Wtip and Vangl2 are required for mitotic spindle orientation and cloaca morphogenesis

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Summary
Defects in cilia and basal bodies function are linked to ciliopathies, which result in kidney cyst formation. Recently, cell division defects have been observed in cystic kidneys, but the underlying mechanisms of such defects remain unclear. Wtip is an LIM domain protein of the Ajuba/Zyxin family, but its role in ciliogenesis during embryonic development has not been previously described. We report Wtip is enriched in the basal body and knockdown of wtip leads to pronephric cyst formation, cloaca malformation, hydrocephalus, body curvature, and pericardial edema. We additionally show that wtip knockdown embryos display segment-specific defects in the pronephros: mitotic spindle orientation defects are observed only in the anterior and middle pronephros; cloaca malformation is accompanied by a reduced number of ciliated cells; and ciliated cells lack the striated rootlet that originates from basal bodies, which results in a lack of cilia motility. Our data suggest that loss of Wtip function phenocopies Vangl2 loss of function, a core planar cell polarity (PCP) protein located in the basal body protein. Furthermore, we demonstrate that wtip and vangl2 interact genetically. Taken together, our results indicate that in zebrafish, Wtip is required for mitotic spindle orientation in the anterior and middle of the pronephros, cloaca morphogenesis, and PCP, which may underlie the molecular etiology of ciliopathies.

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Key words: Ciliopathies, Mitotic spindle, Basal body, Zebrafish

Introduction
Cilia are microtubule-based “hair-like” organelles that are present on the apical surface of most eukaryotic cells. Motile cilia contain a central pair of microtubules, and the basal body anchors the cilium in the cell body. The rootlet is a prominent structure that originates from the proximal end of the basal body (Yang et al., 2002). In recent years, defects in cilia and basal bodies have been linked to a number of important developmental pathways (Veland et al., 2009; Goetz and Anderson, 2010). Defects in cilia assembly and function have been causative with human ciliopathies (Harris, 2007; Hildebrandt et al., 2011). Also, it has been suggested that the basal body is involved in regulating the Wnt pathway, leading ultimately to the generation of kidney cysts (Saburi et al., 2008; Wallingford, 2010; Cao et al., 2010; Skouloudaki et al., 2009; Burcklé et al., 2011; Simons et al., 2005). However, the pathological mechanisms leading to ciliopathies remain incompletely defined.

To understand the function of the genes associated with the human ciliopathies, animal model systems have been instrumental. Many mouse and rat models of ciliopathies have been described (Harris, 2007; Hildebrandt et al., 2011; Menezes and Germino, 2009). These models share features in common with human diseases. In addition to the mammalian model systems, simple vertebrates such as zebrafish, medaka and Xenopus have provided an alternative, more experimentally tractable model systems to study mechanisms responsible for ciliopathies (Wessely and Obara, 2008; Swanhart et al., 2011).

Recently, evidence for the involvement of intraflagellar transport proteins (IFTs), Oif1, and Nphp4 in Wnt-planar cell polarity (PCP) has been reported in zebrafish (Cao et al., 2010; Burcklé et al., 2011; Ferrante et al., 2009). These findings are also supported by the evidence that zebrafish maternal zygotic mutants for the core PCP gene vangl2 lead to pronephric tubule distension and misorientation of the basal body in the neural tube and other tissues (Borovina et al., 2010). PCP signaling processes are involved in a variety of phenotypes (Goetz and Anderson, 2010; Fischer and Pontoglio, 2009; Menezes and Germino, 2009).

The WT-1-interacting protein (WTIP) was originally identified as an interacting partner of the Wilms’ tumor protein 1 (WT-1) in a yeast two-hybrid screen (Srichai et al., 2004). The nuclear export signal domain (NES), the SH3 binding domain (SH3), three LIM binding domains (LIM) and the PDZ binding domain (PDZ) are encoded by wtip. Recent studies suggest the following: (1) Wtip interacts with the C-terminus of the receptor tyrosine kinase Ror2 in yeast and mammalian cells (Verhey van Wijk et al., 2009), and (2) Ajuba LIM proteins (Ajuba, LIMD1 and WTIP) interact with Snail to remodel epithelia dynamics (Langer...
et al., 2008; Das Thakur et al., 2010). However, the subcellular localization of Wtip or its in vivo role during embryonic development is still unknown.

