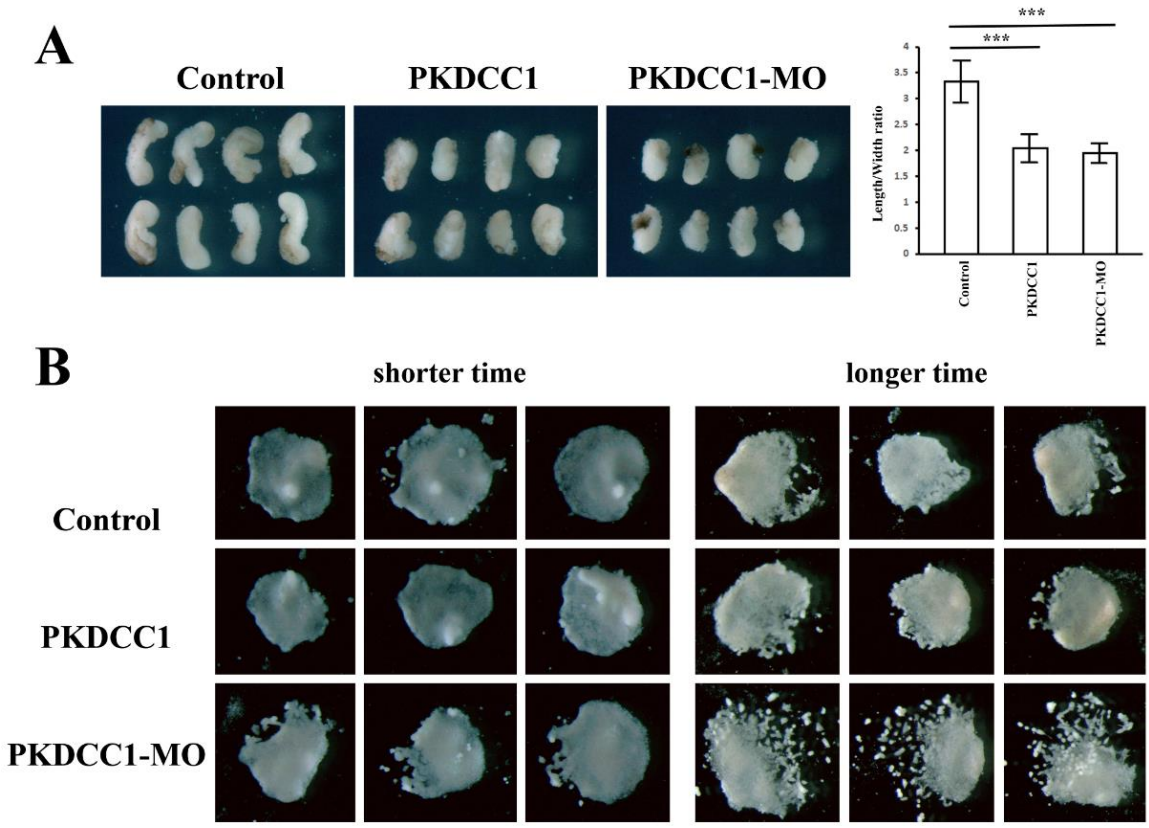

Figure 7. *pkdcc1* regulates gastrulation movements. A) Both ectopic expression and knockdown of Pkdcc1 led to reduction of convergent extension of trunk mesodermal tissues derived from the DMZ. The length over width ratio of the explants decreased significantly by enhanced or reduced expression of *pkdcc1*. B) While altered levels of Pkdcc1 did not prevent collective cell sheet migration on fibronectin, prolonged culture of the explants resulted in more pronounced cell dissociation in the absence of Pkdcc1, suggesting that *pkdcc1* modulated cell cohesion in the anterior mesendoderm.

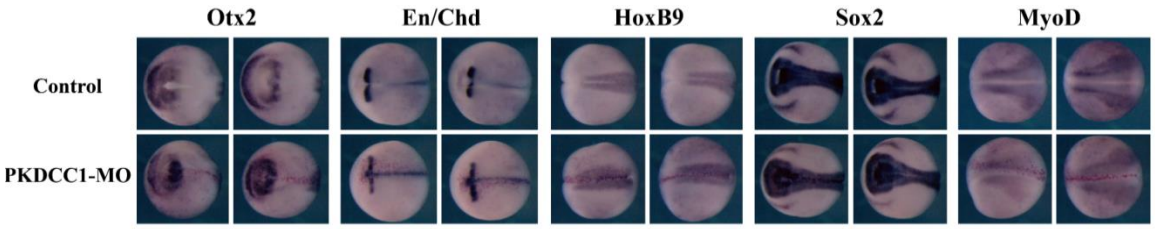

Figure 8. *pkdcc1* regulates anterior neural patterning. Knockdown of Pkdcc1 did not change the expression of the axial and the paraxial mesodermal markers Chd and MyoD or the neural marker Sox2, but reduced the expression domain of the forebrain marker Otx2 and shifted the midbrain marker engrailed (En) anteriorly, indicating that *pkdcc1* modulated anterior-posterior neural patterning.

THE RHOGEF PROTEIN PLEKHG5 REGULATES APICAL CONSTRICTION OF BOTTLE CELLS DURING GASTRULATION

by

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ABSTRACT

Apical constriction regulates epithelial morphogenesis during embryonic development, but how this process is controlled not understood completely. Here, we identify a Rho guanine nucleotide exchange factor *plekhg5* as an essential regulator of apical constriction of bottle cells during *Xenopus* gastrulation. *plekhg5* is expressed in the blastopore lip and its expression can be induced by activin. The protein is localized in the apical cell compartment and can induce apical actomyosin accumulation and cell elongation. Knockdown of Plekhg5 inhibits activin-induced bottle cell and blastopore lip formation in gastrulating frog embryos. Apical accumulation of actomyosin, apical constriction, and bottle cell formation fail to occur in the morphant embryos. Rho signaling is required for Plekhg5 function and blastopore lip formation. Taken together, our data suggest that activin/nodal-dependent *plekhg5* expression at the blastopore lip is essential for local polarized activation of Rho and apical accumulation of actomyosin to induce Rho-dependent apical constriction in bottle cells.

INTRODUCTION

Apical constriction refers to an active reduction of cell apical surface area that then causes further cell shape changes, such as elongation of cells along the apical-basal axis and/or expansion of the basolateral cell compartment. Apical constriction can drive bending of epithelial cell sheets, generate lumens and tubes, facilitate cell ingression and tissue invagination, and promote epithelial-to-mesenchymal transition. Apical constriction is thus a central mechanism underlying epithelial morphogenesis during

multiple developmental contexts, such as gastrulation, neural tube closure, and sensory organ formation (reviewed in Sawyer et al., 2010). In adults, apical constriction is also utilized in distinct conditions, such as wound healing. The reiterative usage of apical constriction in various tissue contexts highlights the importance of understanding the cellular and the molecular mechanisms controlling this fundamental process.

One common theme emerging from studies of apical constriction in different tissue contexts in a wide range of animal models is that polarized positioning and activation of actomyosin cytoskeleton within the constricting cells is crucial (reviewed in Martin and Goldstein, 2014). Both F-actin assembly and myosin accumulation and activation occur preferentially in the apical cell compartment prior to apical constriction, and contractile forces generated by this apical actomyosin network help to shrink apical cell surfaces. The architecture of actin filaments at the cell apex as well as the mechanical loads experienced by these actomyosin networks can differ in different cell types, resulting in apex-minimizing forces generated either by shortening of circumferential actomyosin cables located just below the apex periphery (purse-string) or by contraction of a subapical actomyosin meshwork leading directly to a reduction in apical area (Martin et al., 2009; Ebrahim et al., 2012; Mason et al., 2013). Regardless of the actomyosin architecture, the polarized assembly of the cytoskeleton network near the apical cell membrane needs to be tightly controlled both temporally and spatially to coordinate individual cell shape changes with global tissue morphogenesis (Martin and Goldstein, 2014). Rho family of small GTPases have often been implicated in such precise control of actomyosin dynamics during apical constriction.

The main members of the Rho family GTPases include RhoA, Rac1 and Cdc42. All of them are involved in regulation of actomyosin cytoskeleton, though they exert differential effects on the structure and the dynamics of the actomyosin cytoskeleton. In mammalian cells, RhoA preferentially controls stress fiber and focal adhesion formation, whereas Rac1 and Cdc42 are associated mainly with lamellipodia and filopodia protrusions, respectively (Hall 1998). The Rho proteins switch between a GTP-bound active state and a GDP-bound inactive state. The conversion between the two states is regulated by guanine nucleotide exchange factors (GEFs), which catalyze the exchange of GDP for GTP to activate Rho proteins, and GTPase-activating proteins (GAPs), which enhance the low intrinsic GTPase activity of Rho members to inactivate them. GEFs and GAPs respond to various intra- and inter-cellular signals to control diverse functions of Rho proteins, such as cell division, differentiation and movements (Hodge and Ridley, 2016). Rho members as well as their GEF and GAP regulators have been shown to regulate apical constriction. During *Drosophila* gastrulation, a Rho-specific GEF, DRhoGEF2, is enriched apically in invaginating ventral furrow cells, regulates apical myosin II accumulation and F-actin assembly, and is required for RhoA-dependent cell shape changes and normal tissue invagination (Barrett et al., 1997; Hacker and Perrimon, 1998; Nikolaidou and Barrett, 2004; Barmchi et al., 2005). A requirement for RhoA-dependent apical constriction has also been described during gastrulation of sea urchin and ascidian, though the upstream Rho regulators have not been reported in these species (Beane et al., 2006; Sherrard et al., 2010). In contrast, Cdc42, but not Rho, seems to be crucial during *C. elegans* endodermal internationalization at gastrulation. Cell contact-

induced recruitment of a Cdc42-specific GAP, PAC-1, results in inactivation of Cdc42 at the basolateral cell membrane, leaving active Cdc42 only at the contact-free apical surface. This stimulates the activity of the Cdc42 effector MRCK-1 apically to phosphorylate and activate myosin II for apical constriction of endodermal cells (Lee and Goldstein, 2003; Anderson et al., 2008; Chan and Nance, 2013; Marston et al., 2016). Thus, apical constriction can be driven by different upstream regulators converging on regulation of the apical actomyosin cytoskeleton. The GEFs and GAPs utilized during gastrulation of vertebrate embryos have not been described in detail.

During *Xenopus* gastrulation, a group of surface cells undergo apical constriction and basolateral elongation and expansion to form bottle-shaped cells. Melanosomes become concentrated near the apical cell surface during this process, hence marking the bottle cells with dark pigmentation. The bottle cells first appear on the dorsal side (known as the dorsal lip) and subsequently spread laterally and ventrally to encompass the entire blastopore (blastopore lip). Mesodermal and endodermal tissues involute through the blastopore and thereby internalize. The formation, morphology, and function of the bottle cells have been described by scanning electron microscopy and time lapse video microscopy studies decades ago (Keller 1981, Hardin and Keller, 1988), and the molecular machinery involved in this process is currently being uncovered. It is shown that both actin and microtubule cytoskeletons regulate bottle cell formation, and endocytosis is required to remove apical cell membrane for efficient apical constriction (Lee and Harland, 2007, 2010). Upstream regulators of bottle cell formation include the activin/nodal signaling pathway, which can induce ectopic bottle cells associated with

ectopic mesendoderm in the animal region (Kurth and Hausen, 2000). The components in the Wnt planar cell polarity pathway and the apical-basal polarity protein Lethal-giant-larvae (Lgl) have also been implicated in regulating bottle cell formation (Choi and Sokol, 2009; Ossipova et al., 2015). However, all these factors are expressed more broadly than at the blastopore lip. It is thus unclear how positioning of the bottle cells is regulated in gastrulating embryos and whether the responsible signals control expression and/or activities of any GEFs or GAPs to regulate dynamics of apical cell constriction.

In this study, we report the identification of a RhoGEF, *plekhg5*, as a blastopore lip-specific gene during *Xenopus* gastrulation. Fluorescent protein-tagged Plekhg5 is apically localized in outer epithelial cells and can organize apical actomyosin assembly. Plekhg5 induces ectopic blastopore lip-like morphology in a Rho-dependent fashion in the animal region, and its gene product is required for bottle cell formation in *Xenopus* embryos. Our studies thus reveal that expression of a tissue-specific RhoGEF is necessary and sufficient to induce apical constriction required for bottle cell formation during *Xenopus* gastrulation.

RESULTS

plekhg5 is expressed in cells at the blastopore lip during *Xenopus* gastrulation

In a previous RNA-seq study of differentially expressed genes in distinct tissues of *Xenopus* gastrulae, we identified *plekhg5* as a RhoGEF that is enriched in the organizer of early *Xenopus* embryos (Popov et al., 2017). Whole mount in situ

hybridization (ISH) revealed that *plekhg5* RNA is first detected in early gastrula embryos in the dorsal lip region. Its expression then spreads to encompass the entire blastopore lip during mid-gastrulation and is down-regulated once cells involute inside the embryos and re-spread at late gastrula stages (Fig. 1a-f). Bisected embryos showed that *plekhg5* expression is limited to the surface cells at the blastopore lip (Fig. 1b, d, f). At the neurula and early tailbud stages, *plekhg5* RNA is seen in the head at the hindbrain level and the tail regions (Fig. 1g to k). This pattern of expression persists through late tailbud stages, with additional expression apparent in the otic placodes and the pharyngeal pouches (Fig. 1l and m). Both the notochord and the dorsal neural tube in the tail region contain *plekhg5* transcripts (Fig. 1n). As development proceeds, *plekhg5* expression in the hindbrain region is seen as two distinct domains, with the anterior chevron-shaped domain reminiscent of the rhombic lip structure that contributes to future cerebellum (Fig. 1o). In tadpoles, *plekhg5* expression remains in the hindbrain region, pharyngeal pouches, and the tip of the tails (Fig. 1p and q). In addition, ventral mesodermal cells show increasing *plekhg5* expression from tailbud stages onward (Fig. 1l, p, and q). Bisected embryos reveal notochordal and dorsal neural staining of *plekhg5* transcripts at the tail (Fig. 1r), whereas more anterior regions have transient expression of *plekhg5* in the notochord that disappears at slightly later stages (Fig. 1t to w). Neural crest cells migrating toward and in the dorsal root ganglia also contain *plekhg5* (Fig. 1t to w). Furthermore, embryos bisected along the horizontal plane show specific *plekhg5* signals at the tips of the protruding pharyngeal pouches and in the epithelial cells lining the pharyngeal cavity (Fig. 1s). The dynamic expression of *plekhg5* in tissues undergoing

morphogenesis and in migrating cells suggests that this gene may regulate epithelial bending and tissue morphogenesis during early *Xenopus* development.

