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Trb3 Regulates LR Axis Formation in Zebrafish Embryos

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Tribless family proteins are pseudokinases that lack DFG (Asp-Phe-Gly) motif in the functional kinase domain, regulating Akt and BMP pathways, insulin metabolism, hypoxia, and ubiquitination. This report concerns expression patterns and functional roles of *trb3* in zebrafish embryonic development. *trb3* is evolutionarily well-conserved and located on zebrafish chromosome 11. Spatiotemporal expression studies show that *trb3* transcripts are abundant throughout embryogenesis, but confined to mesendodermal cells during the late blastula phase. Over-expression of *trb3* ventralizes the embryos while a knockdown of *trb3* using morpholino alters positioning of the heart, liver, and pancreatic buds as well as gut looping. Furthermore, constitutive activation of TGF-β signaling with TARAM-A* (TGF-β-related type I receptor) significantly increases the level of *trb3* transcripts during the late blastula phase. Over-expression of *trb3* reduces the level of *smurf1* transcripts, a member of TGF-β signaling. We thus propose that Trb3 governs left-right (LR) axis patterning as a component of TGF-β signaling in vertebrate embryonic development.

INTRODUCTION

Members of the *Tribbles* family, *Tribbles 1, 2 and 3* encode pseudokinases lacking catalytically active kinase domains. They have been highly conserved through the evolutionary process and TRIBBLES proteins are characterized with DLKLRK instead of DLKPEN in kinase motifs (Mayumi-Matsuda et al., 1999). TRIBBLES3 (TRB3) was identified in neuronal cells and is also called NIPK, SKIP3, and SINK (Bowers et al., 2003; Wu et al., 2003). TRB3 modulates NF-κB signaling, lipid metabolism and insulin metabolism (Du et al., 2003; Qi et al., 2006). It also regulates cell cycle *via* negative modulation of Slbo and String in *Drosophila* (Mata et al., 2002). TRB3 acts as an adopter/mediator molecule in the photomorphogenic protein 1 (COP1) that mediates degradation of acetyl-coenzyme A carboxylase (ACC) (Qi et al., 2006) and downregulates SMAD specific E3 ubiquitin regulatory factors 1/2 (Smurf1/2) (Chan at al., 2007; Hua et al., 2011). TRB3 is a key molecule in muscle differentiation *via* PKB/Akt signaling pathways (Kato and Du, 2007), a potent target of various physiological responses like hypoxia (Rzymski et al., 2008), ER stress (Avery et al., 2010), oxidative stress and nutrient deficiency in various cell types (Jousse et al., 2007). *Tribbles* family members have been reported to interact with BMPRII tail domain (BMPRII-TD) in the pulmonary artery smooth muscle cells (PASMCs) (Chan et al., 2007).

Despite the current manifests claiming that TRB3 is involved in BMP signaling and in various physiological roles, its roles in the vertebrate embryogenesis have not been elucidated. In this report we made an attempt to examine functional roles of Trb3 in zebrafish embryogenesis. This report explores the spatiotemporal expression patterns and developmental functions of *trb3* in zebrafish embryos.

MATERIALS AND METHODS

Zebrafish care and embryos

Wild type zebrafish are obtained from the Korea Zebrafish Organogenesis Mutant Bank (ZOMB) and grown at 28.5°C. Embryos are obtained through natural spawning, raised, and staged as described in the Kimmel et al. (1995). Embryonic pigmentation was blocked by treating them with 0.002% PTU from the onset of somitogenesis.

Sequence analysis

Trb3 sequences are subjected for homology as described in the Kim et al. (2008).

Molecular cloning of *trb3*

Total RNA was isolated from the embryos at various stages using an easy BLUE total RNA extraction Kit (iNtRON Bio, Korea) according to manufacturer guidelines. cDNA was synthesized with Superscript™ III reverse transcriptase (Invitrogen Corp) as described in the Maddirevula et al. (2011). RT-PCR is performed using forward primer 5′-ACCATGAGTATGAACCT GTCAACT-3′; reverse primer 5′-GAATTCCTGACAGTCCTCA GTCTGG-3′ with HiPi Pus DNA polymerase (Elpis Bio, Korea). For over-expression studies ORF of *trb3* was sub-cloned into the pCS2+ vector in between *Bam*H1 and *Eco*R1.