In this paper, we have used zebrafish as a model to study Wtip function during embryonic development. We show that the Wtip protein localizes in the basal body. Knockdown of wtip leads to pronephric cysts accompanied by cloaca malformation, hydrocephalus, body curvature and pericardial edema, all phenotypes seen in other zebrafish models of human ciliopathies. Interestingly, wtip knockdown embryos display segment-specific defects in the pronephros: mitotic spindle orientation defects are observed only in the anterior and middle pronephros; cloaca malformation is accompanied by fewer ciliated cells; and ciliated cells lack the striated rootlet that originates from basal bodies, resulting in a lack of cilia motility. Our data suggest that the phenotypes caused by loss of Wtip are due to defects in PCP signaling. We have identified new roles for Wtip in various zebrafish models of human ciliopathies, namely that Wtip contributes to the connection between cystogenesis and cell division in the anterior and middle pronephros, cloaca malformation and PCP in ciliopathy.

Materials and Methods

Fish husbandry

Standard protocols were used to maintain and raise wild-type zebrafish AB strains and embryos at 28.5°C as described by Westerfield (Westerfield, 2000). Embryos were cultured in 0.0045% phenylthiourea in Danieau buffer to inhibit pigmentation and embryos were staged according to Kimmel et al. (Kimmel et al., 1995).

Cloning full-length of zebrafish wtip and w提醒 gene expression by RT-PCR

To obtain the zebrafish wtip gene, BLAST searches were performed using the human WTAP amino-acid sequence as the query (Srichai et al., 2004). A zebrafish full-length wtip cDNA clone was obtained by RT-PCR from total RNA prepared from 48 hpf embryonic posterior (hp) embryos using the RNaseAqous-4PCR Kit (Ambion/Life Technologies, Grand Island, New York, USA). RT-PCR was performed using the SuperScript™ III One-Step RT-PCR System with Platinum® Taq High Fidelity (Invitrogen/Life Technologies, Grand Island, New York, USA) followed by a second PCR using Phusion® High-Fidelity DNA Polymerase (New England BioLabs, Inc., Ipswich, Massachusetts, USA). Primers were used to amplify: AAACCTTGTGACGACGTCGTAT; GCAGGACATGAGTAGAATAGCA; TCATGAACTGCAGACGAAATGAC; and ACTAATCCCGTGTTGTTTT (Eurofins mwg/operon, Huntsville, Alabama, USA). The PCR product was subcloned in pCR®-BluntII-TOPO® vector (Invitrogen) according to the manufacturer’s instructions. RT-PCR of the wtip transcript was performed with total RNA isolated from 1-4 cell stages through 48 hpf embryos using the RNaseAqous-4PCR Kit (Ambion). The primers used to amplify wtip were: GGGATCCATGACGGCCTGAC; GAAGGGAACCTCGCTGACCC; GCTAGGCATATCTGGTCC; and CCGGGCTCAAAAAATGGTGG (Eurofins mwg/operon). Variations in total RNA from each stage were controlled by using gapdh.

In situ hybridization

Whole-mount in situ hybridization was performed as described by Hauptmann and Gerster, (Hauptmann and Gerster, 2000), using the following probes: wtip and evx1 (Piyatilake et al., 2006). For the wtip anti-sense RNA probe, pCR®-BluntII-TOPO®-wtip plasmid was linearized by Nael and Sp6 RNA polymerase (Roche, Indianapolis, USA) to generate the DIG RNA probe. All probes were synthesized using DIG-RNA (Roche) labeling mix according to the manufacturer’s instruction. Alkaline phosphatase-conjugated digoxigenin (Roche) was used to localize the probes. NBT/BCIP (Roche) was used as the chromogenic substrate to produce blue.