Ectopic expression of *plekhg5* induces blastopore lip-like morphology in *Xenopus* embryos

To examine the function of *plekhg5*, we first performed a gain-of-function study. Injection of *plekhg5* RNA into the animal region of 2-cell stage embryos induced ectopic blastopore lip-like morphology. Cells displayed concentrated pigmentation with reduced cell apices, and this became obvious at early blastula stages (Fig. 2A). When comparing this phenotype with that of activin treatment, a transforming growth factor beta family protein that has been shown to induce ectopic bottle cells in the animal region (Kurst and Hausen, 2000), we observed that similar activin injection induced ectopic blastopore lip at early gastrula, but not blastula, stages (Fig. 2A, 2B, and suppl. movie 1). Measurement of apical cell surface areas at the blastula stages demonstrated that *plekhg5* expression reduced cell apices to about one-third of that of neighboring cells or of cells in control embryos (Fig. 2C). Induction of the blastopore lip-like morphology by *plekhg5* was not limited to the animal region, as marginal or vegetal injection of the RNA also induced darkly pigmented cells in those regions (Fig. 2D). In the case of vegetal lip induction, we injected the RNA into one vegetal cell at stages 6 to 7 instead of 2- to 4-cell stages to circumvent the effect of cytoplasmic streaming that carried the injected materials away from the vegetal compartment when they were injected early (Danilchik and Denegre, 1991; data not shown). ISH of mesodermal markers, including Brachyury, Goosecoid, and Chordin, revealed that while activin-dependent formation of the ectopic bottle cells

in the ectoderm was associated with induction of all these markers, none of the genes was turned on by *plekhg5* (data not shown). These results suggest that *plekhg5* regulates cell morphology directly without invoking cell fate changes.

plekhg5 promotes elongation of the superficial epithelial cells

To analyze how *plekhg5* modulates cell shape, we co-injected RNAs of *plekhg5* and membrane-targeted mCherry fluorescent protein. Animal caps were dissected from the injected embryos at early gastrula stages and the outer ectodermal cells were viewed with confocal microscopy. While the caps from control embryos injected with membrane-mCherry RNA alone showed largely similarly sized cells, the caps from *plekhg5*-expressing embryos displayed enhanced mCherry signals in darkly pigmented cells and contained many smaller cells intermingled with several large ones (Fig. 3A). This result implies that overexpression of *plekhg5* may create a mechanical competition between neighboring cells, in which tension generated by apically constricting cells with reduced apical areas stretches adjacent cells with enlarged apical surfaces.

To further investigate cell morphology, we examined the cells in bisected embryos. In control embryos, radial cell intercalation in the ectoderm resulted in thinning of the ectoderm at the gastrula stages, but this did not happen in embryos injected with *plekhg5* RNA. Instead, thick layers of cells accumulated underneath the darkly pigmented epithelial cells, indicating that *plekhg5* blocked radial cell intercalation (Suppl. Fig. 1). Confocal images of the cells injected with membrane mCherry RNA showed that control animal cells displayed a mostly cuboidal morphology in both the

superficial and the deeper layers. In *plekhg5*-expressing embryos, the superficial epithelial cells had an elongated shape whereas the deeper, multi-layered, cells showed a round morphology similar to that in control cells (Fig. 3B). The ratio of apical-basal cell height over apical cell width of the outer ectodermal cells in *plekhg5*-expressing embryos showed a significant increase of 81% over that in control embryos (Fig. 3C). The data indicate that *plekhg5* acts differentially in epithelial and mesenchymal cells and that *plekhg5* regulates apical constriction and cell apical-basal elongation in superficial epithelial cells.

plekhg5 stimulates apical actomyosin accumulation in outer epithelial cells

Apical constriction is often the result of polarized localization and activation of the actomyosin contractile machinery. To test whether Plekhg5 expression modulates actomyosin cytoskeleton, we examined F-actin organization in both animal caps and bisected embryos using Alexa Fluor 488-conjugated phalloidin to stain F-actin. *En face* view of the animal caps revealed that F-actin distribution was normally detected predominantly at the cell-cell contacts, but this pattern was reorganized such that enhanced F-actin signal was observed spanning the surface area of *plekhg5*-expressing cells (Fig. 4A). Side view of the bisected embryos further showed that F-actin signal was strongly enhanced near the apical cell membrane in outer epithelial cells of *plekhg5*-injected embryos (arrows), but showed a contact-associated distribution in the inner cells (arrowheads, Fig. 4B). Fluorescence immunohistochemistry using anti-phosphorylated myosin light chain (pMLC) antibody to detect activated myosin showed a similar apical enrichment of pMLC signal in superficial epithelial cells when *plekhg5* was expressed

(arrows, Fig. 4C). The data thus demonstrate that Plekhg5 facilitates polarized actomyosin assembly in the apical cell compartment in superficial epithelial cells.

Plekhg5 is localized apically in superficial epithelial cells

The specific organization of apical actomyosin cytoskeleton suggests that Plekhg5 protein may be localized in a polarized fashion in epithelial cells. We therefore inspected Plekhg5 protein distribution using a green fluorescent protein (GFP) – tagged Plekhg5 that preserved its ability to induce blastopore lip - like morphology in *Xenopus* embryos (Fig. 5). When expressed in the ectoderm, GFP-Plekhg5 was detected specifically near the apical cell surface of the outer epithelial cells but was diffuse in cells of the deeper layers (arrows, Fig. 5A).

Plekhg5 contains a pleckstrin homology (PH) domain and a PDZ-binding motif (PBM) in addition to the GEF domain (Fig. 5B). To test the role of these domains in protein localization of Plekhg5, we made two deletion mutants that removed the PH and the PBM domains, respectively. Functional studies showed that deletion of the PH domain, but not the PBM motif, rendered the mutant protein unable to induce an ectopic blastopore lip (Fig. 5B). The protein localization assay revealed that deletion of the PBM motif did not alter the apical enrichment of the mutant protein (Fig. 5C, arrows), but deletion of the PH domain led to the loss of apical accumulation of the protein (Fig. 5C, arrowheads). The results establish that the PH domain, but not the PBM motif, is crucial for apical recruitment of Plekhg5 in the superficial epithelial cells.

Induction of the blastopore lip-like morphology by Plekhg5 requires Rho signaling

As Plekhg5 is a Rho-specific guanine nucleotide exchange factor, we examined whether Rho signaling acted downstream of Plekhg5 during its ectopic induction of the blastopore lip. We thus co-expressed RNAs of *plekhg5* and a dominant negative *rhoa* (DN-RhoA, or RhoA-T19N). DN-RhoA greatly reduced the ectopic blastopore lip induction by *plekhg5* (Fig. 6A). In comparison, DN-Rac1 (Rac1-T17N) was less efficient in this activity, though the pigmented cells appeared somewhat more diffuse than when *plekhg5* was expressed alone (Fig. 6B). The data imply that Plekhg5 preferentially activates RhoA to induce ectopic blastopore lips.

Axial defects induced by *plekhg5* splicing-blocking antisense morpholino oligos are rescued by co-expressed *plekhg5* RNA

To examine the endogenous function of *plekhg5* during blastopore lip formation, we designed two splicing-blocking (SB) antisense morpholino oligos (MOs). SB-MO1 targeted the 3' junction of exon 8 and the following intron, whereas SB-MO2 spanned the 3'-end of the exon 7 and the adjacent intron (Fig. 7A). These SB-MOs blocked the splicing donor sites that were conserved between both L and S alloalleles in *Xenopus laevis*, leading to intron retention and premature translational termination. The resulting truncated protein lacked the GEF domain and was expected to be non-functional. RT-PCR analysis of *plekhg5* RNA transcripts obtained from the injected morphant embryos at gastrula stages showed that both SB-MOs worked efficiently to block RNA splicing of both L and S alloalleles (Fig. 7B, C). The morphant embryos often displayed reduced head structures, shortened body axis, and sometimes failure of blastopore closure. These

defects were largely rescued when the SB-MOs were co-injected with the full length *plekhg5* RNA (Fig. 7D, E), demonstrating that the morphant phenotype was specific to the knockdown of the *plekhg5* gene. As the two SB-MOs induced similar phenotypes in all our assays (Fig. 7, Suppl. Fig. 2, and data not shown), we focused our following studies mainly using SB-MO1 (referred to as SB-MO).

Knockdown of Plekhg5 prevents ectopic induction of the blastopore lip by activin

To test whether *plekhg5* regulates blastopore lip formation, we first assessed the role of *plekhg5* in activin-induced blastopore lip formation. Expression of activin alone in the animal region induced ectopic bottle cells and tissue invagination, as reported previously (Kurth and Hausen, 2000). However, in the absence of functional *plekhg5*, activin could no longer induce ectopic blastopore lips (Fig. 8A, Suppl. Fig. 2). The result implies that *plekhg5* might mediate the effect of activin in inducing ectopic blastopore lip. Indeed, ISH revealed that activin turned on the expression of *plekhg5* (Fig. 8B). The SB-MO did not block the induction of *plekhg5* transcription by activin, nor did it prevent induction of the mesodermal markers Goosecoid and Brachyury by activin (Fig. 8B). The results indicate that *plekhg5* is not required for mesodermal fate specification by activin, but its protein product is obligatory in activin-induced blastopore lip formation.

***plekhg5* is required for blastopore lip formation in *Xenopus* gastrulae**

To address whether *plekhg5* is also crucial for endogenous blastopore lip formation, we injected the SB-MOs into the marginal zones of 2 dorsal or 2 ventral cells in 4-cell stage embryos. Examination of the injected embryos at mid-gastrula stages

showed that the absence of the functional Plekhg5 protein prevented blastopore lip formation. Dorsal expression of the MOs blocked dorsal lip formation without affecting the ventral lip, whereas ventral MO injection only inhibited the ventral blastopore lip (Fig. 9A, Suppl. Fig. 2). Marker expression assay revealed that both Goosecoid and Brachyury were expressed in the morphant embryos, but migration of the Goosecoid-expressing head mesoderm toward the anterior region was impaired and involution of the Brachyury-expressing mesodermal tissues was also delayed (Fig. 9B). The data demonstrate that *plekhg5* regulates formation of the blastopore lip around the entire circumference without affecting mesodermal cell fate determination.

Plekhg5 regulates bottle cell morphology and apical actomyosin cytoskeleton during blastopore lip formation

Bottle cells of *Xenopus* gastrulae assume a distinct morphology of a narrow cell apex, an elongated cell body and the expansion of the basolateral cell compartment. This can be visualized with injected membrane fluorescent protein, with surface view revealing slit-like narrowing of the cell apex and the side view showing flask-shaped cell contour (Fig. 10A, B). In *plekhg5* morphant embryos, neither the narrowing of cell apices from the surface view nor the flask-shaped cells from the side view were observed. Instead, cells around the whole marginal zone had a cuboidal or a columnar shape without signs of apical constriction (Fig. 10A, B). Phalloidin staining of the bisected embryos showed an enrichment of F-actin near the apical membrane of the bottle cells in control embryos (arrows, Fig. 10C), but no such F-actin accumulation was detected in *plekhg5* morphant embryos (Fig. 10C). Similarly, enrichment of pMLC around the apical

cell membrane in bottle cells was observed in control embryos (arrows, Fig. 10D), but this pattern was not present in the *plekhg5* morphant embryos (Fig. 10D). The results reveal that *plekhg5* facilitates apical assembly of actomyosin cytoskeleton in the bottle cells to promote efficient apical constriction during blastopore lip formation.

Rho signaling is required for blastopore lip formation during gastrulation

As Rho signaling was required for ectopic blastopore lip induction by *plekhg5* (Fig. 6), we next addressed whether Rho signaling participated in endogenous blastopore lip formation in *Xenopus* embryos. We thus injected RNAs of DN-RhoA or DN-Rac1 into the dorsal marginal zone of 4-cell stage embryos. DN-RhoA blocked formation of the blastopore lip whereas DN-Rac1 induced defects in the shape of the blastopore, but the blastopore lip still formed in the embryos (Fig. 11). The results demonstrate that Rho, but not Rac, signaling regulates apical constriction of bottle cells during *Xenopus* gastrulation.

Gastrulation movements in the absence of the blastopore lip

In our above cell morphology studies, we noticed that some *plekhg5* morphant embryos showed a groove in the marginal zone that hinted at cell internalization in the absence of apical constriction of bottle cells (arrow in Fig. 10A). To further analyze gastrulation movements in the absence of the blastopore lip, we performed time lapse video microscopy to track tissue movements in wild type and *plekhg5* morphant embryos (Fig. 12 and Suppl. movies 2-4). Knockdown of Plekhg5 did not appear to affect epiboly, as animal cells continued to move down and accumulated in several layers above

the marginal zone (arrowhead, Fig. 12). Vegetal rotation also seemed to proceed normally in *plekhg5* morphant embryos, as thinning of the vegetal mass, which was more pronounced on the dorsal side, was observed (arrow, Fig. 12). Surface cell involution was seen to start at the place where cells from epiboly accumulated, and convergent extension became apparent at a delayed time when control siblings had reached mid-neurula stages. Eventually the blastopore closed in a majority of the morphant embryos (Fig. 7, Suppl. movies 2, 3). Blastopore closure was observed even when the embryos were injected with the *plekhg5* SB-MO in all blastomeres of 4-cell stage embryos, resulting in minimal blastopore lip at the mid-gastrula stages (Suppl. movie 3 and data not shown). Convergent extension movements seemed to be the driving force in blastopore closure in these embryos, and this was accomplished at a delayed time point during neurulation (Fig. 12 and Suppl. movies 2-4). Cell movements in the absence of the blastopore lip seemed to be less precise, as about 20% of the embryos failed in blastopore closure, leading to exposed endoderm and the "open back" phenotype (Fig. 7, Suppl. movie 4). Our data suggest that in the absence of the *plekhg5*-dependent formation of the blastopore lip, other morphogenetic movements can compensate to close the blastopore during *Xenopus* gastrulation. However, the delay in blastopore closure and the relaxation in movement precision can introduce developmental defects in the tadpoles.

DISCUSSION

Apical constriction is an important cellular process that regulates cell shape changes during multiple developmental processes in diverse animal species. One crucial driving force in initiating and promoting apical constriction is the activation of the actomyosin contractile machinery specifically at the apical cell compartment. This step is often controlled temporally and spatially to ensure that changes in individual cell morphology coordinate with global tissue morphogenesis patterns. Different animals employ distinct strategies to regulate apical actomyosin cytoskeleton, with most strategies converging at the level of modulating Rho family of small GTPase activity. Understanding the function of tissue-specific regulators of Rho proteins during apical constriction can thus help us to gain insight into cellular and molecular mechanisms regulating this fundamental cell process. In this study, we report that the RhoGEF gene *plekhg5* plays an essential role in controlling apical constriction of the bottle cells during *Xenopus* gastrulation.