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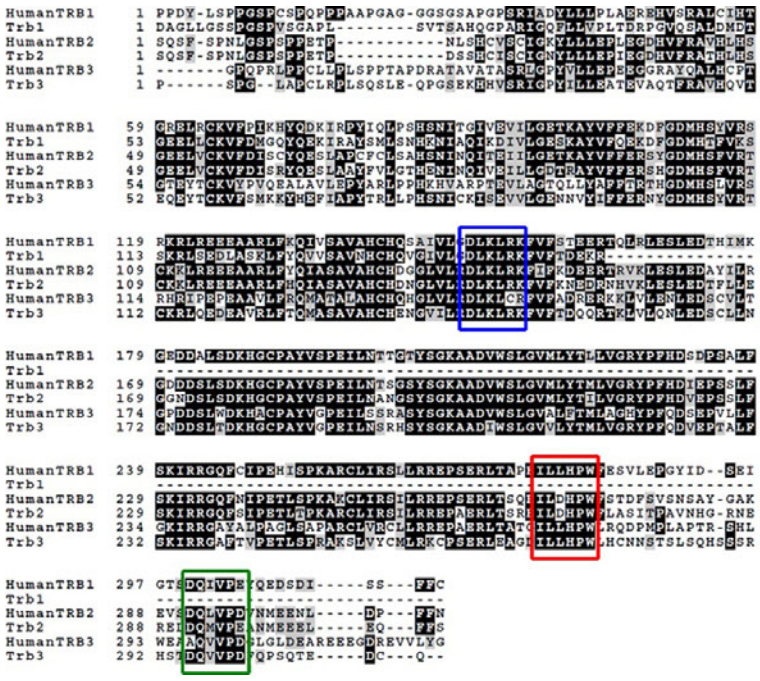


Fig. 1. Amino acid sequence homology study of Tribbles proteins 1, 2 and 3 (ENSDARP00000101435, ENSDARP00000089195, ENS DARP00000036401) in comparison with humans (protein access number: NP_066981.2). Blue box indicates pseudokinas domain.

Morpholinos, *in vitro* transcription, and microinjections

Translation blocking oligonucleotides, morpholinos were obtained from Gene-Tools, USA, and dissolved in water. Ten ng of *trb3*-specific morpholinos [5′-taggagttgacaggttcatactcat-3′], con-trol morpholinos [5′-tagCaCttgaGagCttcataGtcat-3′] were injected into zebrafish embryos at 1 cell stage. *trb3*, *TARAM-A*, lefty1/antivin* and *smurf1-gfp* mRNAs were synthesized with Ambion mMESSAGE mMACHINE kit as per the company directions. The synthesized mRNAs were dissolved in nuclease-free water, diluted in 0.5% phenol red solution for microinjection, which was conducted with Picopump microinjection device (World Precision Instruments).

Whole mount *in situ* Hybridization (WISH)

Embryos were fixed in 4% paraformaldehyde (PFA) overnight, and dehydrated in 100% methanol. Embryos after 24 hpf were digested with 10 μg/ml protease K. WISH is performed with minor modifications in washing as described in (Thisse et al., 1993). *smurf1* antisense probes were synthesized from the partial region (472 bps) within ORF. The partial region is amplified with forward primer 5′-ATGTCGAATCCTGGGACTCG-3′; reverse primer 5′-TCTCTACCAGTCCTCGACA-3′ and cloned into pGEM®-T Vector (Promega). *trb3, cas, ntl, gata5, mixer, cmlc2, foxa3* (Field et al., 2003), *chd* and *evel* (Joly et al., 1993) antisense probes were synthesized with DIG RNA Labeling Kit (SP6/T7) (Roche).