Antibodies and whole-mount immunochemistry

A zebrafish Wtip antisera was raised in rabbits using amino-terminal peptide CGASDLRRLYTHAEVQGHRYS coupled to KLH and the antisera was affinity-purified against the immunizing peptide and used at 1:100 dilution (Covance, Denver, Philadelphia, USA). Other primary antibodies used included: anti-α-tubulin (GTU-88, 1:800 dilution, Sigma, St. Louis, Missouri, USA Cat# T6557); anti-acetylated α-tubulin (6-11B-1, 1:800 dilution, Sigma Cat# T7451); anti-α-tubulin (DM1a, 1:500 dilution, Sigma Cat# T6199); anti-centrin (20H5, 1:1000 dilution, Millipore, Billerica, Massachusetts, USA Cat# 04-1624); and anti-p-Histone H3 (Ser 10) (1:750 dilution, Santa Cruz Biotechnology®, Inc., Santa Cruz, California, USA Cat# sc-8056-R). Secondary antibodies were obtained from Molecular Probes/Invitrogen™Life Technologies, Grand Island, New York, USA and included goat-anti-rabbit-Alexa Fluor®488 (IgG1(H+L), Cat# A11035), goat-anti-mouse-Alexa Fluor®488 (IgG2b, Cat# A21141), goat-anti-mouse-Alexa Fluor®488 (IgG1(γ1), Cat# A21211), goat-anti-mouse-Alexa Fluor®488 (IgG2a(γ2a), Cat# A21313), and goat-anti-mouse-Alexa Fluor®546 (IgGl(γ1), Cat# A21123). Whole-mount immunochemistry was performed on embryos that were fixed in Dent’s fixative (methylene: DMSO=4:1) for anti-Wtip + anti-acetylated α-tubulin, anti-Wtip + anti-γ-tubulin, anti-Wtip + anti-Centrin, anti-phH3 + anti-acetylated α-tubulin + anti-α-tubulin at 4°C overnight. Embryos fixed by Dent’s fixative or PFA/PBS fixed samples were further processed as described by Kim et al. (Kim et al., 2011). Embryos were flat-mounted and visualized using an Olympus IX81-FV500 confocal laser scanning microscope (Olympus, Center Valley, Philadelphia, USA) and analyzed with Fluov View imaging software (Olympus). An Olympus Provis AX-70 photomicroscope equipped with epifluorescence, Normaski, phase contrast optics and a Q-imaging digital camera was connected to a workstation with Q-capture software.

Morpholino and mRNA injections

The following morpholinos were obtained from Gene Tools, LLC, Philomath, Oregon, USA: Translational blocking morpholino elugene13 (MO) that targeted against the 5’UTR of wtip (wtipMO: GATCCTCGTCTGTATCCATGTCGT), or the 5’UTR of vangl2 (vangl2MO: GTACTGGACTGTCGTTATC- CATGTCG) (Cui et al., 2011); a MO that blocked splicing of wtip exon2 by targeting the exon 2/intron 2-3 boundary (wtipMOex2: TGTTATTGTTGAGAAGCTCACCCAGGAT) and a randomized control MO (conMO: CCCTCTTA-CCTCAAGTCAACTTATTA). A volume of 4.6 nl in a concentration of 0.3 mM wtipMO, 2 ng/μl vangl2MO and 20 pg wtip mRNA was injected at one cell stage using a nanoliter 2000 microinjector (World Precision Instruments, Sarasota, Florida, USA). Zebrafish wtip mRNA were synthesized with T7 RNA polymerase (mMessage mMACHINE system) (Ambion), respectively after linearization of the pCR®-BluntII-TOPO®-wtip construct with HindIII (Invitrogen). All mRNA were purified using the RNeasy Mini Kit (Qiagen, Valencia, California, USA). Microinjections into 1-cell embryos were performed as described by Feng et al. (Feng et al., 2008).

Histological analysis

48 hpf embryos were fixed using histology fixative (1% paraformaldehyde, 1.5% glutaraldehyde, 3% sucrose in 70 mM phosphate buffer, (pH 7.2) overnight at 4°C, and dehydrated by a graded series of methanol before embedding in JB4 resin according to the manufacturer’s instructions (Polysciences, Inc., Warrington, Philadelphia, USA). Four micrometer sections were cut by a Leica RM2255 microtome (Leica, Buffalo Grove, Illinois, USA) and stained with hematoyxin and eosin (BBC Biochemical, Dallas, Texas, USA).

Electron microscopy

For transmission electron microscopy analysis, embryos were prepared by previously published protocols (Ichimura et al., 2007). Ultrathin (80–90 nm) sections were then cut and counterstained with uranyl acetate and lead citrate, and observed using a JEOL-1230 transmission electron microscope (JEOL, Tokyo, Japan).

