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PLD1 regulates *Xenopus* convergent extension movements by mediating Frizzled7 endocytosis for Wnt/PCP signal activation

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ABSTRACT

Phospholipase D (PLD) is involved in the regulation of receptor-associated signaling, cell movement, cell adhesion and endocytosis. However, its physiological role in vertebrate development remains poorly understood. In this study, we show that PLD1 is required for the convergent extension (CE) movements during *Xenopus* gastrulation by activating Wnt/PCP signaling. *Xenopus* PLD1 protein is specifically enriched in the dorsal region of *Xenopus* gastrula embryo and loss or gain-of-function of PLD1 induce defects in gastrulation and CE movements. These defective phenotypes are due to impaired regulation of Wnt/PCP signaling pathway. Biochemical and imaging analysis using *Xenopus* tissues reveal that PLD1 is required for Fz7 receptor endocytosis upon Wnt11 stimulation. Moreover, we show that Fz7 endocytosis depends on dynamin and regulation of GAP activity of dynamin by PLD1 via its PX domain is crucial for this process. Taken together, our results suggest that PLD1 acts as a new positive mediator of Wnt/PCP signaling by promoting Wnt11-induced Fz7 endocytosis for precise regulation of *Xenopus* CE movements.

1. Introduction

Morphogenetic movements in gastrulation are crucial for establishing the three basic germ layers and body axis during early embryogenesis. In *Xenopus*, one of the major driving forces for this process is convergent extension (CE) movements, by which dorsal cells polarize and elongate along the mediolateral axis and intercalate toward the midline (convergence), leading to extension along the anterior/posterior axis (Wallingford et al., 2002; Veeman et al., 2003; Wang and Steinbeisser, 2009). The β-catenin-independent non-canonical Wnt pathway is well known to be important for the regulation of CE movements (Kuhl, 2002; Myers et al., 2002; Wallingford and Habas, 2005).

The non-canonical Wnt signaling pathway includes the Rho/JNK-mediated planar cell polarity (PCP) pathway, which regulates various cell behaviors such as cell movements, sensory cell orientation, and cytoskeletal rearrangements that lead to cell polarity (Wallingford, 2012; Sokol, 2000; Habas et al., 2001; Gray et al., 2011; Seifert and Mlodzik, 2007). The Wnt/PCP pathway, which was originally identified in *Drosophila*, is mediated by Frizzled (Fz) and Dishevelled (Dvl), and activates small G proteins such as Rho, Rac, and Cdc42, as well as Rho kinase (ROK) and c-Jun N-terminal kinase (JNK). Endocytosis of the ligand-activated receptor complex is not only a mechanism of signal attenuation by receptor downregulation, it is also an obligatory process underlying the provision of membrane platforms to the endosome for the assembly of signaling complexes (Hupalowska and Miaczynska, 2011; Gagliardi et al., 2008). Internalization of Wnt ligand-activated Fz receptors requires the endocytic activity of β-arrestin 2 (βarr2), Dvl, and clathrin adaptor proteins (APs), which induce specific endosomal targeting and signal activation (Jarrett et al., 2002; Kim and Han, 2007; Yu et al., 2007; Chen et al., 2003). Specificity of activation downstream of Wnt is regulated by receptor-mediated endocytosis and association of cofactors, as well as by specific combinations of Wnt ligands, coreceptors, and lipids (Kikuchi et al., 2009; Sheng et al., 2014).

Phospholipase D (PLD) hydrolyzes phosphatidylcholine (PC) to produce choline and phosphatidic acid (PA), which acts as a second messenger. PLD and its enzymatic product, PA are involved in the regulation of receptor-associated signaling, cell movement and adhesion, tumorigenesis, trafficking of secretory vesicles, and endocytosis (Jenkins and Frohman, 2005; Kang and Min, 2010; Gomez-Cambronero, 2014). Two mammalian PLD isozymes,

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designated PLD1 and PLD2, have been cloned. The catalytic HKD motif is a defining feature of PLD enzymes; highly conserved regions of PLD include a phox consensus sequence (PX), a plekstrin homology (PH) domain, and a phosphatidylinositol-4,5-bisphosphate [PtdIns(4,5)P_2] binding site. PLD1 has a conserved loop region that is not found in PLD2 (Xie et al., 2000; Stahelin et al., 2004; Hodgkin et al., 2000). The PH domain functions for localization of its target proteins, and the PX domain mediates protein-protein interactions and facilitates receptor-mediated endocytosis in epidermal growth factor receptor (EGFR) signaling (Jenkins and Frohman, 2005; Lee et al., 2006, 2009). However, little is known regarding the physiological role of PLD in vertebrate development and Wnt/PCP signaling.

Here, we show that endogenous PLD1 function is required for *Xenopus* CE movements during gastrulation. Furthermore, we suggest that PLD1 acts as a novel positive effector in Wnt/PCP signaling by enhancing Wnt11-induced Fz7 endocytosis via its GTPase-activating protein (GAP) regulatory activity toward dynamin.

2. Results

2.1. Dynamic expression of PLD1 during xenopus gastrulation

The full-length sequence of *Xenopus* PLD1 cDNA has been reported (NCBI Reference Sequence: NM_001136170.1). The encoded protein shows high similarity to orthologs from other vertebrate species (human PLD1a, 75%; human PLD1b, 78%; mouse PLD1, 77%) (Fig. 1A). Notably, *Xenopus* expressed sequence tags (EST) profiles reveal that *Xenopus* PLD1 transcripts are highly expressed in gastrula stages.