RESULTS

Characterization of zebrafish *tribbles3 (trb3)*

Because TRB3 is a critical element in various signaling pathways (Du et al., 2003; Qi et al., 2006) we investigated its embryological roles in a zebrafish animal model. We searched for orthologues of human *TRIBBLES3 (TRB3)* in the zebrafish genome at Zv9 (www.ensembl.org) and identified that zebrafish *tribbles3 (trb3)* contains 3 exons within the range of 4.66 kb. Identified contig region was further searched at the NCBI tBLAST and confirmed as zebrafish Trb3. We isolated the orthologue of human *TRB3* (GenBank accession no; NM_021158), *trb3* (GenBank accession no.; NM_212869) from the cDNA library of zebrafish embryos at 30% epiboly. Comparative analysis of the deduced amino acid sequences of Trb3 versus human TRB3 found that Trb3 shares 52% homology (Fig. 1) with the human TRB3 while it shares 98% homology in the pseudokinase domain located between the 67th and 314th amino acid residues (Fig. 1). It has been reported that human TRB3 contains MEK1 binding domains for control of MEK1/ERK pathways as well as COP1 binding domains for physical interaction with COP1 in lipid metabolism (Lee et al., 2012; Qi et al., 2006; Yokoyama and Nakamura, 2011). Trb3 contains both MEK1 binding domains and COP1 binding domains at the C-Terminal region (Fig. 1; Red and Green box). Taken together, zebrafish *trb3* is a homologue of human *TRB3* and highly conserved in the functional domains pseudokinase, COP1 interacting and MEK1 binding domains.

Spatiotemporal distribution of *trb3* transcripts in the embryos

As an initial approach to circumvent biological functions of Trb3, the spatiotemporal expression profile of *trb3* was analyzed with RT-PCR (Supplementary Fig. 1) and WISH using a DIG-labeled *trb3*-specific anti-sense probe in the first cell stage through 4 days of post-fertilization (dpf) embryos. *trb3* transcripts are present in the first cell stage and in sphere (Figs. 2A and 2B), and become confined to the blastoderm marginal cells during the late blastula (30% epiboly) and early gastrula stage (50% epiboly) (Figs. 2C-2E). At the end of gastrula stage, the transcripts are more specifically restricted to the axial cells and developing notochord at the tail bud (Figs. 2F and 2G). *trb3* transcripts appear more prominent in the notochord and developing nervous system at the 16 somites stage (Fig. 2H), and later in the forebrain, tectum, midbrain-hindbrain boundary, and

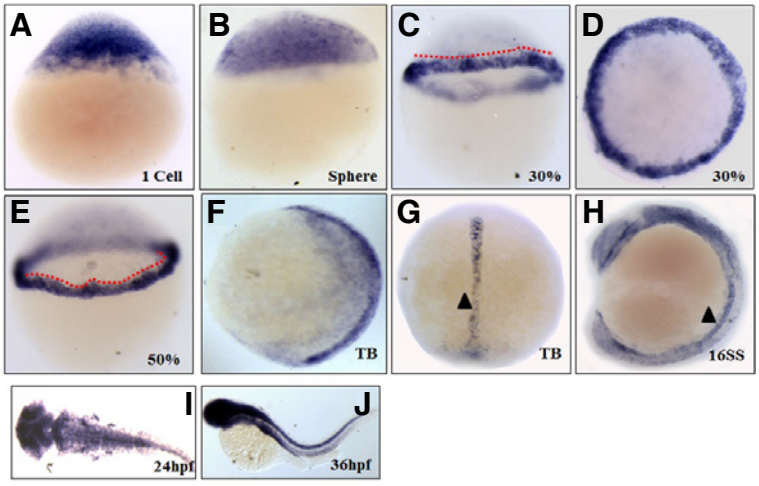


Fig. 2. Spatiotemporal distribution of *trb3* transcripts in the embryos. WISH with anti-sense *trb3* probe at one cell stage (A), Sphere (B), 30% epiboly (C, D), 50% epiboly (E), Tail bud (F, G), 10 somite stage (H), 24 hpf (I) and 36 hpf (J). (A-C, E, F, and H) Lateral views with dorsal side right. (D, I) dorsal views. (J) Lateral views with anterior part left. Dotted lines indicate transcripts restriction to marginal cells. Arrow head indicates notochord.