High-speed video microscopy

Minor modifications were made to a previously published protocol for high-speed video microscopy (Kramer-Zucker et al., 2005). Embryos were incubated in 75 μM phenylthiourea in Danieau buffer. 48 hpf embryos were mounted in 6% methylcellulose containing 50 mM 2,3-butanedione monoxime and 0.02% Tribrom (Triage). Images of cilia motility were taken by using a 60×/1.20 water immersion UPlanSapo DIC T lens on an Olympus AX-70TRF-A microscope (Olympus) equipped with an AOS S-PR1 video camera (AOS Technologies AG, El Cajon, California, USA) under a stream acquisition rate of 200 frames per second in Imaging Studio v 3.3.3 (AOS Technologies) was used. Movies were played at 7 frames per second and analyzed with VirtualDub software.

Statistical analysis

Results were analyzed by the paired Student’s t-test. Variance is displayed as either average absolute deviation (AAD) or as SEM as noted in the figure legend. Differences were considered to be statistically significant at values of p<0.05; p values are as indicated by *p<0.001.
Results
Identification and cloning of zebrafish wtip
Full-length zebrafish wtip was identified and assembled using TBLAST searches with human and mouse WTIP C-terminus sequence (including the three LIM domains) and the PDZ binding domain to identify comparable protein sequences in the zebrafish ENSEMBL database (Sanger Center). The zebrafish wtip gene (Ensembl gene ENSDARG00000063666) is located on chromosome 13; it contains 8 exons and encodes 648 amino acids. Zebrafish Wtip protein amino acid identity with other species ranged from 51.7 to 84.4% (human, mouse, frog, and chicken) (Fig. 1B). Notably, the coding region spanning exons 2–8 (which contains three LIM binding domains and the PDZ domain) was highly conserved. Interestingly, the N-terminal region of Wtip, which is encoded in exon 1 and contains a nuclear export signal and a SH3 domain, was longer in zebrafish, frog and chicken than in human and mouse (supplementary material Fig. S1).

Expression analysis of zebrafish wtip
Next, we analyzed wtip gene expression by RT-PCR and whole-mount in situ hybridization at different stages of embryonic development. RT-PCR was used to detect wtip transcripts from 1-cell stage until 48 hpf at comparable levels (Fig. 1C). A low level of expression was detected by whole-mount in situ hybridization at 256-cell, 30–50% epiboly stages (data not shown). In zebrafish, the onset of zygotic transcription occurs at 512-cell stage (Westerfield, 2000), therefore we demonstrated that wtip mRNA is maternally deposited. From the zygote through the segmentation period, wtip mRNA showed weak ubiquitous expression by whole-mount in situ hybridization (data not shown). After 24 hpf, wtip expression was abundantly detected in the anterior brain, hindbrain, eye, and inner ear but low levels of expression were found in the heart and pronephros (Fig. 1F). In 48 hpf cross-sections, we confirmed punctuated wtip expression in the pronephric glomerulus and tubules (Fig. 1E). Zebrafish wtip expression in the pronephros was also confirmed by RT-PCR using total RNA prepared from isolated pronephros of 48 hpf embryos (Fig. 1D).

Zebrafish Wtip protein is localized to basal body of ciliated tissues
To further investigate how Wtip might function, we examined the subcellular localization of this protein in zebrafish using a zebrafish specific anti-Wtip antibody. Immunostaining using this Wtip antibody together with an antibody recognizing acetylated α-tubulin antibody (green) and Wtip (red). Double immunofluorescence for the cilia marker acetylated α-tubulin (green) and Wtip (red) in confocal projections taken from whole-mount embryos for the middle segment of the pronephros at 24 hpf (G,H), the posterior pronephros at 24 hpf (I,J,K) and KV at the 10-somite stage (L,M,N). DAPI was used to counterstain nuclei (blue). Control embryos for antibody specificity using knockdown mediated by wtipMO of endogeneous Wtip protein translation (H,J,K) and with pre-immune serum (K,N) showed no expression. Localization of Wtip in the basal bodies of cilia was confirmed by double immunostaining with Wtip antibody (red) and either anti-α-tubulin (P,V,green) or anti-centrin (S,Y,green), which are basal body markers in KV (V,Y,10-somite stage) and the middle segment of the pronephros (P,S,24 hpf). Pronephric tubules (pt), acetylated α-tubulin antibody (AT), γ-tubulin antibody (γ-tub), mid-pronephros (pk-mid), and posterior-pronephros (pk-post). Scale bars are 10 μm in E,G,N, 100 μm in F, and 2 μm in O–Z.

