The Subcellular Localization of TRPP2 Modulates Its Function

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ABSTRACT
TRPP2, also known as polycystin-2, is a calcium permeable nonselective cation channel that is mutated in autosomal dominant polycystic kidney disease but has also been implicated in the regulation of cardiac development, renal tubular differentiation, and left-to-right (L-R) axis determination. For obtaining further insight into how TRPP2 exerts tissue-specific functions, this study took advantage of PACS-dependent trafficking of TRPP2 in zebrafish larvae. PACS proteins recognize an acidic cluster within the carboxy-terminal domain of TRPP2 that undergoes phosphorylation and mediate retrieval of TRPP2 to the Golgi and endoplasmic reticulum (ER). The interaction of human TRPP2 with PACS proteins can be inhibited by a Ser812Ala mutation (TRPP2S812A), thereby allowing TRPP2 to reach other subcellular compartments, and enhanced by a Ser812Asp mutation (TRPP2S812D), thereby trapping TRPP2 in the ER. It was found that the TRPP2S812A mutant rescued cyst formation of TRPP2-deficient zebrafish larvae to the same degree as wild-type TRPP2, whereas the TRPP2S812D mutant was significantly more effective in normalizing the distorted body axis of TRPP2-deficient fish. Surprisingly, the TRPP2S812D mutant rescued the abnormalities of L-R asymmetry more effectively than either wild-type or TRPP2S812A, suggesting that the ER localization of TRPP2 plays an important role in the development of normal L-R asymmetry. Taken together, these findings support the hypothesis that TRPP2 assumes distinct subcellular localizations to exert tissue-specific functions.


Mutations of either PKD1, encoding for polycystin-1, or PKD2, encoding for the transient receptor potential (TRP) channel TRPP2 (polycystin-2), are responsible for almost all cases of autosomal dominant polycystic kidney disease (ADPKD). Although the predominant phenotype of this hereditary disease is the progressive formation of renal cysts and loss of renal function, other abnormalities, such as cerebral aneurysms and liver and pancreatic cysts, contribute significantly to the morbidity and mortality of affected patients. The systemic character of the disease becomes even more apparent in mice with a targeted deletion of either PKD1 or PKD2. Homozygote PKD2−/− mice are embryonal lethal and develop progressive total body edema and focal hemorrhage as a result of cardiac malformation. The deletion of PKD1 results in extensive renal cyst formation as well as severe skeletal abnormalities. A striking difference is the situs inversus, present in PKD2-deficient mice but not in PKD1-deficient mice. The left-to-right (L-R) asymmetry in mice critically depends on a ciliated...
TRPP2 is a calcium-permeable nonselective cation channel implicated in cellular functions as diverse as mechanosensation, mating behavior, fertilization, proliferation, apoptosis, and polarity (reviewed by Giamarchi et al.9). TRPP2 shares its overall topology with other members of the TRP family but lacks the N-terminal ankyrin repeats that characterize most other TRP channels. Its biophysical characteristics are surprisingly unremarkable: TRPP2 is a cation channel with a selectivity slightly higher for calcium than for sodium and potassium with both a high single-channel conductance and spontaneous open probability.9 –11 Obviously, such a channel, when inserted into the plasma membranes, requires tight control to prevent detrimental effects for the cellular homeostasis. Perhaps for this reason, TRPP2 is retained in the endoplasmic reticulum (ER) in most cells,12 suggesting that TRPP2 trafficking represents one important mechanism to control the activity of this channel. The acidic cluster phosphorylated by casein kinase 2 (CK2) on serine 812 in human TRPP2 mediates binding to PACS-1 and PACS-2, two connector proteins that link TRPP2 to AP-1 and COPI adaptor proteins, and mediates retrieval of TRPP2 back to the trans-Golgi network (PACS-1) and the ER (PACS-2).13,14 Dephosphorylation of the serine 812 or serine-to-alanine (S812A) mutation inactivates the retrieval mechanism and allows the protein to reach other subcellular compartments. In contrast, a serine-to-aspartic acid (S812D) mutation, mimicking serine phosphorylation, enhances the interaction with PACS molecules and traps the molecule in the ER. Additional adaptor proteins regulate the trafficking of TRPP2. For example, PIGEA-14 augments forward trafficking of TRPP2 from the ER to the cis-Golgi,15 whereas interaction with polycystin-1 facilitates the translocation of TRPP2 to the plasma membrane.16

TRPP2 is also present in the nonmotile cilium of renal tubular epithelial cells. Recent findings strongly support the hypothesis that the ciliary localization of TRPP2 is essential to prevent cyst formation.17 Although direct evidence that TRPP2 is directly activated by mechanical stimuli is still preliminary,18 both polycystin-1 and TRPP2 are indispensable to translate ciliary bending into intracellular calcium transients.9 Because TRPP2 is present at other subcellular localizations during renal development, including the basolateral plasma membrane and the ER, it remains unknown which subcellular localization of TRPP2 contributes to the normal tubular development.