Expression of *plekhg5* in cells undergoing apical constriction

One major issue regarding apical constriction is what factor(s) specify a particular group of cells to undertake the cell shape changes in particular embryonic regions at particular developmental stages. Studies of gastrulation in several animal models help to shed light on this issue. It is found that cell fate determination factors often regulate cell apical constriction. In *C. elegans*, transcription factors responsible for endodermal and mesodermal cell lineages are required for waves of sequential internationalization of the corresponding cells. Ectopic formation of endodermal or mesodermal cells is sufficient

to induce ectopic apical constriction of these cells at the relevant times (Nance and Priess, 2002; Nance et al., 2005; Lee et al., 2006; Rohrschneider and Nance, 2009; Harrell and Goldstein, 2011). Similarly, *Drosophila* mesodermal determination transcription factors Snail and Twist control apical constriction of ventral furrow cells via their downstream targets, such as folded gastrulation (*fog*) and T48, that regulate actomyosin (Leptin and Grunewald, 1990; Martin et al., 2009; Sawyer et al., 2010; Manning and Rogers, 2014). In *Xenopus*, nodal signaling specifies mesodermal and endodermal cell fates in a dose-dependent manner, and ectopic expression of nodal family ligands in the animal region induces ectopic bottle cell formation in conjunction with mesendodermal markers. Both morphological features and cell cycle control of these ectopic bottle cells are indistinguishable from those at the endogenous positions (Kurth and Hausen, 2000; Kurth 2005). Hence *Xenopus* bottle cell formation is also linked to cell fate determination, and nodal signaling can function to connect embryonic patterning and morphogenesis. However, the fact that the bottle cells are present specifically in a narrow ring around the blastopore suggests that nodal downstream factors are likely engaged in positive and negative feedback control to precisely position the bottle cells within a narrow domain. Based on the expression pattern and the function of *plekhg5*, we propose that nodal controls bottle cell formation via transcriptional regulation of *plekhg5*. Once *plekhg5* is turned on, it is sufficient to induce apical constriction in all epithelial tissues regardless of cell fate. The specific expression of *plekhg5* may thus also contribute to distinct cell behaviors after internalization of mesendodermal cells in diverse amphibian species. Bottle cells form in epithelia of both endodermal and mesodermal fate in variable amounts in different species of amphibian, and their timing of undergoing apical

constriction, and if and when they undergo EMT and ingression to form deep mesenchymal mesodermal cells or re-spread to form an epithelial endodermal sheet, also varies according to species (Shook et al., 2002, 2004; Shook and Keller, 2008a, b). *plekhg5* may be the key component in regulation of apical constriction across different nodal-induced tissue fates. Thus, understanding how *plekhg5* expression is controlled becomes critical in comprehending how bottle cells are positioned in gastrulating embryos. Sequence analysis of *plekhg5* promoter and putative enhancer regions reveals multiple transcription factor binding motifs, including those of Smad, Sox proteins, and T-box transcription factors (not shown). Genome-wide ChIP-seq studies indeed show that Smad2/3 and Foxh1, the transcriptional effectors of nodal signaling, can bind to the *plekhg5* enhancer directly (Chiu et al., 2014). Further detailed dissection of the functional DNA elements and their binding factors involved in *plekhg5* expression will be a promising avenue to investigate bottle cell induction at gastrulation. At later stages, dynamic *plekhg5* expression is also observed in tissues undergoing epithelial morphogenesis, such as the forming otic vesicles and the turning points of the protruding pharyngeal pouches. Plekhg5 may thus regulate additional apical constriction events during organogenesis. In addition, expression of *plekhg5* in discrete migratory and mesenchymal cell populations suggest that it may be co-opted into other pathways to regulate cell morphology and directional movements during late embryogenesis.

Apical localization of the Plekhg5 protein

Apical actomyosin activation is a common theme for cell shape changes in gastrulating embryos, but different animals use distinct mechanisms to achieve this

effect. In *Drosophila*, the transmembrane protein T48 and the secreted factor folded gastrulation (*fog*), both downstream targets of the mesodermal specification transcription factor Twist, regulate apical localization of the PDZ-domain-containing DRhoGEF2 in a partially redundant fashion. DRhoGEF2 facilitates apical actomyosin organization through the Rho1/RhoA and Rok/ROCK signaling pathway (Sawyer et al., 2010, Manning and Rogers, 2014). In *C. elegans*, apical activation of actomyosin relies on polarized localization of a Cdc42 GAP protein PAC-1 via cell-cell contact-mediated recruitment of PAC-1 to the basolateral domain, leaving active Cdc42 at the contact-free apical surface to stimulate MRCK-1 activity (Lee and Goldstein, 2003; Anderson et al., 2008; Chan and Nance, 2013; Marston et al., 2016). Our studies reveal a similarity to *Drosophila* development in that *Xenopus* also utilizes an apically localized RhoGEF, Plekhg5, to organize a polarized actomyosin cytoskeleton at the cell apex. However, unlike fly DRhoGEF2, Plekhg5 does not contain a PDZ domain, and no vertebrate T48 or Fog homologs exist. Apical recruitment of Plekhg5 thus relies on a different mechanism. Plekhg5 contains both PH and PBM domains in addition to the GEF motif, and the PBM domain of Plekhg5 homologs has been shown to bind the multiple PDZ-domain-containing factor MUPP1 and its family member Patj in mammalian cells, zebrafish and *C. elegans* (Estevez et al., 2008; Ernkvist et al., 2009; Lin et al., 2012). Since Patj is an apically localized tight junction protein in the Crumbs protein complex (Tepass 2012), it is possible that Plekhg5 is recruited to the apical surface via its interaction with Patj. However, our structure-function analysis reveals that the PBM domain is dispensable for Plekhg5 localization and function, suggesting that other factors are involved in recruiting Plekhg5. Besides the Crumbs protein complex, the Par proteins also have polarized

distribution within epithelial cells. Par3, Par6 and atypical PKC are localized to the apical cell compartment and are required for apical actomyosin assembly and activation during *C. elegans* gastrulation (Nance and Priess, 2002; Nance et al., 2003; Ohno 2001; Goldstein and Macara, 2007). Plekhg5 may potentially interact with these apical Par complex proteins for specific localization. In addition, as the PH domain is required for correct positioning of Plekhg5 and PH domain is shown to bind both phospholipids and proteins, it is possible that interaction with apical membrane lipid phosphotidylinositide 4,5 phosphate (PI2P) helps to recruit Plekhg5 to the apical compartment (Krahn and Wodarz, 2012). Future investigation into specific recruitment of Plekhg5 to the apical surface in epithelial cells promises to yield further insight into molecular mechanisms regulating apical constriction in different animal species.

Rho-signaling-dependent apical constriction

Rho family of GTPases are often activated locally in apically constricting cells to control polarized activation of actomyosin. In *C. elegans*, Cdc42, but not Rho or Rac, is preferentially activated in contact-free surfaces of gastrulating endodermal cells (Anderson et al., 2008). This apically active Cdc42 stimulates the activity of its effector protein MRCK (myotonic dystrophy kinase-related Cdc42-binding kinase), which phosphorylates myosin regulatory light chain to activate the myosin motor function (Marston et al., 2016). In *Drosophila*, Rho1/RhoA protein is likely activated downstream of the apically localized RhoGEF2, as expression of a dominant-negative Rho1/RhoA leads to ventral furrow defects similar to that in *rhogef2* mutants (Barrett et al., 1997; Hacker and Perrimon, 1998). In *Xenopus*, we found that blocking Rho, but not Rac,

signaling prevents ectopic blastopore lip induction by *plekhg5* as well as endogenous blastopore lip formation. This suggests that Plekhg5, a Rho-specific GEF (Marx et al., 2005), locally activates Rho signaling at the apical surface in bottle cells to control apical constriction. Since expression of a dominant-negative Cdc42 construct leads to cell division defects (not shown), we did not further pursue the function of Cdc42 in this process. Several Rho effectors have been identified to regulate actomyosin and microtubule dynamics in different cells, with Rho-dependent protein kinase (ROCK) and Diaphanous (Dia) being shown to regulate apical constriction in Drosophila (Mason et al., 2013). The spatial distribution of ROCK and Dia differs in fly ventral furrow cells, reflecting their differential usages in generating contractile forces and linking the contractile machinery to cell adhesion junctions, respectively. In *Xenopus*, application of the ROCK inhibitor Y-27632 has been shown to be ineffective in blocking blastopore lip formation (Lee and Harland, 2007), and we also confirmed this result. However, if the inhibitor is injected in the region around the presumptive lip cells at late blastula stages rather than added in the culture solution, we did observe the inhibition of blastopore lip formation (data not shown). It is thus possible that the inhibitor is not taken into the *Xenopus* cells efficiently during gastrulation and local high concentration is required to inhibit ROCK activity effectively. Further investigation of the role of ROCK as well as that of Dia, using, for example, constitutively active or dominant-negative constructs, will be informative about the effectors mediating apical constriction downstream of Plekhg5-RhoA signaling in *Xenopus*.

Gastrulation movements in the absence of the bottle cells

Though the appearance of the bottle cells is a striking external indication of gastrulation movements in *Xenopus*, bottle cells per se do not seem to be absolutely required. Both surgical removal of these cells (Hardin and Keller, 1988) and the prevention of bottle cell formation in *plekhg5* morphant embryos can result in complete, albeit delayed, blastopore closure. In the absence of the bottle cells, vegetal rotation - the amoeboid migration movements of the yolky endodermal cells upward and laterally against the blastocoel walls (Winklbauer and Schurfeld, 1999; Wen and Winklbauer, 2017) - proceeds normally, so that a clear area of cells, reflecting thinning of the endoderm, often forms on the dorsal vegetal side. Epiboly movements of the animal cells also occur normally as in the control embryos. Mesodermal cell involution seems to be delayed and may happen at more variable positions in the marginal zone, so that the Goosecoid-expressing domain is positioned at variable distances from the blastopore at late gastrulation. The eventual blastopore closure appears to be driven mainly by convergent extension movements during neurulation, as body elongation helps to push the surface tissues toward the blastopore to facilitate mesodermal internalization. Blastopore closure seems to proceed with somewhat variable speeds among morphant embryos, with a small portion failing, especially those expressing the SB-MO2. The data suggest that although formation of the blastopore lip is not obligatory for gastrulation movements, it may help to facilitate coordination of different cell movements and ensure the robustness and reproducibility of gastrulation. The compensation for lack of apical constriction during gastrulation has also been observed in other animals (Llimargas and Casanova, 2010). In sea urchin, laser ablation of the bottle cells surrounding the vegetal

plate delays, but not abolishes, the invagination of the vegetal plate (Nakajima and Burke, 1996; Kimberly and Hardin, 1998). In *C. elegans*, endodermal cells partially internalize into the embryos in the absence of an apical actomyosin network (Nance et al., 2003). It is thus apparent that multiple mechanisms are involved in gastrulation morphogenesis and they work in partially redundant manner to enable the correct placement of endodermal and mesodermal cells inside the embryos. Apical constriction-mediated cell shape changes help to orchestrate a robust cell movement program for reproducible embryonic patterning and development.

plekhg5 in other tissue contexts

Apical constriction is used reiteratively in multiple developmental contexts. One well studied process is neural tube closure, with the apical constriction of hingepoint cells in the neural plate as a crucial step (Suzuki et al., 2012; Wallingford et al., 2013). *In situ* hybridization of *plekhg5* does not reveal prominent signal in the hingepoint cells, and *plekhg5* morphant embryos do not show obvious neural tube closure defects. This indicates that *plekhg5* may not participate in neural tube closure. Instead, another RhoGEF, GEF-H1/Arhgef2, has been shown to regulate apical constriction of neural cells in *Xenopus* (Itoh et al., 2014). Multiple other factors also participate in control of apical constriction of hingepoint cells in *Xenopus* neural plate (review in Suzuki et al., 2012). Although *plekhg5* is not involved in neural tube closure, its expression in several other places, such as the otic vesicle and cells at the turning points of the protruding pharyngeal pouches, imply that it may regulate apical constriction during organogenesis. In mammalian cell culture and in zebrafish, *plekhg5* homologs are also shown to regulate

directional migration of cancer and endothelial cells and vasculature formation (Liu and Horowitz, 2006; Garnaas et al., 2008; Ernkvist et al., 2009; Dachsel et al., 2013). This suggests that in migrating cells, *plekhg5* may interact with other partners for localized activation of Rho and actomyosin to provide positional cue for directional movement. Further studies will reveal how *plekhg5* controls context-dependent polarization of actomyosin to influence different cell behaviors.

MATERIALS AND METHODS

Obtaining embryos and microinjection

Xenopus laevis frogs were used throughout the study (under the institutional IACUC protocol 09658). Female frogs were primed with 800 units of human chorionic gonadotropin hormone (Sigma) the night before usage. Embryos were obtained by in vitro fertilization, dejellied with 2% cysteine solution, and micro-injected with RNAs or antisense MOs. The animal poles of both blastomeres of 2–cell stage embryos or the marginal zone regions of the two dorsal or two ventral cells of 4-cell stage embryos were injected, as indicated in the text. For vegetal injection, *plekhg5* RNA was injected into one vegetal blastomere at stages 6 to 7 to circumvent transportation of the injected RNA into the marginal area by cytoplasmic streaming.

Plasmids and antisense morpholino oligonucleotides (MOs)

The *plekhg5* coding sequence was PCR-amplified from gastrula stage cDNA, with the N- and the C-terminal primer sequences of Plekhg5-N(NotI): 5’-

AGAAGCGGCCGCACCATGGTATGTCA TCATGCAGACTG-3' and Plekhg5-C(XhoI): 5'-CCGCTCGAG TTACACCTCTGAAGCC GTTAATGTAG-3'. The coding sequence was inserted between the NotI and XhoI sites of the pCS105 vector. GFP-tagged *plekhg5* was constructed by inserting the ligation product of NheI/SalI fragment of pEGFP-C3 and SalI/AscI fragment of *plekhg5* into the XbaI/AscI sites of pCS105 vector. The *plekhg5* mutants were made using a PCR-based method. The primers used in the construction were: plekhg5-PH-del-for: CACACACAATTGGCACAGAATCTCTTGCAAAGAACGAG; plekhg5-PH-del-rev: TGTGTGCAATTGTGTATCTTCAGGAGATGTTCCAATC; plekhg5-DPBM-C(XhoI): CCGCTCGAGTTATGAAGCCGTTAATGTAGAGTT. All the plasmids were linearized with the AscI enzyme before being transcribed with the SP6 RNA polymerase to make RNAs for injection. 100-200pg of *plekhg5* and its mutant RNAs were used for injection. The sequences of *plekhg5* splicing-blocking MOs are: SB-MO1: 5'-ACAAATTACCTCAGGAACCTCAATG-3' and SB-MO2: 5'-AGGCAAATATCTTACCCTTCCAAA-3', both targeting exon-intron junctional sequences to result in intron retention. 20-50ng of MOs were injected in all the experiments.