Fig. 1. Zebrafish wtip gene and protein during development. (A) Zebrafish Wtip domain structure contains one nuclear export signal (NES, orange), one SH3 binding domain (SH3, green), three LIM domains (LIM1-3, yellow) and one PDZ binding domain (PDZ, red). (B) Percentage of amino acid identity between zebrafish Wtip and homologs of WTIP from other species. (C) RT-PCR time course of wtip expression during embryonic development. gapdh was used to control variations in total RNA (C,D). wtip expression was maternally deposited (C) and confirmed in the pronephros by RT-PCR using total RNA from isolated pronephros from 48 hpf embryos (D). (E) Cross-sections of the pronephric glomerulus and tubes. (F) Whole-mount in situ hybridization for wtip on embryos at 24 hpf, 34 hpf, and 48 hpf are side views anterior to the left and dorsal to the top. Zebrafish wtip expression is restricted to the eye, anterior brain, hindbrain, inner ear, and pronephros (E,F). Double immunofluorescence for the cilia marker acetylated α-tubulin (green) and Wtip (red) in confocal projections taken from whole-mount embryos for the middle segment of the pronephros at 24 hpf (G,H), the posterior pronephros at 24 hpf (I,J,K) and KV at the 10-somite stage (L,M,N). DAPI was used to counterstain nuclei (blue). Control embryos for antibody specificity using knockdown mediated by WtipMO of endogeneous Wtip protein translation (H,J,K) and with pre-immune serum (K,N) showed no expression. Localization of Wtip in the basal bodies of cilia was confirmed by double immunostaining with Wtip antibody (red) and either anti-α-tubulin (P,V,green) or anti-centrin (S,Y,green), which are basal body markers in KV (V,Y,10-somite stage) and the middle segment of the pronephros (P,S,24 hpf). Pronephric tubules (pt), acetylated α-tubulin antibody (AT), γ-tubulin antibody (γ-tub), mid-pronephros (pk-mid), and posterior-pronephros (pk-post). Scale bars are 10 μm in E,G,N, 100 μm in F, and 2 μm in O–Z.
μ-tubulin demonstrated that the Wtip protein was localized at the base of cilia in the 24 hpf pronephros, and in Kupffer’s vesicle (KV) in 10-somite stage embryos (Fig. 1G,I,II). The specificity of the staining pattern was verified by pre-incubating with immunized peptide (data not shown) or knockdown of endogeneous wtip expression with antisense morpholino oligonucleotide (wtipMO); both completely abolished the Wtip immunostaining (Fig. 1H,I,M). No staining was observed with the pre-immune serum (Fig. 1K,N). The staining pattern suggested that Wtip was localized to the basal body. To confirm this, we performed double immunostaining with two independent antibodies anti-μ-tubulin and anti-centrin. Indeed, Wtip antibody co-localized with the basal body labeled with either anti-μ-tubulin (Fig. 1O–Q,U–W) or anti-centrin (Fig. 1R–T,X–Z). Wtip was localized to the basal body of all ciliated tissues analyzed (data not shown). Together these data demonstrate that Wtip is expressed in the basal body of cilia in the ciliated tissues.

Knockdown of zebrafish wtip results in pronephric cysts accompanied by cloaca malformation,hydrocephalus, body curvature and pericardial edema.

To address the in vivo function of Wtip during embryonic development, we used anti-sense morpholino (MO) mediated knockdown of wtip (wtipMO) to target the translational initiation site of wtip mRNA (Fig. 2B,E). wtip morphants developed pronephric cysts accompanied by cloaca malformation, hydrocephalus, body curvature, and pericardial edema (Fig. 2B,E,G,I,K). These phenotypes are highly similar to those observed for zebrafish lacking the ADPKD gene pkd2 (Obara et al., 2006), nphp6 (Sayer et al., 2006) and nphp4 (Slanchev et al., 2011; Burcklè et al., 2011). Cross sections demonstrated the presence of glomerular-pronephric tubule cysts (Fig. 2G) accompanied by cloaca malformation (Fig. 2E,K) as well as dilated anterior pronephros (Fig. 2I). To assess if deletion of specific Wtip domains affects the in vivo functions of Wtip, we generated internal deletion allele of Wtip by disrupting mRNA splicing with a wtip exon2-specific donor morpholino (wtipMOex2) (Fig. 3A–C). Targeting the exon2 splice site donor resulted in deletion of exon2, which encodes the first Wtip LIM domain, and results in fusion of exon1 and exon3 (Fig. 3B,C). The wtipMOex2 morphants displayed pronephric cysts accompanied by cloaca malformation, hydrocephalus, body curvature, and pericardial edema (Fig. 3D–G).