Because serine 812 has a major impact on the subcellular localization of human TRPP2, we examined how TRPP2 S812 mutants affect the function of TRPP2 in zebrafish. The zebrafish TRPP2 homologue has been located on chromosome 19,20; it is closely related to human TRPP2, containing a phosphorylatable acidic cluster in its carboxy-terminal domain. In a 2-d-old zebrafish larva, immunostainings show a strong expression of TRPP2 in the trunk muscles and in the pronephric duct but also in the ependymal cells of the central nervous system, the olfactory placode, cells of the inner ear, and the lateral line organ. In the zebrafish pronephros, TRPP2 is localized to specific compartments of the cell along the different nephron segments: In the anterior duct, TRPP2 is associated with basolateral membranes and luminal bundles of cilia. In the posterior duct close to the cloaca region, TRPP2 accumulates in intracellular vesicles and in cilia, mainly at their base, but is almost absent from basal cell surfaces.21 Depletion of TRPP2 in zebrafish results in three striking phenotypes: Cyst formation of the pronephric duct, a curved body axis, and a randomized organ situ.19–22 Using TRPP2 mutants with de-

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**Figure 1.** Sequence comparison of the acidic cluster of TRPP2 and the furin binding regions of PACS-1 and -2. (A) The acidic clusters (boxed) are conserved between human and zebrafish TRPP2/polycystin-2 but are lacking in C. elegans TRPP2. (B) Human PACS-1 furin-binding region (FBR; NM_018026) and zebrafish PACS-1 FBR (EF531598). (C) Human PACS-2 FBR (NM_015197) and zebrafish PACS-2 FBR (EF531599). The human and zebrafish PACS-1 and -2 FBR are highly conserved. ClustalW and boxshade were used for the alignment.
fined subcellular localizations to rescue the phenotypic changes caused by depletion of zebrafish TRPP2, we attempted to characterize the localization-dependent functions of TRPP2.

RESULTS

The ER and Golgi Retrieval Mechanisms of TRPP2 Are Evolutionarily Conserved

Zebrafish TRPP2/polycystin-2 shares the conserved acidic cluster of the carboxy-terminal domain with the mammalian homologues but not with the Caenorhabditis elegans TRPP2 (Figure 1A). Blast analysis of mammalian PACS-1 and PACS-2 identified two closely related proteins that we termed zPACS-1 and zPACS-2. Both proteins contain a furin-binding region (Figure 1, B and C); the overall amino acid sequence similarity compared with the human homologues is 62% identity and 76% similarity for zPACS-1 and 73% identity and 84% similarity for zPACS-2 (Supplemental Figure 1, B and C). We predicted, therefore, that these two connector proteins would exert the same properties as their mammalian counterparts. As shown in Figure 2, the furin-binding region of zPACS-1 and zPACS-2 recognized and bound zebrafish (Figure 2A) and human TRPP2 (Figure 2A) and human TRPP2 (Figure 2, B and C), requiring a phosphorylatable serine at position 812.13 As has been reported for human TRPP2, the zebrafish TRPP2, expressed in HeLa cells, was almost completely retained in the ER (Figure 2D), where it co-localized with the ER protein BAP31.23,24 A similar result can be obtained when zebrafish TRPP2 is expressed in the zebrafish fibroblast cell line PAC2 that is propagated at 28°C; zTRPP2 co-localized with the ER-marker protein calnexin (Figure 2E).