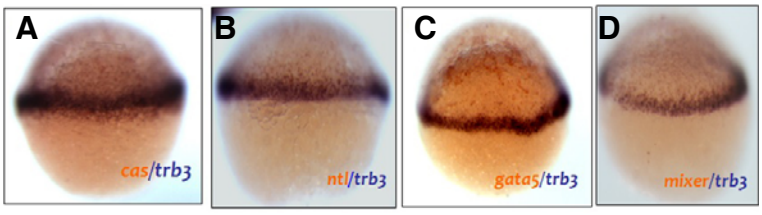


Fig. 3. Localization of *trb3* transcripts in the blastodermal marginal cells at late blastula. Two color *WISH* was performed with DIG labeled *trb3* antisense probe and fluorescent labeled DIG antisense probes of *cas* (A), *ntl* (B), *gata5* (C) and *mixer* (D) 30% epiboly (4.7 hpf). (A-C) lateral views. Control embryo at 30% epiboly (E).

hindbrain at 24 hpf (Fig. 2I). Interestingly, they become intensified in the nervous system and developing endoderm-derived organs, such as the intestine at 36 hpf (Fig. 2J). These cell- and tissue-specific expression patterns of *trb3* in the embryos suggest that Trb3 may have a critical role in early embryogenesis.

Because *trb3* transcripts are restricted to the blastodermal marginal cells at 30% epiboly (Fig. 2C) we analyzed in-depth expression profiles of *trb3* in cells using two color WISH germ layered specific markers, such as a *cas* (endodermal marker), *ntl* (mesoderm marker), *gata5* and *bon/mixer* (mesendodermal markers). Expression domains of *trb3* are partially overlapped with those of *cas* and *ntl* (Figs. 3A and 3B) but had more overlap with those of *gata5* and *bon* (Figs. 3C and 3D), confirming its expression in the mesendodermal cells.

Trb3 is a component of TGF-β signaling

It is known that Nodals, members of the TGF-β pathway, play a critical role in LR axis patterning (Schier, 2009) whereas *lefty1/antivin* works as an antagonist to Nodal signaling in zebrafish embryogenesis (Bisgrove et al., 1999). To test the functional relationship between TGF-β signaling and Trb3, we initially overexpressed *TARAM-A** which structurally resembles Alk4, a receptor of TGF-β related ligands in the *Xenopus* embryos (Chen et al., 2004) that constitutively induces TGF-β signals (Peyrieras et al., 1998; Renucci et al., 1996). We then measured the level of *trb3* transcripts in the *TARAM-A** overexpressed embryos at 30% epiboly, and found that its level was significantly elevated while at the same time extending out to the blastodermal cells (Figs. 4A and 4B). In contrast to the TRARAM-A* effect, overexpression of *antivin/lefty1*, a Nodal inhibitor, clearly reduces the level of *trb3* transcripts (Figs. 4A and 4C). It is thereby evident that TGF-β signaling positively regulates the transcription of *trb3*.

Forced expression of *trb3* ventralizes the embryos

To assess embryological roles of Trb3 in embryogenesis, we over-expressed *trb3* by microinjecting 250 pg of 5′-capped *trb3* mRNAs (Figs. 5A-5H). Over-expression of *trb3* induces ventralization in 65% (n =1 10) of *trb3* mRNA injected embryos, giving rise to a shortened axis at 11 hpf (Figs. 5A and 5B), small eyes, elaborated somites, cardiac edema, and a thickened yolk plug at 36 hpf (Figs. 5C and 5D).

Embryos overexpressing *trb3* are further assayed with the dorsal marker, *chordin* (*chd*) encoding a BMP4 inhibitor (Sasai et al., 1995) and the ventral marker, *even-skipped-like1* (*eve1*) (Joly et al., 1993). At 60% epiboly, *eve1* transcripts are increased in the ventral marginal cells of the embryos as to those of the control embryos (Figs. 5E and 5F). The dorsal marker, *chd* expression domains are markedly reduced in the dorsal side cells of the *trb3* injected embryos in comparison with the control embryos at 6 hpf (Figs. 5G and 5H). All these observations support that Trb3 is an essential regulator for the ventral patterning during the late gastrula stage and thereafter until it reaches 36 hpf (Figs. 5A-5D).

