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TRPM7 regulates gastrulation during vertebrate embryogenesis

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ABSTRACT

During gastrulation, cells in the dorsal marginal zone polarize, elongate, align and intercalate to establish the physical body axis of the developing embryo. Here we demonstrate that the bifunctional channel-kinase TRPM7 is specifically required for vertebrate gastrulation. TRPM7 is temporally expressed maternally and throughout development, and is spatially enriched in tissues undergoing convergent extension during gastrulation. Functional studies reveal that TRPM7's ion channel, but not its kinase domain, specifically affects cell polarity and convergent extension movements during gastrulation, independent of mesodermal specification. During gastrulation, the non-canonical Wnt pathway via Dishevelled (Dvl) orchestrates the activities of the GTPases Rho and Rac to control convergent extension movements. We find that TRPM7 functions synergistically with non-canonical Wnt signaling to regulate Rac activity. The phenotype caused by depletion of the Ca^{2+}- and Mg^{2+}-permeant TRPM7 is suppressed by expression of a dominant negative form of Rac, as well as by Mg^{2+} supplementation or by expression of the Mg^{2+} transporter SLC41A2. Together, these studies demonstrate an essential role for the ion channel TRPM7 and Mg^{2+} in Rac-dependent polarized cell movements during vertebrate gastrulation.

Introduction

Vertebrate gastrulation is comprised of a series of dynamic cell shape changes that results in part via modulation of the actin cytoskeleton (Keller, 2002). A key breakthrough in our understanding of the molecular mechanisms regulating gastrulation was the seminal findings that components of the non-canonical Wnt signaling pathway regulate this process (Wallingford et al., 2002; Yin et al., 2009). Understanding the molecular, cellular, and biochemical mechanisms that regulate gastrulation continues to remain a central focus of developmental studies. To date, a number of signaling pathways including Wnt, FGF, and BMP have been implicated in this process (Roszko et al., 2009).

Non-canonical Wnt signaling is currently loosely applied to a number of intracellular branches that function to regulate cell polarization and motility through modification of the actin cytoskeleton. This pathway appears to be independent of transcription and the well-elucidated canonical or β-catenin-dependent pathway (Semenov et al., 2007). The Wnt signal is mediated through the Frizzled (Fz) receptor and the LRP5/6 co-receptor, leading to activation of the cytoplasmic phosphoprotein Dishevelled (Dvl) (Wallingford and Habas, 2005). Of a number of branches downstream of Dvl, the activation of the small GTPases Rho and Rac plays central roles during gastrulation (Komiya and Habas, 2008). Activation of Rho occurs through the molecule Daam1, which complexes with Dvl in response to Wnt stimulation (Habas et al., 2001). This Rho pathway is coupled to the activation of the Rho associated kinase Rock that subsequently functions to regulate cytoskeletal changes through phosphorylation of myosin's light chains (Marlow et al., 2002; Veeman et al., 2003; Wallingford et al., 2002). Activation of Rac occurs downstream of Dvl, which in turn stimulates JNK activity to also cause cytoskeletal changes (Habas et al., 2003; Li et al., 1999; Yamanaka et al., 2002). The mechanism of action of a host of identified factors including Strabismus, Prickle, and Diversin, among others, and their integration to subsequently govern cell polarization for directional cell migration remains unresolved (Semenov et al., 2007).

Interestingly, a number of studies have pointed to a non-canonical Wnt/Ca^{2+} pathway in which Wnt stimulation leads to an increase in intracellular Ca^{2+} levels (Kohn and Moon, 2005). This Ca^{2+} pathway has been shown to function during vertebrate gastrulation, and Ca^{2+} is a potent divalent second messenger that can further negatively influence canonical Wnt signaling (Slusarski and Pelegri, 2007). Indeed, while many studies have uncovered changes in Ca^{2+} levels in

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cells undergoing gastrulation, it appears that much of these intracellular fluxes are due to the release of Ca^{2+} from intracellular stores (Slusarski and Pelegri, 2007). The identity of calcium channels that may be expressed on the plasma membrane and functionally traffic extracellular Ca^{2+} into cells during early stages of development remains unknown. Moreover, whether other ion channels or divalent cations such as Mg^{2+} play any functional roles during gastrulation remains an understudied area of investigation.

The Transient Receptor Potential (TRP) superfamily is composed of cation-permeant ion channels that have varied, but poorly understood functions, ranging from thermosensation, chemosensation, and visual transduction (Clapham, 2003). Unique among ion channels, the ubiquitously expressed TRPM7 was discovered as the first ion channel to possess its own kinase domain (Nadler et al., 2001; Runnels et al., 2001). While most divalent cations block ion fluxes through Ca^{2+}-permeable ion channels, TRPM7 exhibits high permeation of Ca^{2+} as well as other essential divalent cations, including Mg^{2+} and Zn^{2+} (Monteilh-Zoller et al., 2003). Consequently, an array of functions has been assigned to TRPM7, presumably owing to the diversity of cations permeated by the channel. Loss of TRPM7 has been reported to cause skeletal, pigmentation, and kidney defects in zebrafish (Elizondo et al., 2005). More recently, it was found that deletion of *TRPM7* in mice disrupted embryonic development, resulting in lethality between E 6.5 and 7.5, although the reason for this lethality remains unknown (Dorovkov et al., 2005; Jin et al., 2008).