To provide further evidence that PACS molecules are a major regulator of zTRPP2 localization, we expressed zebrafish PACS-1 and -2 in zebrafish larvae, speculating that abundant PACS-1 and PACS-2 should facilitate the retention of endogenous zTRPP2 in the ER and Golgi. According to the severity of the alterations, the larvae were classified into three groups (Figure 3A). As shown in Figure 3, A and B, excess amounts of PACS resulted in hydrocephalus, pericardial edema, aberration of the body axis, and cyst formation in the pronephros in zebrafish larvae at 55 hours post fertilization (hpf) similar to the depletion of TRPP2 by morpholino injection (Figure 4B). The effect was dosage-dependent (data not shown) and was more pronounced with zPACS-2 (50 pg; cyst formation in 41.2%), the PACS family member that interacted more strongly with zTRPP2 (Figure 2A) than zPACS-1 (180 pg; 10.5% cysts). The co-injection of zPACS-1 and -2 RNA was only slightly additive in their effect on the rate of dysmorphic changes (Figure 3C) but not on cyst formation (43%), pointing to saturation of the effect of zPACS-2. These results suggest that overexpression of zPACS prevents zTRPP2 from reaching its normal destination.

For further substantiation of these data, endogenous TRPP2 was stained in the zPACS-RNA–injected zebrafish larvae. In the anterior ducts of wild-type (WT) larvae, TRPP2 is expressed in cilia and associated with basolateral cell membranes and infoldings (Supplemental Figure 3, A through C) as

![Figure 2. The FBR of zPACS-1/2 interacts with zebrafish TRPP2 and human TRPP2. The interaction is enhanced for TRPP2S812D but reduced for TRPP2S812A. Zebrafish TRPP2 is retained in the ER. (A) HEK 293T cells were transiently transfected with the flag-tagged cytoplasmic domain of zebrafish TRPP2 (F9.zTRPP2.cyt) and with V5-tagged FBR of zPACS-1 and -2 (V5.zPACS-1 or -2.FBR). F9.zTRPP2.cyt co-precipitated with V5.zPACS-1/2.FBR but not with V5.GFP. (B) Transient transfection of HEK 293T cells with V5.zPACS-1.FBR and flag-tagged cytoplasmic domain of human TRPP2 (F9.hTRPP2.cyt) WT or mutated serine 812 (WT, S812A, S812D). F9.hTRPP2.cyt co-precipitated with V5.zPACS-1.FBR and flag-tagged cytoplasmic domain of human TRPP2 (F9.hTRPP2.cyt) WT or mutated serine 812 (WT, S812A, S812D). V5.zPACS-1.FBR bound and co-precipitated the C-terminus of human TRPP2 WT and TRPP2S812D but less strongly with TRPP2S812A. Similar results were obtained by pull-down experiments (Supplemental Figure 2). (D) Overexpression of F9.zTRPP2 and BAP31.EGFP in HeLa cells shows an overlapping distribution of both proteins in the ER. (E) Overexpression of F9.zTRPP2 in zebrafish PAC2 cells (kept at 28°C) shows a corresponding overlap with calnexin in the ER.]
Previously shown by Obara et al., PACS overexpression abrogates the distinct basolateral staining pattern, leading to a more homogeneous intracellular distribution of TRPP2 (Supplemental Figure 3, D, G, and J). Also, the cilia co-localization is disturbed in the co-injected larvae; therefore, PACS molecules play a crucial role in TRPP2 trafficking and maintenance of tubular integrity.

Depletion of PACS molecules by morpholino knockdown causes dysmorphic changes in 55-hpf-old larvae (Supplemental Figure 4) that were classified similarly to the aforementioned experiment of PACS overexpression. Morphant larvae showed pronephric cysts in 41% (pacs-1 MO), 26% (pacs-2 MO), and 64% (combined knockdown).

Modulation of TRPP2 Expression Levels in Zebrafish Causes Cyst Formation, Body Curvature, and Situs Inversus

We hypothesized that TRPP2 uses distinct subcellular localization to exert tissue-specific functions. To establish a robust model system to address this question, we examined the effects of zTRPP2 depletion by morpholino oligos (pkd2 MO), targeting the splice donor site of coding exon 3 or exon 5 as recently described. As expected, zTRPP2 depletion caused cyst formation of the pronephros, a characteristic dorsal body axis curvature, and a random organ situs (Figure 4A and 6C). The pronephric cysts were validated by histologic cross-sections.
Differential Rescue of Cyst Formation and Body Axis Curvature by WT and Mutant TRPP2