RT-PCR

To assay for the efficiency of *plekhg5* splicing-blocking MOs, several primer pairs were designed. The sequences of the primers 1 to 6 (Fig. 7A) are: primer 1 (exon 8, forward): 5'-CAAGTTGCATTCATACAGTATGTTTG-3'; primer 2 (exon 10, reverse): 5'-TCCGGACTCTTGTAGATTCAACAG-3'; primer 3 (intron 8 of plekhg5.L, forward):

5’-GAACAGATTTAGGATTGATAGGTCAG-3’; primer 4 (intron 8 of plekhg5.S, forward): 5’-GAACAtATTTAGAATTGATAAGTCAG-3’; primer 5 (exon 7, forward): 5’-GACGCAAGTATTCCGGTACAAGATC-3’; primer 6 (intron 7, reverse): 5’-GGCAATTTTAGCAGTTTGTATAGAAA-3’. The expected sizes of the PCR products are: primers 1+2 (no intron): 277bp; primers 3+2 (plekhg5.L intron retention with SB-MO1): 508bp; primers 4+2 (plekhg5.S intron retention with SB-MO1): 385bp; primers 5+2 (no intron): 441/447bp (L/S alloalleles); primers 5+6 (intron retention with SB-MO2): ~270bp (S alloallele is not annotated clearly).

In situ hybridization (ISH)

ISH was performed as described by Harland (1991). For *plekhg5 in situ*, the C-terminal fragment of the coding sequence was used as the probe. The embryos were bisected before or after staining to reveal internal signals.

F-actin staining and Immunofluorescence (IF)

For F-actin staining, embryos or explants were fixed in MEMFA for 30 minutes, washed with PBS three times, and stained with 5 units/ml Alexa Fluor 488-conjugated phalloidin (Invitrogen) in PBS with 0.1% Tween 20 for 3 hours at room temperature or overnight at 4^{o}C. For immunocytochemistry of phosphorylated myosin light chain, we adopted the protocol described in Lee and Harland (2007). Anti-phospho-Ser19 myosin light chain mouse Ab (Cell Signaling Technology, CST, 1:250) and anti-phospho-Ser20 myosin light chain rabbit antibody (Abcam, 1:500) were used in dorsal marginal zone and animal tissues, respectively (Fig. 10 and 4, respectively). The Abcam antibody produced

somewhat higher background in the marginal zone tissues, whereas the CST antibody seemed to work best when tissues were treated with methanol first, although this resulted in destroy of the membrane mCherry signal. Alexa Fluor 488-conjugated secondary antibody (1:200) was used to stain the embryos before they were imaged with the confocal microscope.

Imaging

For stereo imaging of embryonic phenotypes and in situ hybridization, Zeiss M2Bio and Nikon AZ100 microscopes were used. For time-lapse movies, embryos were positioned to the correct orientations (animal or vegetal side up) using modeling clay, and 6 to 8 hour time-lapse imaging was performed with 3-minute intervals. For fluorescence microscopy, an Olympus Fluoview 2000 upright confocal microscope was used. Most of the images were taken using a 20X (NA0.95) lens. Maximum projections of Z-stack images were used for the figures shown in the manuscript.

Morphometric and statistical analysis

The surface areas of blastula stage embryos and the height-to-width (H/W) ratio of the outer epithelial animal cells were measured using NIH ImageJ software. A total of 362 cells from 31 control embryos, 347 darkly pigmented cells from 32 *plekhg5*-injected embryos, and 350 normal pigmented cells from 32 *plekhg5*-injected embryos from 4 independent experiments were used for measurement of the surface areas of the blastula embryos. The ratio of the average areas of the darkly pigmented cells over those of the control or normal pigmented cells were calculated for each experiment and student t-test

was performed to assess the statistical significance. In all 4 experiments, there was a significant reduction of the cell surface areas when *plekhg5* induced darkly pigmented cells in the animal region. For the final plot, the fold differences from the 4 independent experiments were compared and the average fold difference, the standard deviation, and the student t-test for these experiments were calculated to determine the statistical significance. The *p*-value of the t-test against darkly pigmented versus control cells was 0.00073. For H/W ratio, a total of 153 cells from 28 control embryos and 129 cells from 26 *plekhg5*-injected embryos from 3 independent experiments were measured. For each experiment, the differences in H/W ratio between the samples were calculated and shown to be statistically different. For the final plot, all the cells were included to calculate the average H/W ratio and standard deviation and used for the student t-test analysis. The *p*-value was less than 0.0001.

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Competing Interests

None.

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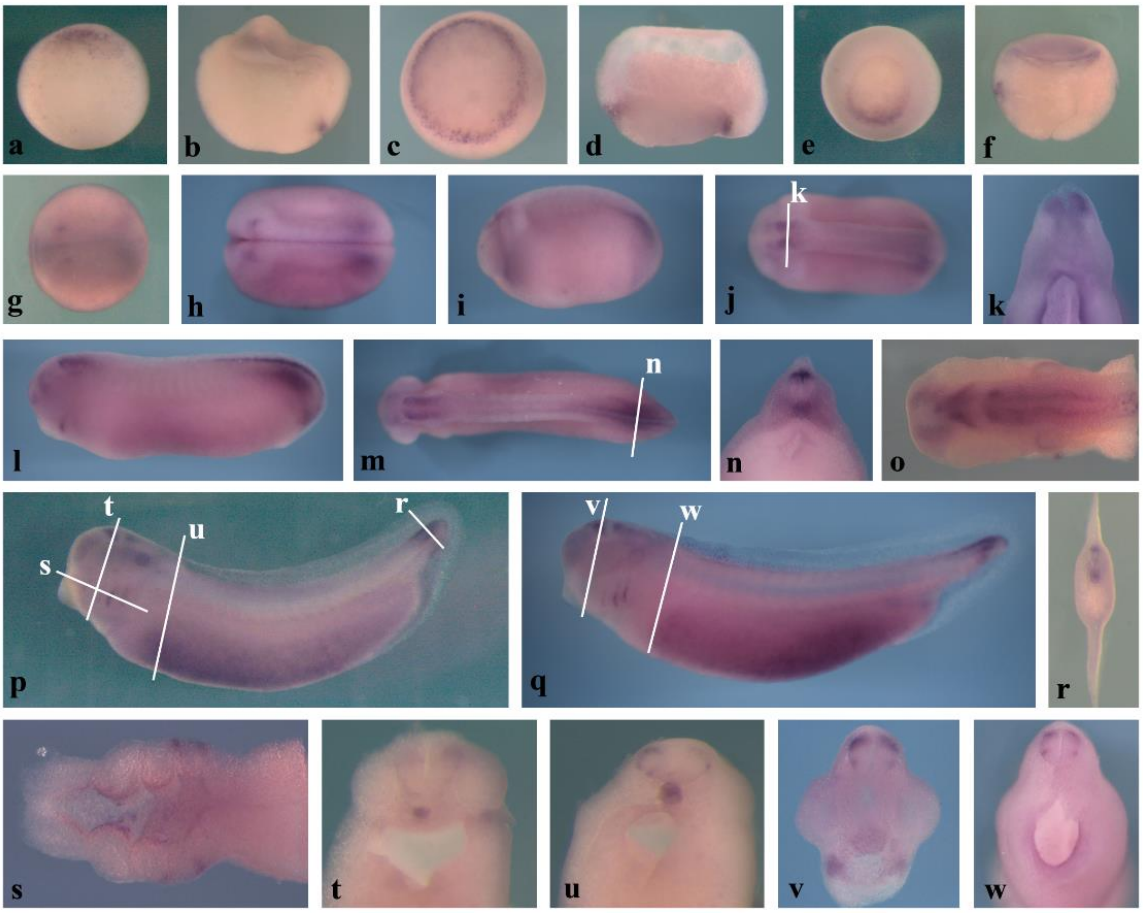


Figure 1. Expression of *plekhg5* during early *Xenopus* embryogenesis. a-f) *plekhg5* is expressed in the blastopore lip during gastrulation. Vegetal view (panels a, c, e) and side view of bisected embryos (panels b, d, f) are shown. g-o) During neurula and tailbud stages, *plekhg5* is expressed in the tail, hindbrain, otic placode, and pharyngeal pouch. Sections of the embryos reveal *plekhg5* signal in the dorsal neural tube and the notochord. p and q) Expression of *plekhg5* at the tadpole stages is detected in the hindbrain, otic vesicles, dorsal fin, tail, pharyngeal pouch, and the ventral-lateral mesoderm. r to w) Sections of the tadpole embryos show expression of *plekhg5* in the notochord - transiently in the trunk but persisting in the tail, the migrating neural crest

cells along the ventral route and in the dorsal root ganglia, the dorsal neural tube in the tail, and the turning points of the pharyngeal pouches.

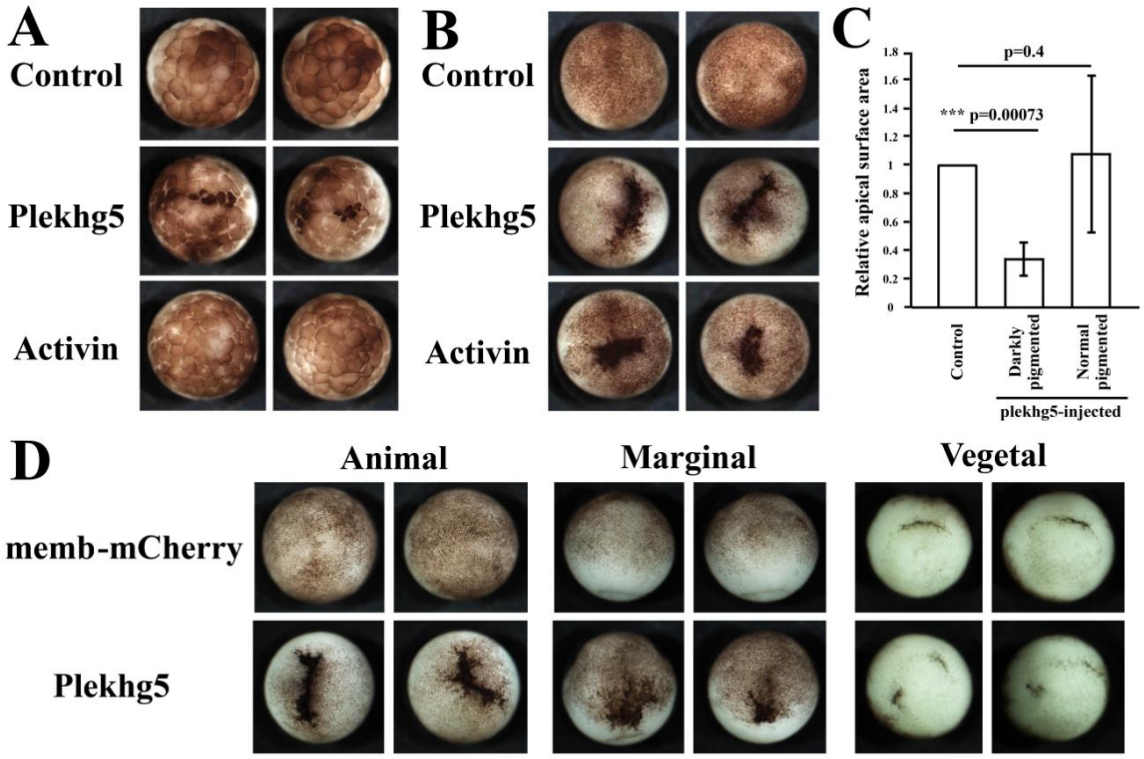


Figure 2. Ectopic expression of *plekhg5* induces blastopore lip-like morphology. A) *plekhg5*, but not activin, induces apical cell constriction in ectodermal cells at early blastula stages. B) Both *plekhg5* and activin induce ectopic blastopore lip at gastrula stages. C) Apical cell surface of *plekhg5*-expressing darkly pigmented cells is reduced to about 1/3 of that in control cells. Student t-test shows a significant difference between surface areas of the dark and normal cells. A total of over 300 cells each from 31 control embryos and 32 *plekhg5*-injected embryos from 4 independent experiments were analyzed. D) *plekhg5* induces blastopore lip-like morphology when injected either in animal, marginal zone, or vegetal regions. In all the experiments, 100-200pg of *plekhg5* and 5pg of activin RNAs were used.