Importantly, the wtipMO or wtipMOex2 morphants phenotype could be rescued by the co-injection of wtip mRNA, demonstrating that the phenotypes specifically result from wtip knockdown (Fig. 2C; data not shown).

It has been reported that mechanical obstruction of the cloaca generates pronephric glomerulus cysts (Kramer-Zucker et al., 2005). Recently, cloacal malformation has also been associated with pronephric cysts in other zebrafish knockdown models of ciliary gene function, including pkd2 (Obara et al., 2006), nphp6 (Sayer et al., 2006) and nphp4 (Burcklè et al., 2011; Slanchev et al., 2011). Indeed, we found a good correlation between the formation of pronephric glomerular cysts at 48 hpf and cloaca malformation at 24 hpf. When we observed lower concentrations of wtip morphants (0.5–1 ng / embryo), a normal cloaca was formed and no pronephric glomerulus cysts were developed. Conversely, higher concentrations of morpholino (4 ng / embryo) induced more severe phenotypes including the formation of pronephric glomerulus cysts accompanied by cloaca malformation. Based on these data, we decided to further analyze the specific cloacal malformation, which caused pronephric cyst formation in wtip knockdown zebrafish. To do so, we first determined if cloacal malformation was associated with changes in cloaca-specific gene expression. Specifically, we examined evx1 expression by whole-mount in situ hybridization at 34 hpf (Fig. 4C,D), the time point when structural defects can first be seen at the gross morphological level. The evx1 transcription factor is normally expressed in the posterior pronephros, cloaca, and ectodermal cells (Thaéron et al., 2000) (Fig. 4A,C). In the wtip knockdown embryos, evx1 expression was still present in the posterior pronephros and cloaca, but the altered shape of this region was clearly marked by evx1 expression in 34 hpf embryos (Fig. 4D). Interestingly, we found that changes in the evx1 expression domain existed already at 24 hpf in the wtip morphants (Fig. 4B). At this stage evx1 expression in wtip morphants has expanded towards the caudal of posterior pronephros (Fig. 4B). Together, these results suggest that Wtip is required for early caudal pronephros morphogenesis, similar to the phenotypes described in pkd2- (Obara et al., 2006), nphp6 (Sayer et al., 2006) and nphp4 (Slanchev et al., 2011; Burcklè et al., 2011) knockdown zebrafish. Similar to nphp4 knockdown, we also observed in wtip morphants that programmed cell death of ectodermal cells that form the cloaca opening is blocked (data not shown).

Cilia formation in wtip morphants.

Mutations in genes, which are important for cilia and basal body development, have been reported to cause pronephric cyst formation in zebrafish (Wessely and Obara, 2008; Swanhart et al., 2011). To examine the effects of wtip knockdown on
ciliogenesis, we examined the posterior pronephric duct by immunostaining with antibodies against the cilia marker, acetylated α-tubulin and the basal body marker γ-tubulin. As shown in Fig. 4E,F, cilia in the posterior pronephros and cloaca region of wtip morphants at 24 hpf were shorter than in control embryos (1.42±0.43 μm, (n=24) versus 6.31±1.23 μm, (n=32); p<0.001). Moreover, the number of cells forming cilia was decreased by 50% in the posterior pronephros and cloaca (Fig. 4E,F). We also examined the motility of 9+2 cilia using high-speed video microscopy analysis (supplementary material Movies 1, 2). In control 48 hpf embryos, cilia in the posterior pronephros and cloaca were motile, beating with a frequency of 63±11 Hz, n=5 (supplementary material Movie 1). By comparison, the cilia of wtip morphants were completely non-motile (supplementary material Movie 2).