To assess tissue-specific differences, we selected a zpkd2 morpholino oligo that resulted in cyst formation, suggesting that a delicate balance of polycystin-1/TRPP2 levels is necessary to maintain tubular integrity. We tested, therefore, whether overexpression of WT and mutant hTRPP2 causes pronephric cyst formation. As a control, we overexpressed GFP by injection of capped RNA: With up to 460 pg/embryo, there was a homogenous fluorescence, but no dysmorphic features were detectable (n = 71) (Supplemental Figure 5). Lower amounts of hTRPP2 capped RNA (100 pg) had only minor or no detectable effects. The amount of capped RNA injected was chosen in the high range (200 pg) to provoke overexpression effects. At higher concentrations (200 pg), all three mRNA, WT hTRPP2, hTRPP2S812A, and hTRPP2S812D, resulted in alterations of the body shape and axis of the larvae (Figure 4C). According to the severity of the alterations, the larvae were classified into three groups (Figure 4C). More than 70% of the larvae looked like WT. The percentages for the different dysmorphic classes and the number of embryos that died after 7 hpf are given in Figure 4D; lethality was highest in the hTRPP2S812A group. Cyst formation was observed in approximately 1% of the larvae overexpressing hTRPP2WT and hTRPP2S812A but not with hTRPP2S812D.

To show that the rescue effect by injection of hTRPP2 capped RNA is not caused by different expression levels of the mutant forms, we expressed these constructs in the zebrafish fibroblast cell line PAC2 by DNA transfection: Equal expression levels were found in relation to co-transfected GFP and endogenous actin (Supplemental Figure 6). Using the C-terminal cytosolic domain of human TRPP2 attached to the membrane tag CD16.7 as described previously, we showed that the intracellular localization of the mutant forms corresponds to the observations in mammalian cell lines,13 CD16.7.hTRPP2S812A reaches the plasma membrane in PAC2 cells and co-localizes with the cell surface protein P75.GFP (Supplemental Figure 6B), whereas CD16.7.hTRPP2S812D and CD16.7.hTRPP2WT do not.

Figure 5. Rescue of the zpkd2 morphant phenotype by coinjection of capped mRNA of WT human TRPP2 and mutant hTRPP2S812A and hTRPP2S812D. All larvae were examined at 55 hpf. (A) The zpkd2 morphant larvae had a dorsally bent body axis, hydrocephalus (arrowhead), and pronephric cysts (arrow), as described in Figure 4. Co-injection of pcd2MO with 100 pg of hTRPP2 WT capped RNA inhibited the formation of pronephric cysts, hydrocephalus, and body axis curvature (judged by trunk-tail angle of <90°, because most of the morphant larvae showed an angle of considerably more than 90°). Co-injection of pcd2MO with hTRPP2S812A (SA) mRNA showed a similar effect as WT mRNA. Co-injection of pcd2MO with hTRPP2S812D (SD) capped RNA could not inhibit cyst formation as efficiently as WT hTRPP2 or hTRPP2S812A capped RNA (arrow) but had a stronger rescue effect on the body curvature. (B) Graphical presentation of the rescue experiments by co-injection of pcd2MO and 100 pg of hTRPP2WT, S812A, S812D capped RNA compared with injection of pcd2MO alone with regard to cyst formation and body axis curvature (judged by trunk-tail angle of <90°): The rescue with hTRPP2WT was statistically significant in both aspects of the evaluation (* in C); a rescue to the same extent was seen with hTRPP2S812A, whereas hTRPP2S812D had a significant effect only on body axis curvature. (C) Net effect of rescue relative to the percentage of phenotype: There was no difference in the rescue of cyst formation between hTRPP2WT (31.2%) and hTRPP2S812A (34.3%) mRNA, but the degree of rescue was less with hTRPP2S812D mRNA (10.5%). With regard to the rescue of the body axis, there was also no significant difference between hTRPP2WT and hTRPP2S812A mRNA, but the effect of hTRPP2S812D RNA (43.4%) that is held back in the ER/Golgi by the interaction with PACS molecules was significantly better. Data of seven independent experiments were pooled. Compared with injection of morpholino alone, statistically significant differences obtained by applying the χ² test are marked with *.
Differential Effects on the L-R Axis Determination by WT and Mutant TRPP2 and Overexpression or Depletion of PACS Molecules

The determination of the L-R asymmetry of the body axis has been linked to motile cilia and a chemical gradient. In the mouse embryo, motile cilia are present on the ventral node, a transient embryonal structure at the end of the primitive streak, and generate a leftward flow that carries vesicles with retinoic acid and Sonic hedgehog to the left side of the node to initiate asymmetry. In zebrafish, dorsal forerunner cells form a ciliated Kupffer’s vesicle and seem to amplify L-R information established during an earlier developmental stage. The importance of TRPP2 in L-R patterning of the zebrafish has been shown before.