Trb3 is required for left-right (LR) axis orientation of the organs

Specific expression of *trb3* in the mesendodermal cells (Figs. 3A-3D) raises the possibility that Trb3 is of critical importance to the development of organs derived from the mesendodermal cells. To test this hypothesis we performed loss-of-function studies of *trb3* by injecting *trb3* specific translation blocking morpholino into the embryos at cell stage one. To assess molecular events associated with the loss-of-function of Trb3 in the embryos at 48 hpf we performed WISH with the LR axis patterning marker for heart development, *cardiac myosin light chain-2* (*cmlc2*). In contrast to the control embryos (n = 75), the

Table 1. Phenotype rescue of heart laterality by coinjecting *trb3* mRNAs and *trb3* morpholino

Trb3 MO (ng)	mRNA (pg)	*n*	Heart laterality defects (%)
10	-	108	39
10	250	94	6

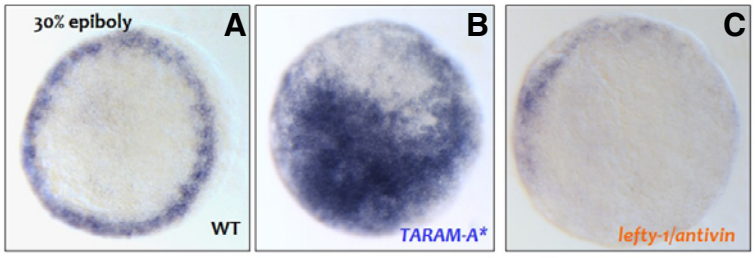


Fig. 4. Embryo injected with 100 pg of TARAM-A* at two cell stage (B). Embryo injected with 50 pg of *lefty-1* at two cell stage (C).

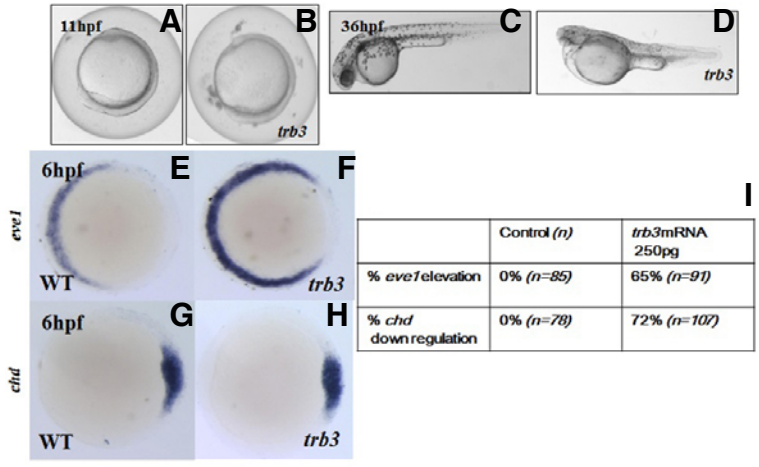


Fig. 5. Gain-of-function studies of *trb3*. Forced expression of *trb3* induces ventralization of the embryos (A-H). Control embryos (A, C), injected embryos (B, D) are at 11 hpf and 36 hpf, respectively. (E-H) Molecular marker studies with *eve1* (E, F) and *chd* (G, H); control embryos (E, G). Quantification of molecular marker study of *trb3* overexpressed embryos (I).

molecular labeling found that morphological defects in the cardiac looping and positioning, reversed looping (D-looping) and caused failure in the remaining ventricle looping to the midline (39%; *n* = 108) (Figs. 6A-6C). *trb3* morphants at 55 hpf were further assayed with *forkhead box A3 (foxa3)*, an endoderm marker, which specifies visceral organs, such as the liver and the pancreatic bud (Field et al., 2003). Spatiotemporal expression patterns of *foxa3* detect heterotaxy in the gut looping as well as positional changes of the liver and pancreatic bud. Morphants show randomized positioning of the liver and pancreatic bud as well as organ duplication (Figs. 6D-6F). In order to conform to the specificity of *trb3* and its morpholino we performed rescue experiments by coinjection of *trb3* mRNA and *trb3* MO, which showed phenotype with rescued heart laterality defects (Table 1). These findings strongly suggest that Trb3 is an essential element in regulating LR axis orientation of the heart, gut, pancreas and liver in organogenesis.