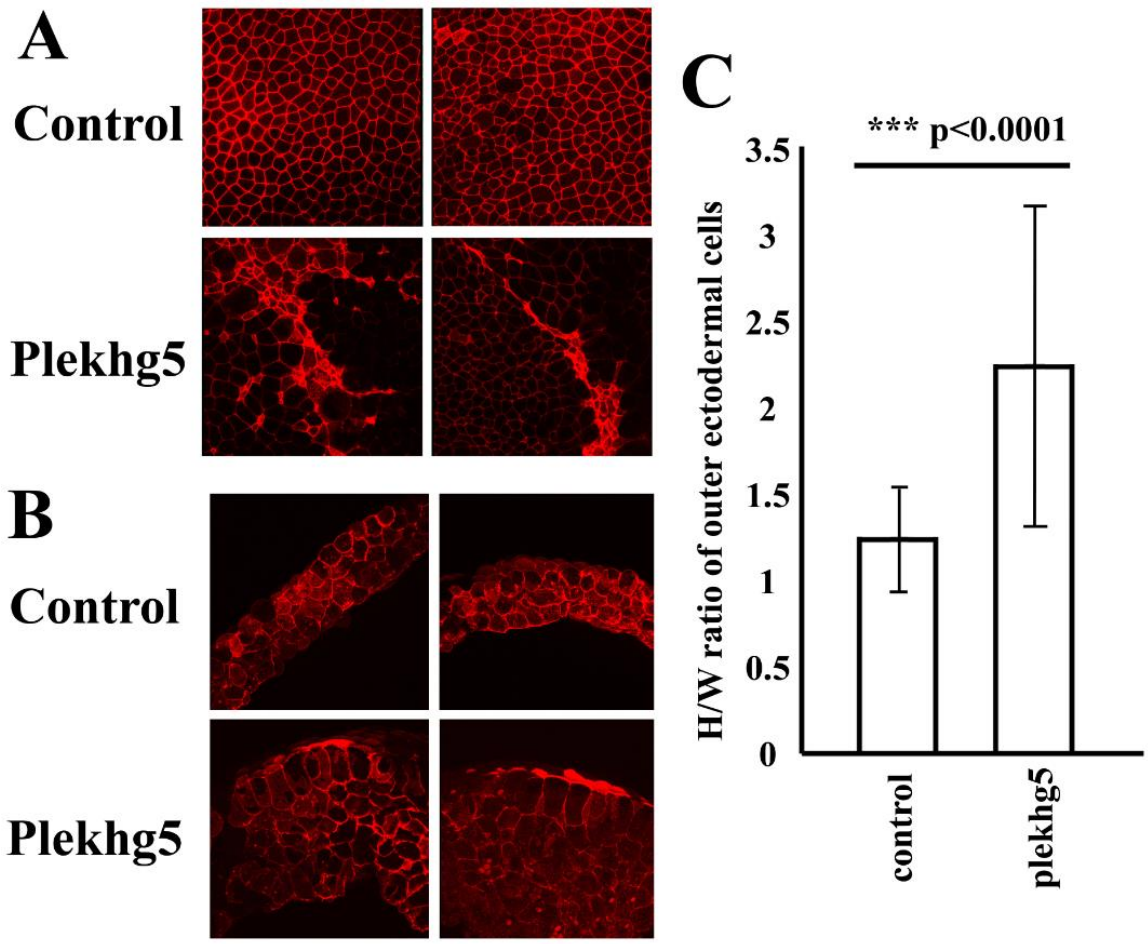
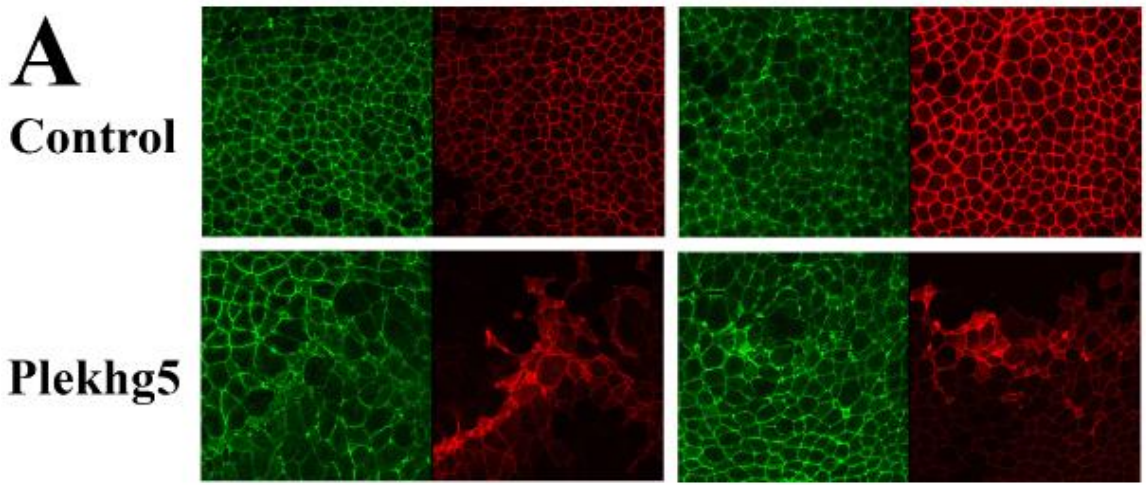
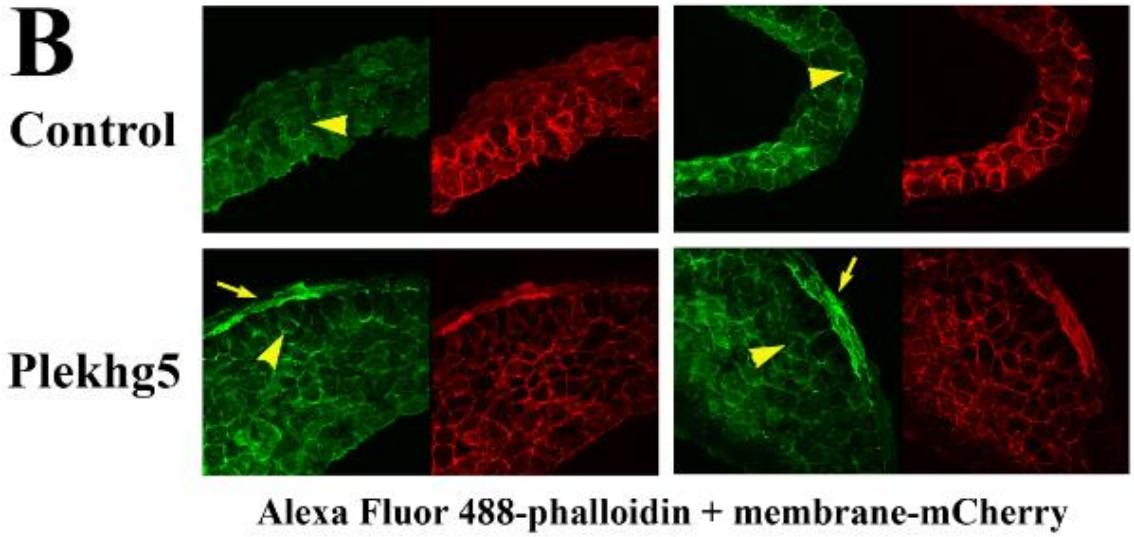


Figure 3. *plekhg5* induces apical constriction and cell elongation in outer epithelial cells. A) At early gastrula stages, a majority of *plekhg5*-expressing cells with reduced cell surface are mixed with several cells with enlarged surface areas, implying a mechanical competition among the *plekhg5*-expressing cells. B) Side views of bisected embryos show elongation of superficial epithelial cells from *plekhg5*-injected embryos. C) Height (H) over apical width (W) ratio analysis shows that *plekhg5*-expressing outer epithelial cells have a significant increase in H/W ratio. A total of 153 cells from 28 membrane-mCherry-injected embryos and 129 cells from 26 *plekhg5*-injected embryos from 3 independent experiments were used for statistics analysis.


A
Control
Plekhg5
Alexa Fluor 488-phalloidin + membrane-mCherry


B
Control
Plekhg5
Alexa Fluor 488-phalloidin + membrane-mCherry

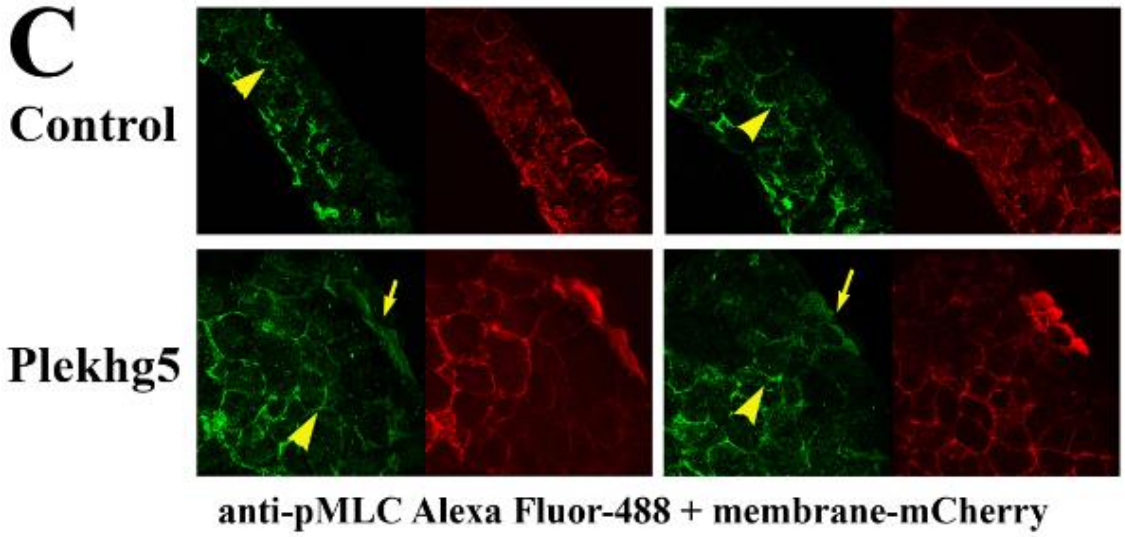

C
Control
Plekhg5
anti-pMLC Alexa Fluor-488 + membrane-mCherry

Figure 4. *plekhg5* stimulates apical actomyosin accumulation. A) F-actin is distributed mainly at cell-cell contacts in the ectodermal cells of control embryos, but is observed spanning the surface areas in *plekhg5*-expressing cells. B) Side view of bisected embryos shows that *plekhg5* promotes F-actin assembly in the apical compartment of outer epithelial cells (arrows), whereas F-actin is localized mainly at the cell-cell contacts in control cells (arrowheads). C) Anti-phosphorylated myosin regulatory light chain (pMLC) antibody staining reveals that pMLC is located mainly at the basolateral surfaces of control ectodermal cells (arrowheads), but its signal is enhanced in the apical cell compartment in *plekhg5*-expressing cells (arrows).

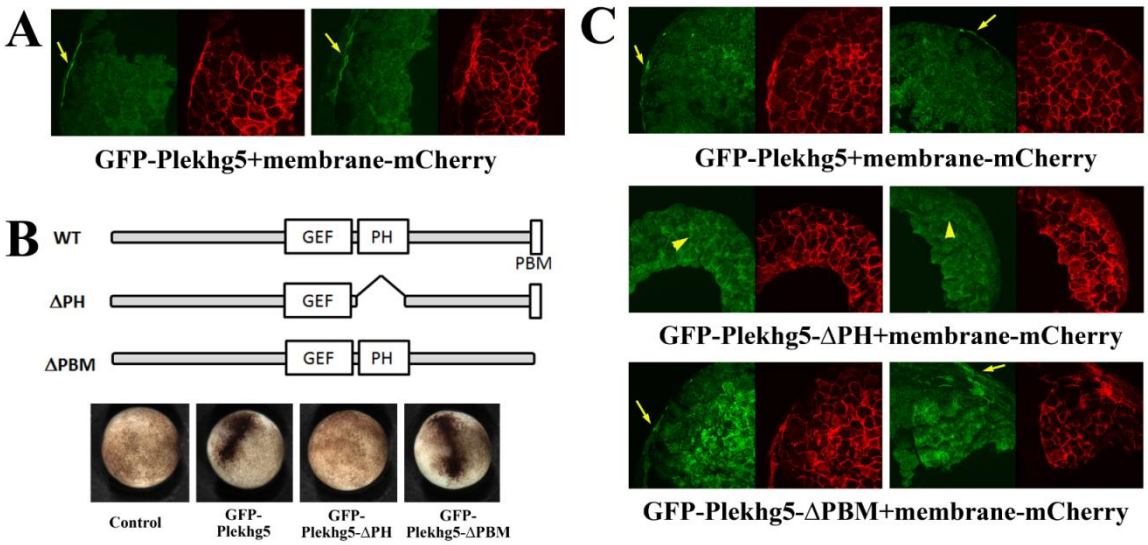
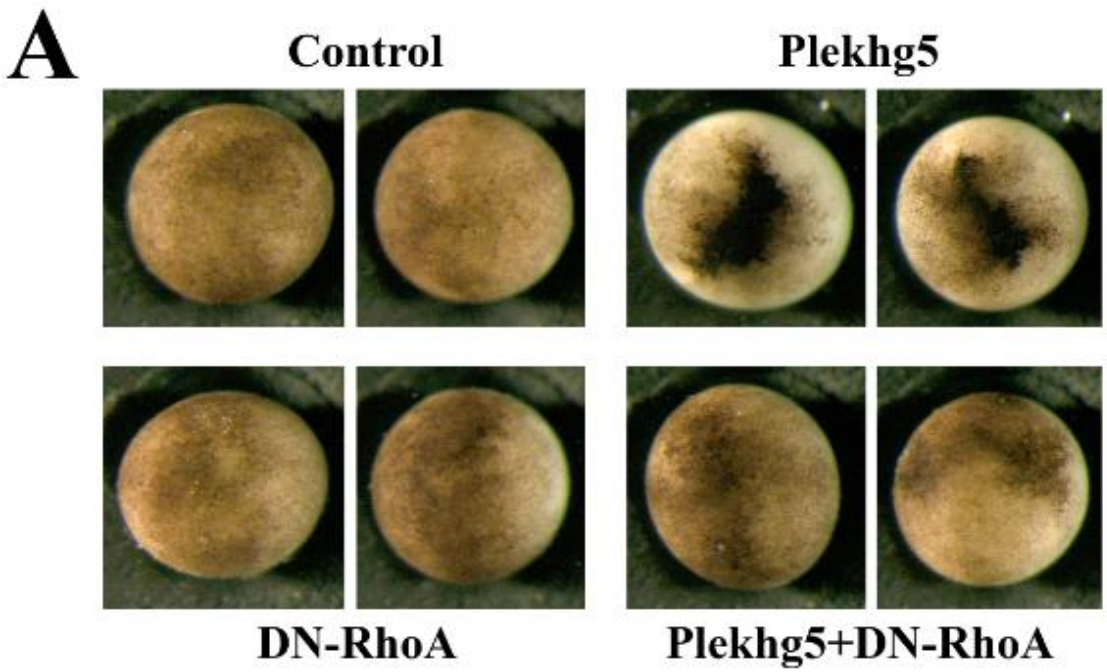


Figure 5. Plekhg5 is localized in the apical cell compartment via its PH domain. A) GFP-tagged Plekhg5 protein is detected at the apical cell surface in superficial epithelial cells (arrows), but is diffuse in deeper ectodermal cells. B) Plekhg5 contains a pleckstrin homology (PH) domain and a PDZ-binding motif (PBM) in addition to the GEF domain. Analyses of the deletion mutants that lack one of the domains reveal that removal of the PH domain, but not the PBM motif, abolishes the ability of the protein to induce ectopic blastopore lip-like morphology. C) Deletion of the PH, but not the PBM, domain results in dislodging of the protein from the apical surfaces. Arrows point to the apical cell compartments, and arrowheads points to the cell-cell contact areas.