To determine the extent of laterality defects, we analyzed the position of the heart, liver, and pancreas at 55 hpf using RNA antisense in situ hybridization. The in situ marker for the heart was myl7 (myosin, light polypeptide 7), for the liver and pancreas was fkd2 (forkhead-2), and for the pancreatic islet was preproinsulin (ins). In a WT zebrafish larva, the ventricle of the heart loops toward the right and the atrium loops toward the left, whereas the liver is positioned to the left of the midline and the pancreas lies to right of the midline (Figure 6A, top and left, whereas the liver is positioned to the left of the midline and the pancreas lies to right of the midline (Figure 6A, top and bottom left). The situ inversus in a zpkd2 morphant larva is shown in Figure 6A, top and bottom right; other disturbances of L-R patterning (organ midline position and bilateral organ anlage) are depicted in Figure 6B. Knockdown of zTRPP2 caused an inversed organ situ in approximately 40% and a midline organ position or bilateral organ anlage in approximately 10% (Figure 6C). For determination of the differential effects of mutant PKD2, capped RNA was injected together with pkd2MO as described previously. The situ was determined for each organ (heart, liver, and pancreas) separately (Figure 6C). The results for both situ abnormalities (inversus and midline/bilateral position), pooled and averaged for the three organs, are given in Figure 6D. The relative rescue for WT hTRPP2 was 19.1% (significantly different from pkd2MO; P < 0.05) and for hTRPP2s812A was 9.6%, reducing the percentage of situ inversus and midline position. Surprising, the ER-contained hTRPP2s812D normalized the amount of situ abnormalities significantly more effective (28.9%; P < 0.01) compared with pkd2 morphants than either WT or the hTRPP2s812A mutant. Disturbance of the subcellular compartmentalization of TRPP2 by either overexpression or knockdown of PACS molecules also causes laterality defects (Supplemental Figure 7).

DISCUSSION

TRPP2 belongs to the family of TRP channels that has been implicated in cellular processes as diverse as mechanosensation, cellular proliferation, polarity, apoptosis, mating behavior, and directed sperm movement (reviewed by Köttgen). It acts as a nonselective cation channel that uses calcium as a ubiquitous second messenger. Although polycystin-1 may regulate the activity of TRPP2 in some tissues, it remains largely unknown how TRPP2 exerts the multiple tissue-specific functions that have been attributed to this ion channel.

The function of ion channels is typically regulated at multiple levels, including the interaction with binding partner that alters the electrochemical properties of the ion channel or posttranslational modifications that affect the localization and/or stability of the molecule. Human TRPP2 contains a phosphorylatable serine 812 embedded in an acidic cluster within the carboxy-terminal cytoplasmic domain. Phosphorylation of serine 812 by CK2 triggers the interaction with the proteins PACS-1 and PACS-2, two connector proteins that couple cargo proteins of the secretory pathway to either AP-1/3 or COP1 adaptor proteins. PACS-1, originally identified as a connector protein involved in the trafficking of furin, mediates retrieval of TRPP2 to the trans-Golgi network (TGN), whereas PACS-2 retains TRPP2 in the ER. Inhibition of either PACS function or CK2 activity releases TRPP2 from these interactions and mediates forward trafficking of TRPP2. On the basis of our in vitro data and the demonstration that zPACS interacts with zebrafish and human TRPP2, we postulated that PACS molecules play a major role in the subcellular localization and function of TRPP2. Because PACS molecules prevent TRPP2 from reaching the cell surface, we postulated that overexpression of zPACS family members trap zTRPP2 and promote the formation of pronephric cysts. As shown in Figure 3, overexpression of zPACS-1 and -2 alone and in combination indeed induced the development of pronephric cysts, which most likely is caused by mislocalization of endogenous TRPP2 as judged by the reduction of the basolateral and cilial immunostaining (Supplemental Figure 3). This further supports the role of PACS in the regulation of TRPP2 localization and function.