Over-expression of *trb3* reduces level of *smurf1* transcripts

Studies in the pulmonary artery smooth muscle cells (PASMCs) show that TRB3 regulates Smurf1 in association with BMP signaling components (Chan et al., 2007). We thus examined the putative correlation between Smurf1 and Trb3 in zebrafish embryogenesis. We first measured endogenous level of *smurf1* transcripts in the embryos at 14 hpf, in which *trb3* transcripts were microinjected at one cell stage for over-expression. Over-expression of *trb3* remarkably decreases the level of *smurf1* transcripts in the embryos (Figs. 7A-7F). However, during embryonic development expression domains of *trb3* (Figs. 2F and 2H) are largely overlapped with the expression domains of *smurf1* (Figs. 7A and 2B). Two different amounts (200 pg or 300 pg per embryo) of *trb3* mRNA reduce the transcriptional level of *smurf1* in a concentration-dependent manner (Figs. 7C-7F) in the 80% (*n = 98)* of *trb3* mRNA injected embryos, evidently demonstrating that Trb3 is a critical regulator of Smurf1 to modulate TGF-β signaling in embryos.

DISCUSSION

Pseudokinases are functionally important in various cellular signaling processes although they are a functionally inactive in phosphorylation (Reviewd by Boudeau et al., 2006). Tribbles proteins are initially identified in *Drosophila* and are critical for cell division and mitosis (Rorth et al., 2000). Three isoforms of TRB are reported with unique function in *Drosophila* while their

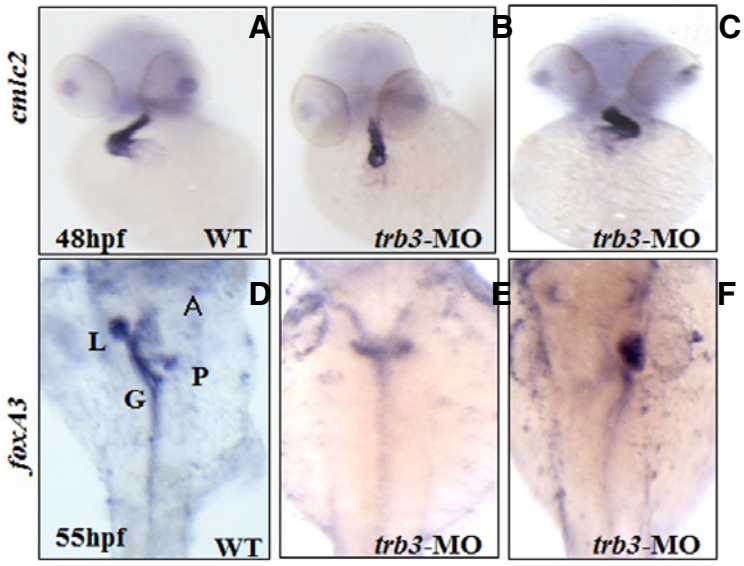
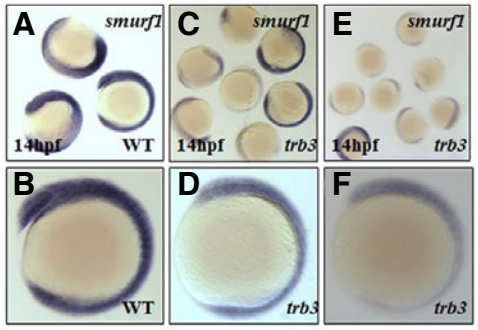


Fig. 6. Knock down of *trb3* alters LR axis orientation of organs. Morphant embryos are analyzed with organ specific markers, heart (A-C), liver, gut and pancreatic bud (D-F); heart position was iden-tified with cardiac specific marker, *cmlc2* while the positions of liver, gut, pancreatic bud with *foxa3*.