To further understand cilia and basal body function, we examined their ultrastructure by transmission electron microscopy (Fig. 5G–I). In the posterior pronephros and cloaca of 34 hpf control embryos, the 9+2 axenome was associated with basal feet, transition fibers and a striated rootlet structure (Fig. 4H,J). It is noteworthy, that this unique rootlet structure, which is required for cilia motility. Wtip is not required to form the 9+2 axenome, but is required for ciliogenesis in the posterior pronephros and cloaca. Specifically, Wtip is not required to form the 9+2 axenome, but is required for the rootlet structure, which is required for cilia motility.

Some gene products involved in human kidney diseases localized to basal bodies are known to contribute to Wnt/PCP signaling in cystic kidneys (May-Simera et al., 2010; Burcklé et al., 2011; Jonassen et al., 2008). However, phenotypic detail defects (cloaca morphogenesis, ciliogenesis) among these models are not completely identical. Therefore, we decided to examine specific defects caused by wtip knockdown in the posterior pronephros related to Wnt/PCP signaling. The Vangl2 protein is a core component of the PCP pathway (Ross et al., 2005). We first analyzed if vangl2 morphants have a phenotype similar to wtip morphants with regard to cloaca morphogenesis, ciliogenesis and evx1 expression pattern (Fig. 2B,E, Fig. 4B,D,F, Fig. 5A,C,E,H,K). Interestingly, vangl2 morphants did develop cloaca malformation (Fig. 5A,C) and generated fewer cells with cilia (Fig. 5E), similar to wtip morphants (Fig. 2B,E, Fig. 4F). We next performed a combined knockdown of wtip and vangl2 using sub-threshold doses of both morpholinos to assess whether a genetic interaction between these proteins would alter morphogenesis of the posterior pronephros (Fig. 5B,D). Indeed, in the wtip and vangl2 double morphants, cilia were further shortened relative to controls or to wtip knockdown, wtipMOex2 morphants resulted in pronephric cyst formation accompanied by cloaca malformation. (A) Predicted domain structure of zebrafish Wtip protein. The putative nuclear export sequence (NES), SH3 binding domains, three LIM domains and the PDZ binding domain (PDZ) are shown in boxes. Anti-sense morpholino to target the translational initiation site of wtip mRNA (wtipMO) was used for wtip knockdown and wtip exon2 specific MO (wtipMOex2) was used to target the exon2/intron2 junction. (B) To test the function of wtip in zebrafish, we targeted splice donor sites in wtip exon2 with MO. The extent to which steric blockade of mRNA splicing caused alterations in wtip mRNA processing was quantified by RT-PCR using flanking exon primers. Injection of wtipMOex2 at the one- to two-cell stage resulted in an in-frame deletion of exon 2 and resulted in the joining of exon3 to exon1, thus deleting most of the first LIM domain. (C) The efficacy of the injected wtipMOex2 was quantified at 48 hpf by RT-PCR. The upper panel shows the result of RT-PCR with primers in exons1 and 4 of wtip. The lower panel shows the result of RT-PCR with primers for gapdh, used to control RNA amounts. (D) Side view of 48 hpf wtipMOex2 morphants displaying pronephric cysts, hydrocephalus, body curvature and pericardial edema. (E) Lateral view of cloaca at 48 hpf shows cloaca malformation. Histological transverse-sections of 48 hpf wtipMOex2 morphant embryos are 4 μm JB-4 plastic sections stained with hematoxylin and eosin (F,G) at the level of the glomerulus cysts (F, black arrow), tubular cysts (F, asterisks) and cloaca (G, black arrow). Scale bars are 200 μm in D and 10 μm in E–G.

To the control embryos, where all cells were ciliated (Fig. 4I). Interestingly, in these non-ciliated cells centrosomes could still be observed, but their mother centriole/basal bodies were separated from the inner leaflet of the apical cell membrane (Fig. 4K). From these data, we conclude that Wtip is required for ciliogenesis in the posterior pronephros and cloaca. Specifically, Wtip is not required to form the 9+2 axenome, but is required for the rootlet structure, which is required for cilia motility.