A
Control
Plekhg5
DN-RhoA
Plekhg5+DN-RhoA

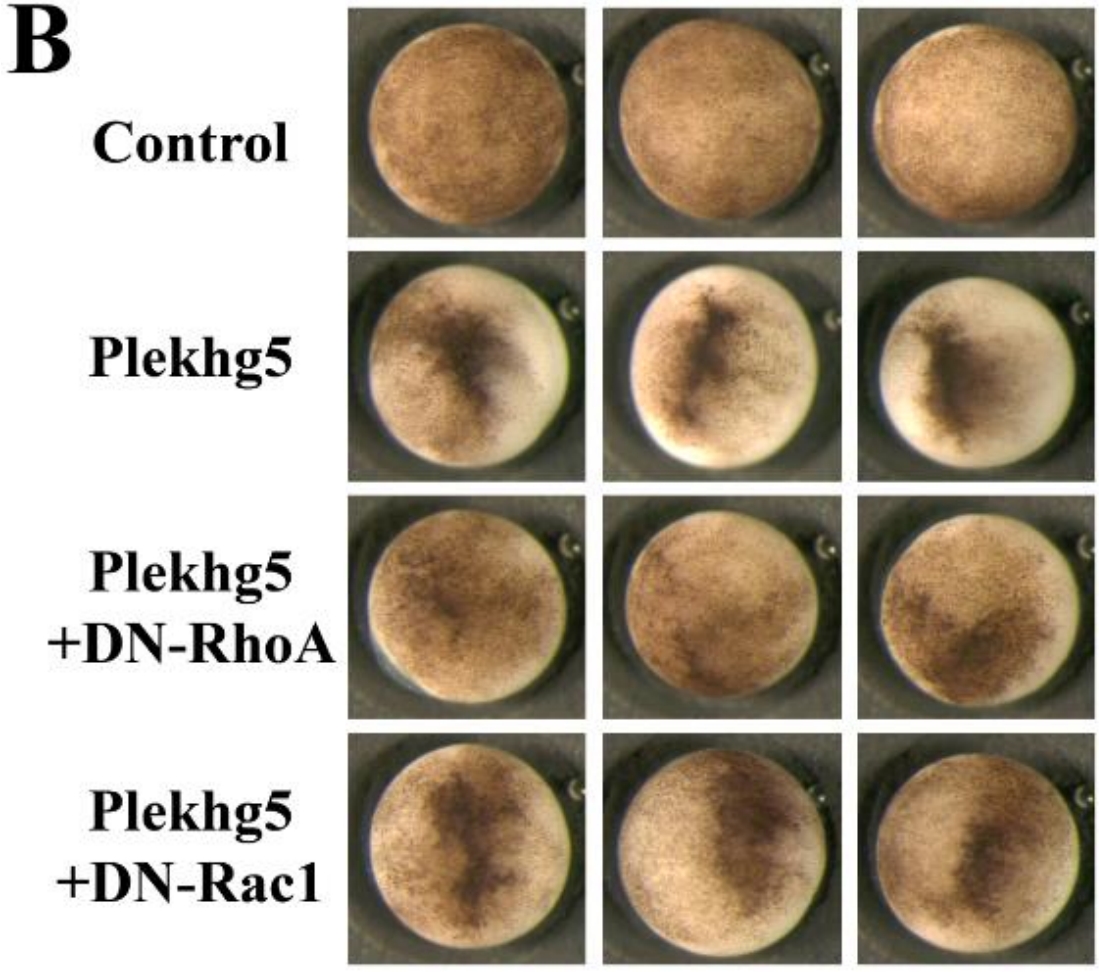

B
Control
Plekhg5
Plekhg5
+DN-RhoA
Plekhg5
+DN-Rac1

Figure 6. Plekhg5 requires RhoA signaling to induce ectopic blastopore lip-like morphology. A) Co-expression of *plekhg5* with a dominant negative (DN) RhoA construct (RhoA T19N) blocks the ability of *plekhg5* to induce ectopic blastopore lip. B) Unlike DN-RhoA, dominant negative Rac1 (Rac1 T17N) is inefficient in blocking *plekhg5* from inducing ectopic blastopore lip.

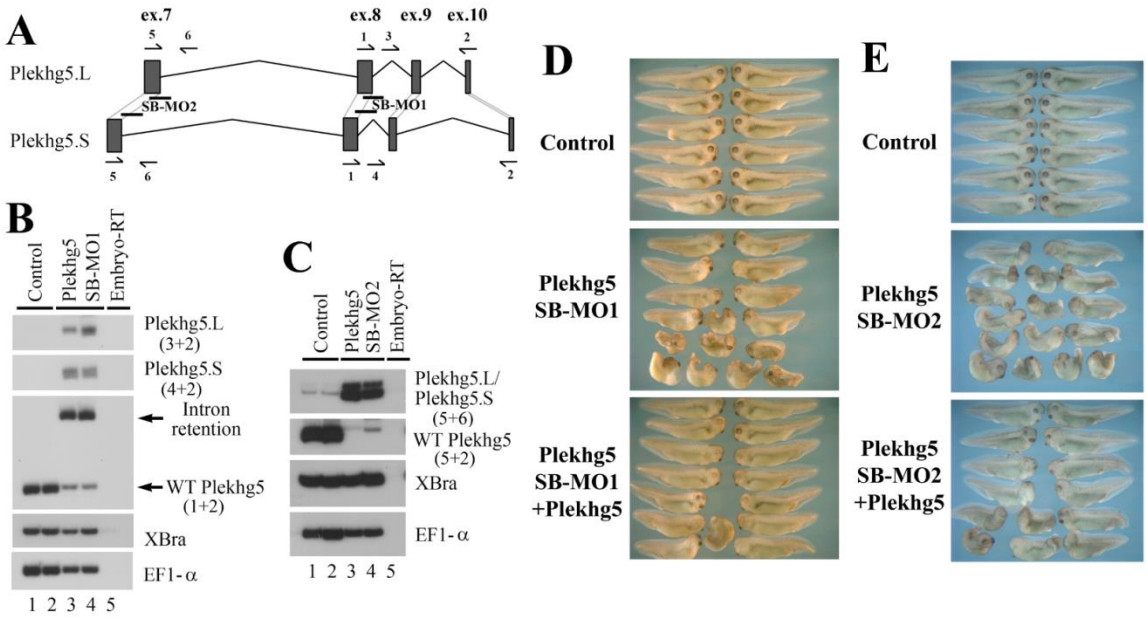


Figure 7. *plekhg5* splicing-blocking (SB) MOs efficiently block RNA splicing of both L and S alloalleles and induce axial defects that are largely rescued with the wild type *plekhg5* RNA. A) Schematic representation of the genomic regions of L and S alloalleles of *plekhg5* that are targeted by the SB MOs and the positions of the primers used for RT-PCR analysis of splicing efficiency. B, C) Both SB-MO1 and SB-MO2 are efficient in blocking splicing of both L and S alloalleles, as indicated by the presence of intron-retention products in *plekhg5* morphant embryos. The primers used in the PCR reactions are indicated in parentheses. D, E) *Plekhg5* SB-MOs induce axial defects, including small head, shortened axis, and some with failure in blastopore closure. The defects are largely rescued when SB-MOs are co-expressed with wild type *plekhkg5* RNA.

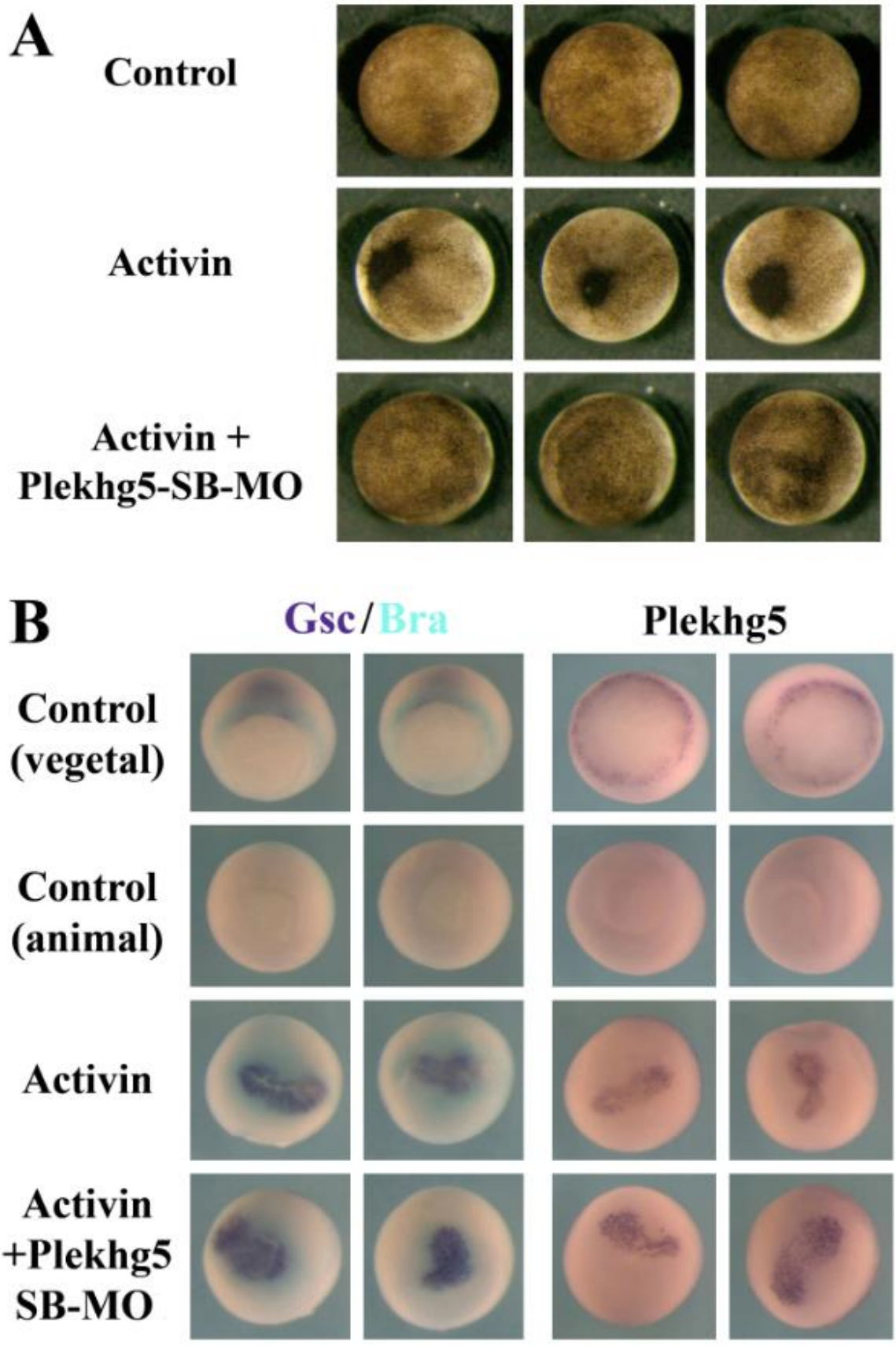

A
Control
Activin
Activin +
Plekhg5-SB-MO
B
Gsc/Bra
Plekhg5
Control
(vegetal)
Control
(animal)
Activin
Activin
+Plekhg5
SB-MO

Figure 8. *plekhg5* is required for activin-induced blastopore lip formation. A) Co-expression of activin RNA with *plekhg5* SB-MO blocks ectopic blastopore lip induction by activin. B) *In situ* hybridization shows that activin induces *plekhg5* expression together with the mesodermal markers Goosecoid (*gsc*) and Brachyury (*bra*) in the ectoderm. The presence of *plekhg5* SB-MO does not prevent induction of any of these genes by activin.

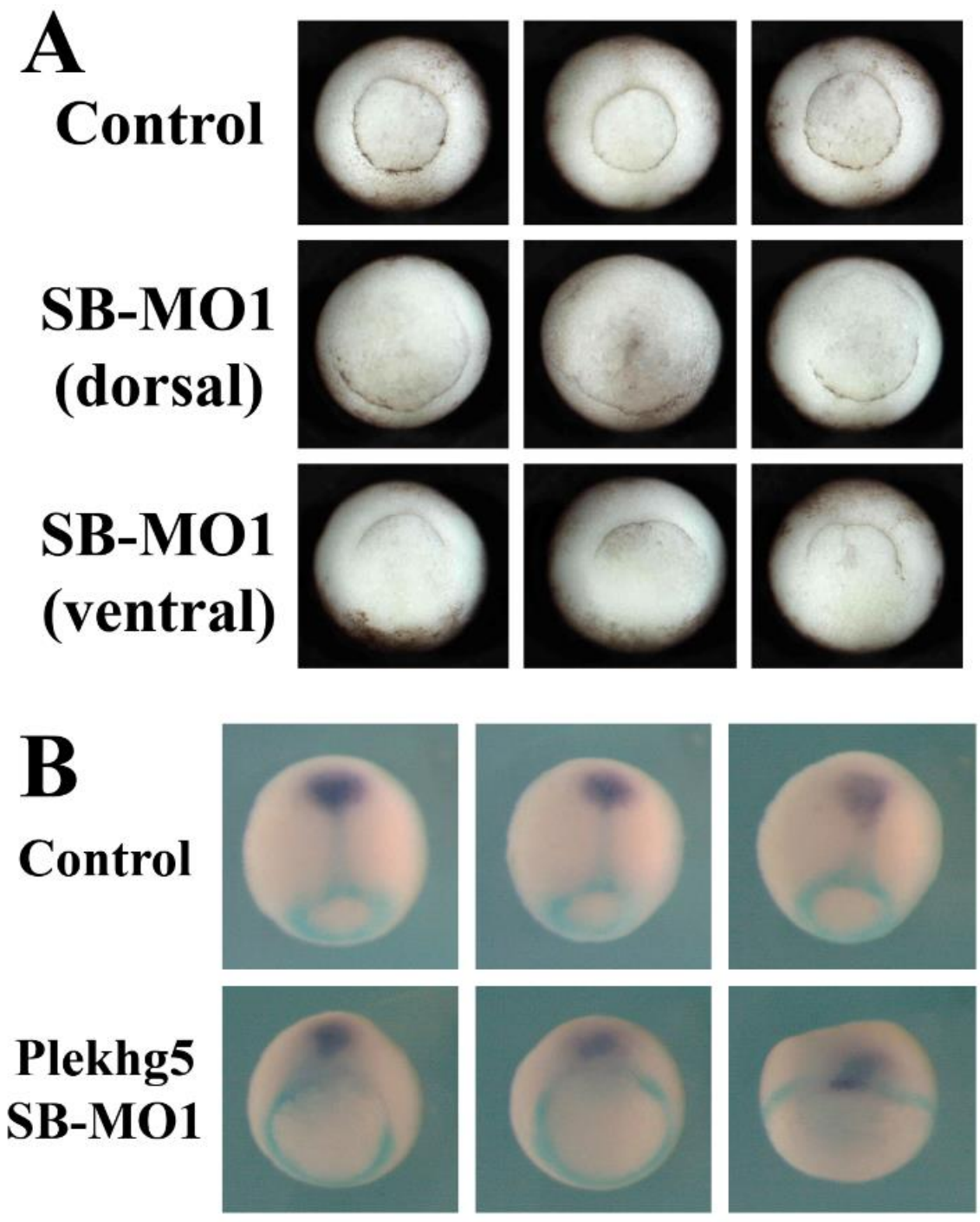


Figure 9. *plekhg5* is required for endogenous blastopore lip formation. A) Injection of *plekhg5* SB-MO into dorsal or ventral marginal zones blocks blastopore lip formation dorsally or ventrally, respectively. B) *In situ* hybridization of the mesodermal markers

gsc and *bra* shows that *plekhg5* SB-MO does not prevent mesodermal induction in the morphant embryos, but the movements of the mesoderm are impaired.