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	Control *(n)*	*smurf1* downregulation
*trb3*mRNA 200pg	0% (*n=65*)	80% (*n=99*)
*trb3*mRNA 300pg	0% (n=*81*)	90% (*n=86*)

Fig. 7. Forced expression of *trb3* alters level of *smurf1* transcripts. Level of *smurf1* transcripts are measured in *trb3* overexpressed embryos (A-F). (A, B) Control embryos. (C, D) 200 pg *trb3* mRNA injected embryos. (E, F) 300 pg *trb3* mRNA injected embryos. Quantification of the number of embryos with reduced *smurf1* levels in the *trb3* overexpressed embryos (G).

functional roles in developing embryos remain unclear. In this report we aimed to analyze the functional roles of a pseudokinase, *tribbles3* in a vertebrate animal model zebrafish *(Danio rerio)* embryogenesis. We identified *tribbles3* in the zebrafish genome and isolated zebrafish *trb3* which is an orthologue of human TRB3. We found that *trb3* is expressed throughout embryogenesis. In particular, *trb3* transcripts are restricted to mesendodermal cells, the precursor cells for the heart, liver, gut and pancreatic bud. Transforming growth factor (TGF-β) signaling has been demonstrated as a pivotal regulator for the induction of mesendoderm, a tissue specific to the Spemann organizer (Rodaway and Patient, 2001). Overexpression of the TGF-β activator (TARAM-A*) and the antagonist, Lefty1 confirmed that TGF-β signaling is required for *trb3* expression in the embryos.

Studies have shown that Trb3 physically interacts with the BMP type II receptor in mammalian cells and thereby regulates BMP signaling (Chan et al., 2007). BMPs and their receptors belong to the TGF-β super family and are critical for ventral patterning in vertebrates (von Bubnoff and Cho, 2001). Overexpression of *trb3* induced ventralized phenotypes, which were further confirmed by molecular marker studies. Existing evidence from animal models revealed that the TGF-β super family contributes to the left-right asymmetry (LR) process, notably Nodal, Pitx2, Lefty 1 and Lefty2 (Meno et al., 1998). Bone morphogenic protein (BMP) signaling also governs LR patterning; it subdues *nodal* expression in the left lateral plate mesoderm (LPM) to regulate heart laterality and dorsoventral fate in *Xenopus* and zebrafish embryos (Monterio et al., 2008; Ramsdell, 1998; Tucker et al., 2008). Secreted BMPs belonging to the TGF-β super-family transduce the signals by interacting with BMP type I and II receptors (Chen at al., 2004; Hua et al., 2011). Considering these combined features of the signaling pathways including Trb3, *trb3* expression in the mesendodermal cells (Figs. 2C and 4), and the altered expression pattern of *cmlc2* and *foxa3* in the *trb3* MO (Fig. 6), it is conceivable that Trb3 plays a pivotal role in controlling LR axis patterning.

The evolutionarily conserved LR patterning process is tightly regulated during embryogenesis and involves various elements such as ubiquitin proteasome system (UPS) components. Ubiquitin E3 ligases, Smad ubiquitination-related factor 1 (Smurf1) and Smurf2 control TGF-β signaling by regulating cellular levels of Smad molecules (Izzi and Attisano, 2004). SMADs are vital regulators of the TGF-β signaling with numerous interactions in the cytoplasm and nucleus (Rebagliati et al., 1998) and SMADs levels are modulated by Smad ubiquitination regulatory factor 1 (Smurf1) and Smurf2. Smurfs are E3 ubiquitin ligases and master regulators of SMADs (Moustakas et al., 2001). Over-expression of *trb3* reduces the level of endogenous Smurf1 levels in the embryos (Fig. 7). Expression and functional studies on

trb3 in the zebrafish embryos demonstrated that *trb3* is expressed in mesendodermal cells during the late blastula stage and is a vital component of TGF-β signaling (Figs. 2 and 4). Over-expression of *trb3* induces ventralization and reduces the *smurf1* transcripts (Fig. 7) whereas the knock-down of *trb3* using *trb3*-specific morpholino alters the positions of the heart, liver, pancreatic bud, and gut loop (Fig. 6). It is most likely that Trb3 exerts its biological roles *via* Smurf in developing vertebrate embryos. It would be of great interest to identify target molecules of Trb3 using proteomic approaches for extension of the Trb3 network during vertebrate embryonic development.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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