In this study, we report the cloning and functional analysis of TRPM7 in the *Xenopus* embryo. We show that XTRPM7 plays a previously undefined role in convergent extension movements during gastrulation. Importantly, our studies point to a key role of the channel, but not the kinase domain of this protein, and for the Mg^{2+} cation in regulating polarized cell movements during gastrulation, independent of mesodermal specification. Additionally, we show that XTRPM7 regulates gastrulation via its ability to modulate the activation levels of the small GTPase Rac and not Rho. These studies underscore a key role for this ion channel in regulating gastrulation, and furthermore uncover a central role for Mg^{2+} in this crucial embryonic process.

Materials and methods

Embryo manipulations

Embryo manipulations and convergent extension assays in explants were performed as described (Khadka et al., 2009; Sato et al., 2006). Embryo injections were performed using *in vitro* transcribed RNAs, cDNAs, or Morpholino oligonucleotides (MO). Microinjection and microdissection of *Xenopus* embryos were performed as described (Khadka et al., 2009; Liu et al., 2008). Briefly, RNAs (500 pg) encoding GFP-CAAX and membrane-tethered Cherry were microinjected separately into dorsal blastomeres of four-cell stage embryos, alone or with RNAs encoding TRPM7 (2 ng) or XTRPM7 MOs (75 ng). "Shaved" Keller explants and analysis of cell shape and alignment were performed as described (Khadka et al., 2009; Liu et al., 2008). Detection of activated Rho and Rac was accomplished using a modified GST-pulldown purification assay (Habas and He, 2006). The relative level of activated GTPase was calculated by comparing the total amount of activated protein in each band to the total amount of the protein in the lysate, and then normalizing this number to the amount of activated protein captured in the GST-pulldown assay from uninjected embryos.

Plasmids and oligonucleotides

To obtain the complete *Xenopus* TRPM7 (XTRPM7) sequence (accession number: GQ304750), we employed a PCR based approach to amplify and clone this cDNA from a stage 10.5 *Xenopus* library. Details are available upon request. pBS–SV40–TRPM7 containing full length *Mus musculus* TRPM7 was generated by subcloning a KpnI/NotI fragment containing the TRPM7 ORF from pTracerCMV2–TRP-PLIK (Runnels et al., 2001) into pBS–SV40. pBS–SV40 contains the SV40 polyadenylation sequence from pCS2+, which was introduced by PCR subcloning an amplified 220 bp PCR fragment into the NotI/SacII sites of pBS using the following primers: 5′-GCG GCC GCG TAG ATC CAG ACA TGA TAA GAT ACA TTG ATG AGT TTG G-3′ and 5′-CCG CGG AAT TAA AAA ACC TCC CAC ACC TCC CC-3′. To create the "kinase-inactive" mutant of TRPM7 containing G1618D, we employed QuikChange (Stratagene) using the primers previously described (Su et al., 2006). The pOG1 vector encoding human TRPM6α was kindly provided by Dr. Vladimir Chubanov (Philipps Universität Marburg) (Chubanov et al., 2007). The Mg^{2+} transporter SLC41A2 was subcloned into pCS2+ from the FLAG-SLC41A2/pcDNA4/TO vector, generously donated by Dr. Andrew Scharenberg (University of Washington) (Sahni et al., 2007). pCS2+ containing C-Daam1, ΔDIX-Dvl, ΔDEP-Dvl, and Xdd1 have been previously described (Habas et al., 2001) as has pCS2+ containing Rho and Rac derived constructs (Habas et al., 2003).

Results

XTRPM7 is temporally and spatially expressed in embryonic tissues undergoing morphogenesis

To investigate the role of TRPM7 during early embryogenesis, we first cloned *Xenopus laevis* TRPM7 (XTRPM7), a protein that shares a sequence identity greater than 77% with the human protein. XTRPM7 possesses a domain architecture similar to that of its vertebrate orthologues, exhibiting high sequence identity within its NH_2-terminal TRPM homology region, its pore domain, and its COOH-terminal kinase domain (Supplementary Figs. 1A–C). We then examined the temporal and spatial expression patterns of XTRPM7 mRNA during *Xenopus* development. Two primer sets were designed to amplify fragments from the 5′ UTR and the 3′ UTR regions of the XTRPM7 mRNA, and RT-PCR analysis revealed expression of XTRPM7 maternally to the tadpole stage (Supplementary Fig. 2A). *In situ* hybridization demonstrated a relatively low level of XTRPM7 maternally through the gastrula stages of the developing embryo (Fig. 1A). During the gastrula stage, XTRPM7 RNA was most highly expressed in the dorsal ectodermal and mesodermal regions. By the neurula stage, XTRPM7 expression was higher in the neural plate, anterior neural fold, and neural hinge point regions. Later in development, XTRPM7 expression became restricted to the developing brain, kidney, heart, and notochord (Fig. 1A). This expression pattern is suggestive of a broad role for XTRPM7 during development.

Dorsal expression of TRPM7 disrupts Xenopus gastrulation

To determine the function of TRPM7 *in vivo*, we first expressed TRPM7 to elucidate its effect during *Xenopus* development. Injection of *murine* TRPM7 RNA into the ventral marginal zones of the four-cell embryo had no effect on *Xenopus* development. However, injection of TRPM7 RNA into the dorsal marginal zones of the four-cell embryo resulted in severe gastrulation defects in a dose-dependent manner, while injection of a similar dose of β-galactosidase (β-gal) RNA had no significant effect (Figs. 1B–C). In the TRPM7-injected embryos, the resulting gastrulation defect phenotypes were classified into two classes: severe and mild. In the severe class, anterior structures, including the head, eyes and cement glands, were reduced or absent; in addition, axial extension was impaired resulting in a severe dorsal-flexure in the embryos, and the blastopore and neural folds failed to close. In the mild class, there was a reduction in the size of the head,