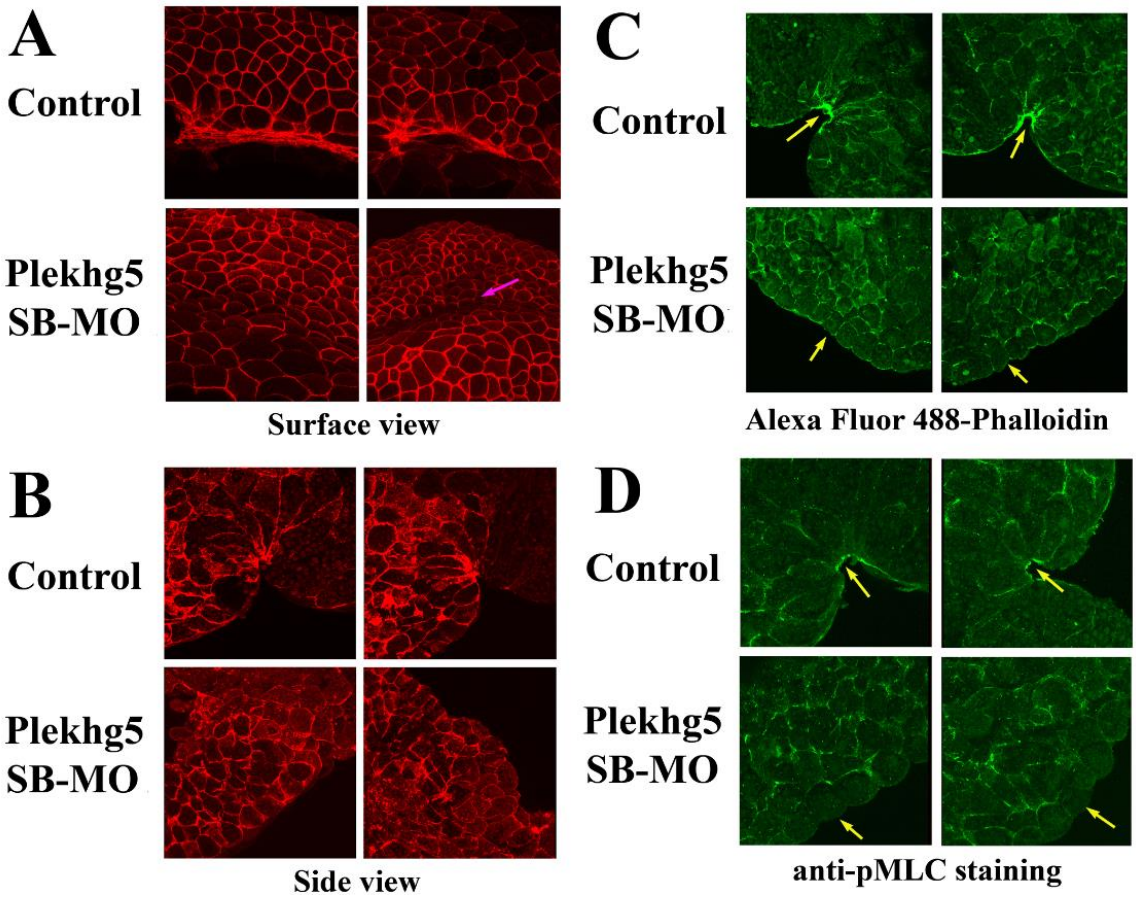


Figure 10. *plekhg5* regulates bottle cell morphology and apical actomyosin assembly. A) Surface view of the cell shapes around the blastopore lip. Apical constriction leads to narrowed cell surface at the blastopore lip in control embryos, but no such drastic reduction of surface area is apparent in *plekhg5* morphant embryos. The arrow points to the groove in the morphant embryos through which cells seem to internalize. B) Side view of the dorsal marginal zone shows flask-shaped bottle cells at the blastopore lip, but cells in the morphant embryos fail to constrict apically to form such bottle cells. C) Phalloidin staining of bisected embryos reveals F-actin enrichment at the apical cell surface of bottle cells in control blastopore lip (arrows), but no apical F-actin accumulation is obvious in *plekhg5* morphant embryos. D) Anti-pMLC antibody staining

demonstrates an apically concentrated pMLC pattern in control blastopore lip (arrows), but pMLC does not accumulate at the apical surface in *plekhg5* morphant embryos.

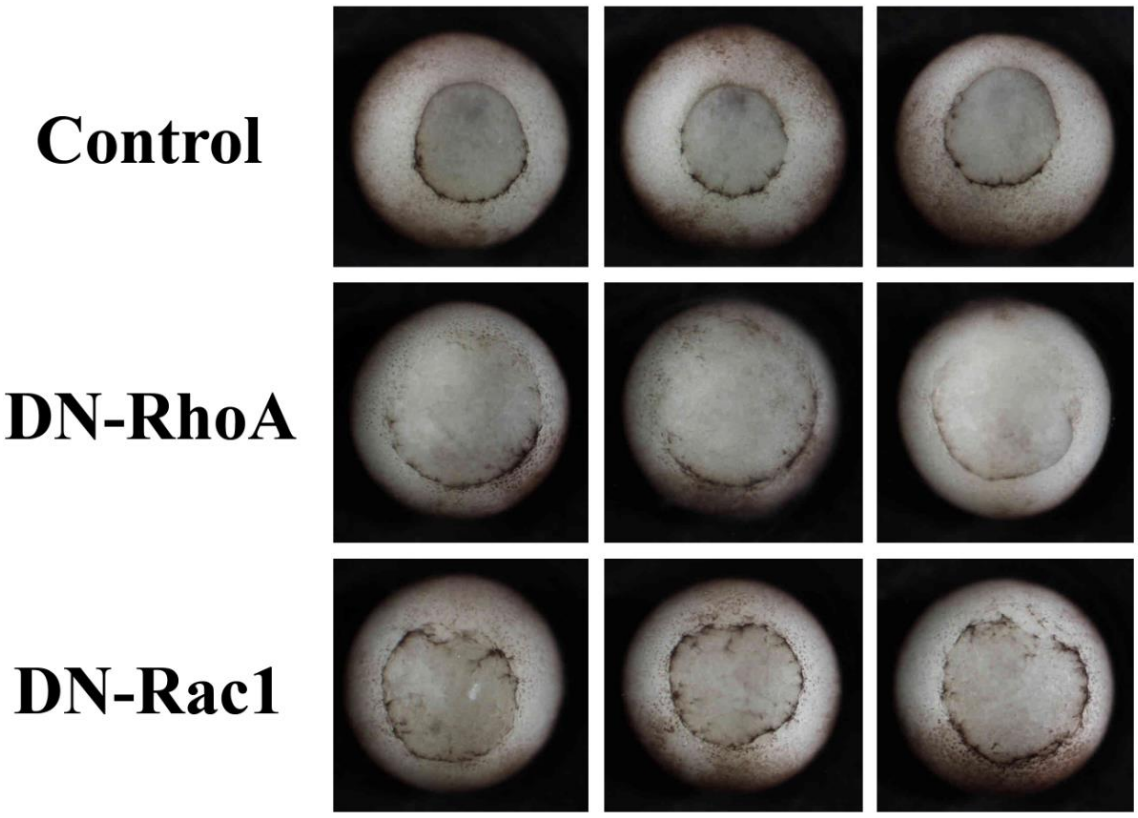


Figure 11. Rho signaling is required for blastopore lip formation in *Xenopus* gastrulae. DN-RhoA, but not DN-Rac1, blocks blastopore lip formation in *Xenopus* embryos.

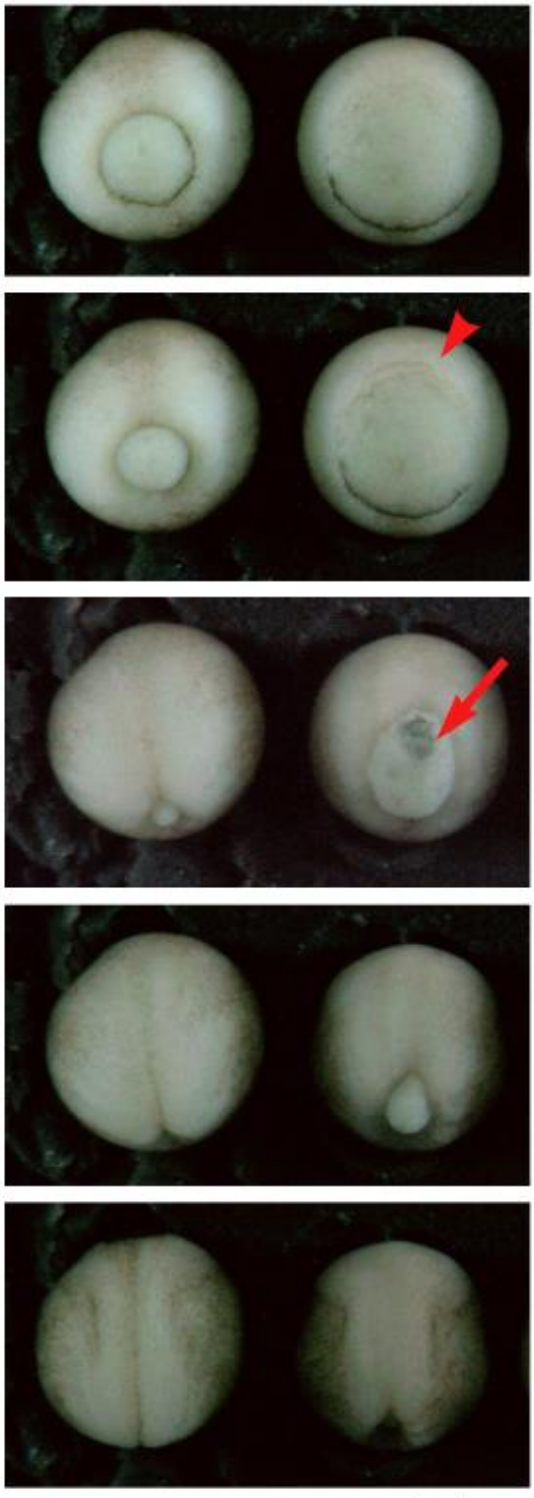

Control
Plekhg5
SB-MO

Figure 12. Gastrulation movements in the absence of the blastopore lip. Selected still frames from a time lapse video of gastrulation of control and *plekhg5* morphant embryos are shown here. Continued epiboly in the morphant embryos results in accumulation of cells in the marginal region in the absence of the blastopore lip (arrowhead) before cells move inside the embryos without the lip, and vegetal rotation in the absence of the dorsal lip leads to thinning of the vegetal mass with an apparent clear area that is often located on the dorsal side (arrow). Convergent extension movements eventually help to close the blastopore in most morphant embryos when control siblings reach the neurula stages.

SUMMARY

Regional gene expression patterns account for disparate cell behaviors seen in gastrulation

While the signaling pathways and mediators of cell fate specification in early *Xenopus* development have been well characterized, the genes utilized to accomplish gastrulation cell movements are generally less well studied. RNA sequencing allows prioritization of enrichment of gene expression relative to other regions, allowing for the opportunity to tie specific genes with a specific region of the embryo exhibiting a particular behavior during gastrulation. Hopefully, this will furnish a list of genes relevant to and necessary for the execution of those behaviors by those tissues in that developmental context. We isolated sections of the presumptive head mesendoderm shortly after stage 10, which displays the behaviors of directional collective migration and bottle cell formation, as well as sections of presumptive trunk tissue from the dorsal and ventral marginal zones at around stage 11, when these tissues undergo convergence and extension or convergence and thickening, respectively (Keller, 1975; Keller, 1976). We sought to identify all of the genes that make up the transcriptional profile of these tissues, and sort out those genes most highly enriched in the tissues exhibiting the behavior of interest relative to the tissues which do not exhibit that behavior. Many of the ventrally enriched genes are associated with cell signaling and transcriptional regulation

of cell fate, whereas the set of genes enriched on the dorsal side of the embryo contains many genes potentially associated with cell movement. Ventral marginal zone does not show enrichment of genes that might be associated with cell motility or cytoskeleton reorganization, though that does not mean it does not express such genes, only that there is no particular battery of genes associated with the cell behavior – in this case convergent thickening – that is enriched within this tissue. This is perhaps indicative of a scenario where convergent thickening behavior is a consequence of the absence from the ventral side of the embryo of those factors driving extension behavior in the dorsal side.