The serine-mediated interaction of TRPP2 with PACS connector proteins allowed us to test how a defined subcellular localization of TRPP2 affects the function of TRPP2. In the zebrafish embryo, knockdown of TRPP2 results in three phenotypes that can be readily analyzed: Pronephric cysts, dorsal body axis curvature, and abnormalities of the L-R asymmetry. Renal cyst formation not only occurs in the absence of polycystin-1 or TRPP2 but also critically depends on the balance between the two molecules, and overexpression of either polycystin-1 or TRPP2 is cystogenic. Overexpression of human TRPP2 led to cyst formation, albeit in a very small percentage only. Knockdown of PACS molecules could be regarded as mimicking overexpression of TRPP2 in certain cellular compartments, in the way that the control of subcellular compartmentalization exerted by PACS-1 and -2 is abolished. The knockdown causes cyst formation in single-morphant and even more in double-morphant larvae (Supplemental Figure 4). Double morpholino knockdown is thought to increase overexpression of TRPP2 dramatically at the outer cell membrane and by that is shifting the balance between TRPP2 and polycystin-1.
The serine^{812}-to-alanine mutation of TRPP2 inhibits the interaction with PACS molecules and promotes forward trafficking of TRPP2, whereas the serine^{812}-to-aspartic acid mutation augments binding of PACS, retaining TRPP2 in the ER and TGN (Supplemental Figure 6). Because PACS molecules control the subcellular localization of TRPP2, we used these two mutants to replace the function of WT TRPP2. Using an antisense morpholino-oligonucleotide to deplete TRPP2, we generated zebrafish with mild to moderate phenotypic changes and scored the presence of abnormal body curvature, cyst formation, and heterotaxia. All three rescue approaches, using WT TRPP2, TRPP2^{S812A}, or TRPP2^{S812D}, ameliorated the abnormal phenotypes but uncovered interesting differences. Whereas the S812A mutant effectively rescued pronephric cysts, the S812D mutant was much more effective to reverse the abnormal body axis than cyst formation. These results have significant implications. First, they suggest that TRPP2 acts at both compartments (i.e., the ER and the plasma membrane/cilium) to block cyst formation. Second, TRPP2 localized in the ER, where it potentially acts as a calcium release channel,^{37} is strikingly effective to normalize the curved body axis but hardly prevents cyst formation. Reduced TRPP2 ER retention may also underlie the higher incidence of body axis curvature and dysmorphia in relation to cyst formation in pacs-2 morphants compared with pacs-1 morphants (Supplemental Figure 4).

A curved body axis is observed in many morphant zebrafish; however, it is closely correlated with TRPP2-MO–mediated cyst formation; this tight association has been used to identify novel zebrafish mutants that cause pronephric cysts.^{19} Endogenous zTRPP2 is present in muscle cells of the trunk myotomes in an intracellularly repeating pattern along the sarcomeric bands of the myofibrillae, most likely corresponding to the tubular sarcoplasmic reticulum at the sarcomeric M-line.^{21} The underlying pathogenesis of the axis curvature is unknown. Because tail morphogenesis is almost completed by the time the axis curvature becomes detectable,^{38} the analysis of the curly tail phenotype suggests that the axis curvature might be caused by late events of tail elongation after 33 hpf and/or by the dorsal contraction of tail muscles along the distal trunk.^{20} Human TRPP2 is expressed in the skeletal muscle.
TRPP2 and Zebrafish

Because the sarcoplasmic reticulum functions as a calcium store in skeletal muscle cells, it is conceivable that ER-TRPP2 modulates muscle tone along the zebrafish somites as has been described for TRPP2 in smooth muscle cells. 40–42 Cai et al. 43 found that the TRPP2 channel activity is modulated by phosphorylation of Ser812; therefore, we cannot exclude that differences in ion channel activity contribute to the differences seen between the TRPP2 mutants and WT form in their ability to rescue the phenotype of TRPP2-depleted zebrafish.