To investigate the potential role of PLD1 during *Xenopus* embryogenesis, we first analyzed the spatiotemporal expression of PLD1 transcripts and protein. Consistent with the EST profiles, whole-mount *in situ* hybridization showed that PLD1 transcripts were specifically localized to involuting mesoderm and the overlying ectoderm of *Xenopus* gastrula embryos (Fig. 1B). We then examined the localization of endogenous PLD1 protein by immunoblotting during *Xenopus* early development. Before gastrulation (st.8.5), PLD1 protein was equally distributed between dorsal and ventral sides of the embryo (Fig. 1C). Interestingly, as gastrulation proceeded (st.10.5), PLD1 protein became strongly enriched in the dorsal region (Fig. 1C). These data show that *Xenopus* PLD1 is dynamically expressed and may act in the dorsal region of the embryo to play a role in gastrulation.

2.2. Loss or gain of PLD1 function causes gastrulation defects in Xenopus

To fully understand the endogenous function of PLD1 during *Xenopus* embryogenesis suggested by its local enrichment in the dorsal marginal region of the embryo, we used dorsal region-specific loss-of-function and gain-of-function approaches. For loss-of-function analyses, we knocked down endogenous *Xenopus* PLD1 by injecting PLD1 antisense morpholino oligonucleotides (MOs), which can efficiently inhibit the translation of *Xenopus* PLD1 mRNA by binding to a complementary 5'-region of the mRNA containing the untranslated region (UTR) and ATG start codon. The specificity of the PLD1 MO was first verified using *Xenopus* gastrula embryos (Fig. 2A). Injection of PLD1 MOs effectively blocked the translation of PLD1 mRNA by binding to its target sequence (UTR-PLD1-Myc), whereas PLD1 mRNA lacking the MO target sequence (ORF-PLD1-Myc) was resistant to the MO (Fig. 2A). We found that injection of PLD1 MOs at the dorsal side of the embryo induced dose-dependent defects in the gastrulation processes, including a shortened axial body structure and spina bifida (Fig. 2B and C). These phenotypes were rescued by add-back of the MO-resistant form of *Xenopus* PLD1 mRNA (Fig. 2B and C), a result that also confirms the specificity of the MO effects. Similarly, overexpression of human PLD1a or PLD1b also induced gastrulation defects (Fig. 2D and E). Moreover, gastrulation defects resulting from human PLD1b were rescued by co-expression of PLD1 MO, indicating the importance of precise regulation of PLD1 function for gastrulation and confirming the functional conservation of PLD1 across species (Fig. 2D and E).

2.3. PLD1 is required for CE movements in xenopus

Dorsally enriched expressions of *Xenopus* PLD1 and defective phenotypes of PLD1 morphants are characteristics for genes that

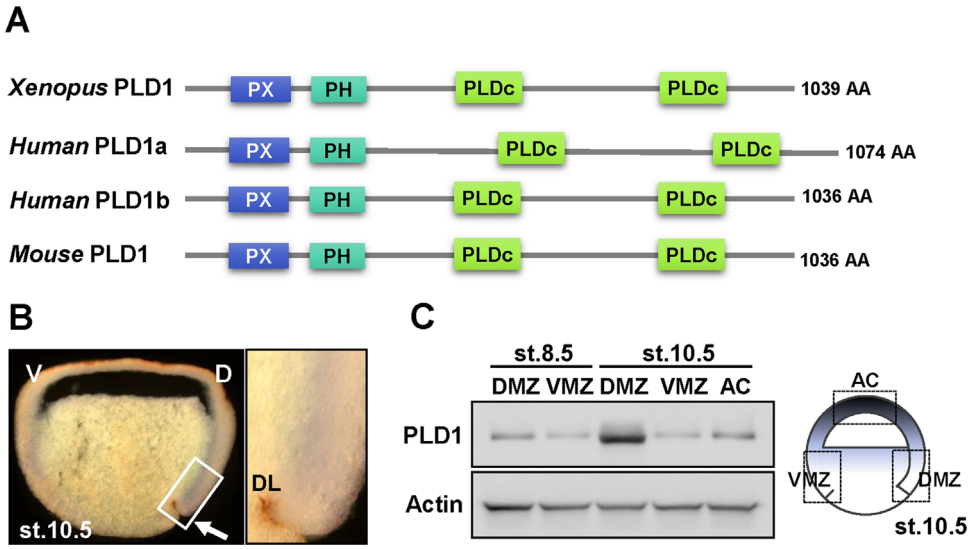

Fig. 1. PLD1 is dynamically expressed during *Xenopus* development. (A) Schematic diagrams of PLD1 in various species. PLD1 is well conserved among vertebrates. The number of amino acids in each PLD1 protein is indicated. (B) *In situ* hybridization assay with PLD1 and sagittal section views. Endogenous PLD1 was expressed in the involuting mesoderm and the overlying ectoderm. A white arrow indicates dorsal lips of stage 10.5 embryos and right panel is a magnified view of a white rectangle. (C) PLD1 protein was asymmetrically enriched in the dorsal region. Dorsal Marginal Zone (DMZ), Ventral Marginal Zone (VMZ), and Animal Cap (AC) were subjected to western blotting with anti-PLD1 antibody.

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