Several differentially enriched genes potentially associated with modulation of cell movement were demonstrated to alter cell behaviors without influencing cell fate. The ability of a gene to influence convergent extension can be assayed by overexpression in animal caps treated with Activin to induce dorsal mesodermal cell fate and convergent extension to produce an elongated explant. The effect of co-expression of the gene on Activin-induced tissue elongation will indicate whether the gene plays a role in the convergent extension process. We analyzed several signaling receptors, regulators of Rho GTPases, effectors of Rho family members, and transcription factors during this process. Among those differentially expressed genes, two G-protein coupled receptors that were enriched in the dorsal marginal zone (DMZ), *cxcr7* and *gprc5c*, interfered with animal cap elongation when overexpressed. Cxcr7 is reported to be a pseudoreceptor for Cxcl12 (Venkiteswaran et. al, 2013), and is proposed to act as a sink to generate a gradient of Cxcl12 ligand to direct migration of a Cxcr4-positive cell population in zebrafish lateral line development (Donà et. al, 2013). *Gprc5c* is one of several Gprc5 family members expressed during gastrulation. It has been shown to bind to Fzd proteins and disrupt

elongation of animal caps, though this effect could not be recapitulated by morpholino-mediated depletion of Gprc5 proteins (Harada et. al, 2007). Among the differentially expressed guanine nucleotide exchange factors (GEFs) for RhoA family GTPases, only one, the DMZ-enriched *arhgef3*, was found to impede convergent extension when overexpressed. While both Arhgef3 and Plekhg5 are known to be exchange factors for RhoA (Arthur et. al, 2002; Goh and Manser, 2010), and RhoA is an important downstream effector of the non-canonical Wnt signaling pathway in convergent extension (Habas et. al, 2001), the difference in outcome observed upon injection of these genes suggest diversity in regulation of RhoGEF function. Interaction with polarity proteins for membrane recruitment of Plekhg5 in epithelial but not mesodermal cells (Ernkvist et. al, 2009) may render it inactive outside of epithelial cells. Alternatively, Plekhg5 may preferentially activate RhoA effector Diaph1 rather than RhoA effector kinases (Dachsel et. al, 2013) and thus be incapable of affecting convergent extension. The three downstream effectors of the small GTPase Cdc42; Cdc42ep2, Cdc42ep3, and Cdc42se2, are able to impair elongation of Activin-treated animal caps. While it is possible that this inhibition is an artifact of overexpression, such that the Cdc42 effector proteins outcompete endogenous targets of the Cdc42 pathway for binding to a limited pool of GTP-loaded Cdc42, injection of moderate amounts of dominant-negative Cdc42 T17N was previously shown to have little effect on elongation of Activin-treated animal caps (Shibata et. al, 2005). Nevertheless, confirmation of a role for these proteins in convergent extension awaits morpholino-mediated knockdown. An additional note from this set of experiments was the observation that the transcription factors *atf3* and *fos* were also able to inhibit convergent extension when overexpressed. This is interesting in that

Fos cooperates with c-Jun in activation of transcriptional targets, and c-Jun phosphorylation by c-Jun N-terminal kinase is an oft used readout for activation of the non-canonical Wnt signaling pathway (Habas et. al, 2003). Furthermore, *atf2* is reported to be a transcriptional target of the non-canonical Wnt signaling pathway (Ohkawara and Niehrs, 2011). Given the observed enrichment of *atf3* within the DMZ, it is tempting to speculate that it too may serve as a transcriptional target of this pathway. If Jun/Fos and Atf3 indeed mediate activation of transcriptional targets of the non-canonical Wnt signaling pathway, it is likely that certain feedback inhibitors of pathway may be activated downstream of these transcription factors which causes convergent extension defects when they are over-expressed.

Finally, we sought to characterize in more depth the developmental function of *pkdcc1*, an ortholog of the mammalian vertebrate lonesome kinase (Vlk), which was present in the prospective head mesendoderm and DMZ samples. VLK was observed to function as a secreted tyrosine kinase with a broad range of substrates, including matrix metalloproteases and extracellular matrix components (Bordoli et. al, 2014). Pkdcc1 expression is present at the dorsal marginal zone at the start of gastrulation, and its expression domain appears to travel toward the anterior of the embryo over the course of development. Both overexpression and depletion of Pkdcc1 with antisense morpholino produced defects in convergent extension of dorsal marginal zone explants, and knockdown of Pkdcc1 protein seems to impair adhesion of head mesendoderm explants, which tend to scatter rather than remaining as one cohesive unit. This fits well with the observations that Pkdcc1 is capable of inhibiting the non-canonical Wnt pathway which is central to convergent extension movement (Vitorino et. al, 2015), and that the Vlk

knockout mouse phenotype bears superficial resemblance to Wnt5a and Ror2 mutants (Goncalves, et. al, 2011; Imuta et. al, 2009). The results also raise the possibility that mutations of *pkdcc1* orthologs might manifest as autosomal recessive Robinow syndrome in humans (van Bokhoven et. al, 2000; Roifman et. al, 2015).

Plekhg5 is required for bottle cell formation in *Xenopus*

As we had previously identified *plekhg5* as a gene highly enriched in the prospective head mesendoderm at the start of gastrulation, and suspected that it might be involved in cell behaviors associated with this tissue, we sought to characterize it further. In situ hybridization revealed that *plekhg5* was expressed in a ring at the vegetal hemisphere in a pattern that roughly presages the site of formation of the blastopore groove. Ectopic expression of Plekhg5 was able to induce a cell shape change resembling apical constriction of superficial cells, and this cell shape change was dependent on RhoA signaling, as it was abrogated by coinjection of dominant-negative RhoA T19N. Plekhg5 is localized to the apical surface in superficial cells, and this localization is dependent upon an intact pleckstrin homology domain. Morpholino-mediated depletion of Plekhg5 protein resulted in a loss of apical constriction typically seen in the region where bottle cells are induced, and a loss of invagination of the prospective endoderm associated with blastopore formation. However, most embryos were able to close their blastopore at a time point corresponding to late neurula stage in control embryos, and displayed defects very similar to what has been described for embryos following micro-surgical removal of the bottle cells (Keller, 1981).

Our data would indicate that Plekhg5 is both necessary and sufficient for apical constriction of bottle cells. While our studies have focused primarily on the role of *plekhg5* in gastrulation, we did observe expression of *plekhg5* in the brain at later stages of development, similar to what has been seen in rodents (Marx et. al, 2005). Given the reported link of mutations in PLEKHG5 to Charcot-Marie-Tooth disease and distal spinal muscular atrophy (Maystadt et. al, 2006; Maystadt et. al, 2007; Azzedine et. al, 2013; Kim et. al, 2013), efforts to further characterize the role of *plekhg5* in these tissues may prove worthy of pursuit.

FUTURE STUDIES

Recruitment of Plekhg5 to the apical surface is important for its function in mediating apical constriction, identification of the factors involved, and determination of whether these are proteinaceous factors or phosphoinositides would be valuable in understanding how Plekhg5 activity is regulated. Plekhg5 interacts with PDZ domain containing proteins Mpdz/MUPP1 and Inadl/Patj through its C-terminal PDZ binding motif (Ernkvist et. al, 2009; Estevez et. al, 2008), and this motif has been postulated to be required for localization of Plekhg5 to the apical surface (Liu and Horowitz, 2006). We have not observed any defect in apical localization of Plekhg5 lacking the PDZ binding motif; however, deletion of the pleckstrin homology domain was sufficient to abrogate recruitment of Plekhg5 to the apical membrane and rendered Plekhg5 unable to induce apical constriction. Pleckstrin homology domains are known to bind to phosphoinositides (Ferguson et. al, 1995), warranting investigation of phosphoinositide binding by Plekhg5,

and suggesting that Plekhg5 recruitment may depend on Phosphatidylinositol 3'-Kinase or PI4P 5'-Kinase.

While induction of actomyosin contraction downstream of RhoA is usually attributed to phosphorylation of myosin regulatory light chain by RhoA effector kinases, there are no data to indicate a role for RhoA effector kinases in bottle cells (Lee and Harland, 2007). What then explains the necessity for a RhoA guanine nucleotide exchange factor in bottle cell formation? Having established the importance of RhoA signaling for bottle cell formation, we could couple morpholino-mediated depletion of RhoA with rescue by RhoA mutants that are defective in binding certain effectors resulting in activation of only a select subset of effectors (Sahai et. al, 1998). This can help us to narrow down the link between Plekhg5 and distinct RhoA effectors during apical constriction.

There are data to suggest that Plekhg5 may direct GTP-loaded RhoA toward activation of the formin Diaph1 and simultaneously actively suppress RhoA effector kinases to promote reorganization of the actin cytoskeleton in mammalian cells (Dachsel et. al, 2013). This implies that Diaph1 may be the primary effector downstream of Plekhg5. Diaph1 is an actin filament barbed end capping protein, and is thought to incorporate new actin monomers to nucleate or lengthen filaments through recruitment of Profilin-associated globular actin via the FH1 domain of Diaph1 (Evangelista et al., 2002). However, the only *diaph1* clone we have been able to recover from cDNA of gastrulating *Xenopus* embryos lacked an FH1 domain, which is perhaps indicative of roles for Diaphanous proteins outside of actin polymerization. Apart from its role in actin assembly and turnover, Diaph1 can bind to and stabilize microtubules (Ishizaki et. al,

2001). While microtubules can play a role in apical constriction in some contexts (Booth et. al, 2014), this is likely to involve anchoring of microtubule minus ends by orthologs of CAMSAP family proteins and MACF1 (Toya et. al, 2016), rather than plus end capture by Diaphanous proteins, which is associated more frequently with protrusion formation at the leading edge of migratory cells (Kaverina and Straube, 2011). Nevertheless, we can employ a Diaph1 mutant incapable of interaction with microtubules (Wen et. al, 2004) to discriminate between actin capping activity of Diaph1 and microtubule recruitment, and their contribution to apical constriction.

Regulation of the positioning of the *plekhg5* expression domain is central to its role in *Xenopus* gastrulation, so it is important to identify the regulatory elements responsible for restriction of *plekhg5* transcription to the site of blastopore formation. The transcription factors Smad 2/3, Foxh1, and Tcf3 are essential for bottle cell formation (Chiu et. al, 2014; Wills and Baker, 2015). Since the binding sites for these factors within the *plekhg5* gene have been identified, we can target these sites with CRISPR, either to delete them or use dCas9 to occupy those sites and prevent transcription factor binding. This should allow us to confirm that these transcription factor binding sites represent functional enhancers regulating the expression of *plekhg5*.

Changes in cell shape resembling bottle cell formation have been observed in the avian and mammalian primitive streak (Nakaya and Sheng, 2009; Tam et. al, 1993; Viebahn et. al, 1995). These cell types are not likely to play an analogous role in morphogenesis, as they delaminate from the epithelium and undergo EMT whereas *Xenopus* bottle cells maintain cell-cell contacts and exert pulling force on surrounding cells (Solnica-Krezel and Sepich, 2012). However, as both delaminating and bottle cells

need to reduce their apices, it is conceivable that Plekhg5 may also play a role in shaping cells undergoing EMT in amniotes. Expression of Plekhg5 in these apically constricting cells could indicate a conserved function of this gene in gastrulation movements in species other than *Xenopus*. This issue thus merits investigation.

Mammalian Vlk (vertebrate lonesome kinase) has not one but two orthologs in *Xenopus laevis*; *pkdcc1* and *pkdcc2*. In support for a divergent role for these two proteins, it is reported that *pkdcc1* inhibits activation of the non-canonical Wnt pathway whereas *pkdcc2* does not (Vitorino et. al, 2015). That Pkdcc1 has been demonstrated to alter both canonical (Ding et. al, 2016) and non-canonical Wnt signaling implies that it probably exerts an effect on components common to both pathways. The localization of Pkdcc1 in the secretory pathway indicates that its targets are likely secreted. This would imply that Pkdcc1 acts on either Wnt ligands, Fzd receptors, or both; and mediates its effects either through changes in the rate of secretion of these proteins or through modulation of their interaction with one another.

In contrast with Pkdcc2, the Pkdcc1 protein expressed in isolation would appear to be retained in the Golgi apparatus, though when both Pkdcc1 and Pkdcc2 are present within the same cell, hetero-dimerization may allow for secretion of Pkdcc1 via its association with Pkdcc2 (Ding et. al, 2016). Bordoli et. al noted that catalytically inactive VLK is not secreted from the cell, raising the question of whether Pkdcc1 retains tyrosine kinase activity. Additionally, the authors report the presence of a Proline-Glycine-rich (PG-rich) region at the N-terminus of VLK which is conserved among mammalian Vlk orthologs, and deletion of this PG-rich region renders the kinase catalytically inert. This

region does not appear to be conserved in *Xenopus* Pkdcc1 or Pkdcc2. While it is unclear whether the phosphorylation targets of VLK in activated platelets are the same targets of Pkdcc1 and 2 in the dorsal marginal zone, VLK can autophosphorylate upon activation and this may be used to assess catalytic activity (Bordoli et. al, 2014).

Vlk (Pkdcc2) was found to exert an effect on progression of proteins through the secretory pathway independent of its ability to phosphorylate substrates (Kinoshita et. al, 2009). In *Xenopus*, overexpression of a kinase-dead Pkdcc1 mutant does not appear to induce a phenotype distinct from that induced by the wildtype protein, whereas a difference is apparent for Pkdcc2. Moreover, the kinase activity may not be necessary for Pkdcc1 function in Wnt signaling regulation (Ding et. al, 2016). If Pkdcc1 is not a functional kinase and is not secreted, this could indicate that it acts locally within the marginal zone to modulate availability of cell surface receptors and/or their ligands for effective interaction. One question regarding the kinase-defective Pkdcc1 is whether it is capable of physically interacting with the ligand or the receptor components. Thus, the follow-up experiments should include, in addition to looking at potential changes in glycosylation patterns of Fzd or Wnt proteins (indicative of protein positions along the secretory pathway), co-immunoprecipitation of Pkdcc1 with Wnt5a or Fzd7 and examination for changes in interaction between Wnt5a and Fzd7 in the presence or absence of Pkdcc1. The results should aid in elucidating the mechanism whereby Pkdcc1 influences Wnt signaling during gastrulation.

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APPENDIX A

IACUC APPROVAL

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

MEMORANDUM

DATE: 16-Jun-2016

TO: Chang, Chenbei

FROM: Robert A. Kesterson, Ph.D., Chair

Institutional Animal Care and Use Committee (IACUC)

SUBJECT: **NOTICE OF APPROVAL**

The following application was approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on 16-Jun-2016.

Protocol PI: Chang, Chenbei

Title: Epigenetic regulation of early development of the vertebrate nervous system

Sponsor: National Science Foundation

Animal Project Number (APN): IACUC-20462

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

Institutional Animal Care and Use Committee (IACUC)
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Birmingham, AL 35294-0019

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM
Institutional Animal Care and Use Committee (IACUC)

MEMORANDUM

DATE: 31-May-2017

TO: Wang, Jianbo

FROM:

Robert A. Kesterson, Ph.D., Chair

Institutional Animal Care and Use Committee (IACUC)

SUBJECT: **NOTICE OF APPROVAL**

The following application was approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on 31-May-2017.

Protocol PI:	Wang, Jianbo
Title:	The Role of Planar Cell Polarity Signaling in Outflow Tract Malformation in 22q11.2 Deletion Syndrome
Sponsor:	NIH - National Institutes of Health/DHHS
Animal Project Number (APN):	IACUC-20938

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

This protocol is due for full review by 30-May-2020.

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