It is puzzling that the ER/TGN-TRPP2 S812D mutant molecules rescue L-R patterning defects induced by depletion of zebrafish TRPP2 more efficiently than WT TRPP2. L-R asymmetry specification, probably best characterized for the mouse animal model (reviewed by Hirokawa et al. 4 and Ray and Belmont 80), is initiated by a leftward-directed flow at the ventral node. This flow has been shown to propel vesicles of retinoic acid and sonic hedgehog that initiate asymmetric cellular signaling and axis determination. 29 Nodal flow induces a calcium transient at the left periphery of the ventral node that is absent in TRPP2-deficient animals. 7 In zebrafish, symmetry-breaking events seem to occur earlier and initially depend on the differential activity of the H+/K+-ATPase. 30,44 Later in development, the ciliated Kupffer’s vesicle (the equivalent to the murine ventral node) is formed, and a leftward-directed fluid flow generated by motile cilia is essential to translate the early asymmetry-breaking events into the correct determination of the L-R axis. 45,46 Zebrafish TRPP2 is clearly required for this process, because morpholino-mediated knockdown of zTRPP2 results in laterality defects. 21,22 According to the two-cilia model, 47 TRPP2 participates in ciliary mechanosensation at the left side of the ventral node and translates the flow-mediated ciliary deflection into intracellular calcium transients. According to the chemical gradient model, 6,29 a transported morphogen is responsible for eliciting the calcium transient. This left-sided calcium transient has also been shown in zebrafish embryos. 48 Our data suggest that TRPP2, located in the ER/TGN (as expected for the mutant TRPP2 S812D), plays an important role, perhaps by modifying second messengers that are triggered by either flow- or ligand-induced signaling. Indeed, this hypothesis is supported by the curly up zebrafish mutant, expressing a mutant form of TRPP2. 20 Schottenfeld et al. 20 found that the symmetric expression of the nodal-related gene southpaw, around the Kupffer’s vesicles, was not affected in the curly up mutant zebrafish. In contrast, the expression of southpaw is impaired along the left lateral plate mesoderm in the mutant embryos, indicating that zTRPP2 is required for the correct expansion and propagation of the southpaw signal and the signals downstream of southpaw, such as leftys and pitx2. If the left-sided expansion of southpaw expression is mediated by a signaling cascade involving calcium, then ER-TRPP2, operating as a calcium-release channel, could be part of this signaling event. Both PACS overexpression and depletion caused defects in laterality by compromising the balanced subcellular compartmentalization of endogenous TRPP2. These studies are underlining the importance of both the intracellularly and the cell membranous (e.g., cilia) located pools of TRPP2 for L-R axis determination.

Taken together, our results confirm the role of PACS-dependent TRPP2 trafficking in an in vivo model and provide a first insight into the localization-dependent functions of TRPP2. During renal development, TRPP2 assumes at least three different subcellular localizations: The ER/TGN, the basolateral plasma membrane, and the cilium. In some tissues, for example muscle cells, TRPP2 seems to reside predominantly in intracellular compartments such as the ER/TGN. A similar localization characterizes the subcellular distribution of TRPP2 in tubular epithelial cells of the adult kidney in mammals. On the basis of our findings, we speculate that TRPP2 is required at both the plasma membrane/cilium and the ER/TGN to prevent cyst formation, whereas the ER localization may suffice to regulate muscle tone. Plasma membrane/cilium localizations and, provokingly, intracellular localizations of TRPP2 may contribute to the efficient execution of symmetry-breaking events at the Kupffer’s vesicle and lateral plate mesoderm in zebrafish.

CONCISE METHODS

Zebrafish Lines and Cloning of Zebrafish PACS
WT AB, ABTL, or TLEK zebrafish were maintained and raised in adherence to the National Institutes of Health Guide for Care and Use of Laboratory Animals as described previously. 45,49 Zebrafish PACS-1/2 cDNA were amplified from total RNA by reverse transcriptase–PCR. For more information, refer to the online supplemental information.

Morpholino Antisense Oligonucleotide and mRNA Injections
Injections were performed as described previously. 45 See online supplemental information for details.

Co-immunoprecipitations, MBP Pulldown Experiments, and Western Blot Analysis
HEK 293T and zebrafish PAC2 cells were propagated and transfected, and the cell lysate was used for the biochemical assays. 31,52 For more details, see online supplemental information.

Immunofluorescence and Confocal Microscopy
HeLa cells, PAC2 cells, and whole zebrafish larvae overexpressing the proteins of interest were fixed and stained, and images were obtained using a confocal microscope (LSM 510; Zeiss, Jena, Germany). For details, refer to the online supplemental information.

Statistical Analysis
The χ2-test and the t-test were applied to analyze the results.
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DISCLOSURES

None.

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