Some gene products involved in human kidney diseases localized to basal bodies are known to contribute to Wnt/PCP signaling in cystic kidneys (May-Simera et al., 2010; Burcklé et al., 2011; Jonassen et al., 2008). However, phenotypic detail defects (cloaca morphogenesis, ciliogenesis) among these models are not completely identical. Therefore, we decided to examine specific defects caused by wtip knockdown in the posterior pronephros related to Wnt/PCP signaling. The Vangl2 protein is a core component of the PCP pathway (Ross et al., 2005). We first analyzed if vangl2 morphants have a phenotype similar to wtip morphants with regard to cloaca morphogenesis, ciliogenesis and evx1 expression pattern (Fig. 2B,E, Fig. 4B,D,F, Fig. 5A,C,E,H,K). Interestingly, vangl2 morphants did develop cloaca malformation (Fig. 5A,C) and generated fewer cells with cilia (Fig. 5E), similar to wtip morphants (Fig. 2B,E, Fig. 4F). We next performed a combined knockdown of wtip and vangl2 using sub-threshold doses of both morpholinos to assess whether a genetic interaction between these proteins would alter morphogenesis of the posterior pronephros (Fig. 5B,D). Indeed, in the wtip and vangl2 double morphants, cilia were further shortened relative to controls or to wtip
Zebrafish Wtip is a new player in ciliopathy

Fig. 4. In wtip knockdown embryos, cloaca malformation is associated with fewer ciliated cells and the lack of rootlet structures, which may compromise the motility of cilia. Lateral views of evx1 expression in control (A, C) and wtip morphants (B, D) in the posterior pronephros and cloaca at 24 hpf (A, B) and 34 hpf (C, D). In 24 hpf (A) and 34 hpf (C) control embryos, evx1 was expressed in the epidermis and cloaca. In wtip morphants, evx1 was expressed broadly around the area of the posterior pronephros and cloaca at 24 hpf (B). evx1 continued to be expressed at 34 hpf in the malformed cloaca (D).

Confocal projections of a double immunofluorescence of the posterior pronephros and cloaca in 24 hpf control (E) and wtip morphant (F) embryos labeled with anti-\(\gamma\)-tubulin (basal body, red) and anti-acetylated \(\alpha\)-tubulin (cilia, green) revealed fewer ciliated cells in the wtip morphants (F). The white arrow points to the cloaca area. Transmission electron micrographs of the transverse sections in 34 hpf control embryos (G) and wtip morphants (H) revealed an axoneme with 9+2 structure. Longitudinal sections in control embryos displayed a striated rootlet structure (arrowheads) located adjacent to the basal body (I). In wtip morphants, ciliated cells exhibited a basal body but lacked rootlet structure (J), and non-ciliated (double arrow) cells exhibited mother centrioles/basal bodies, which were separated from the inner leaflet of the apical cell membrane (K). Scale bars are 25 \(\mu\)m in A–D, 100 \(\mu\)m in E, F, 50 \(\mu\)m in G, H, and 100 \(\mu\)m in I–K.

or vangl2 single morphant embryos (1.05 ± 0.43 \(\mu\)m, \(n=18\); \(p<0.001\)) in the posterior pronephros and cloaca at 24 hpf (Fig. 4F, Fig. 5E,F). In addition, cilia formed in 30% fewer cells by immunostaining with the cilia marker anti-acetylated \(\alpha\)-tubulin and basal body marker anti-\(\gamma\)-tubulin. Finally, the expression of evx1 in wtip and vangl2 double morphants embryos (Fig. 5F) as assessed by immunostaining with the cilia marker anti-acetylated \(\alpha\)-tubulin and basal body marker anti-\(\gamma\)-tubulin. The results suggest that Wtip is associated with Vangl2, and that PCP defects can influence posterior pronephros morphogenesis and ciliogenesis.

Wtip is required to maintain the correct angle of the mitotic spindle in the anterior and middle pronephros

As shown above, Wtip localizes to the basal body, and lack of Wtip results in pronephric cyst formation. One working hypothesis for the formation of PKD is that the plane of cell division is altered (Jonassen et al., 2008; Fischer and Pontoglio, 2009; Delaval et al., 2011; Jonassen et al., 2012). To test whether Wtip in zebrafish embryos affects mitotic function, wtip morphants were examined for spindle and cell division orientation in the 48 hpf pronephros by labeling the pronephric cilia with the anti-acetylated \(\alpha\)-tubulin antibody and co-labeling mitotic spindle fibers with the anti-\(\alpha\)-tubulin antibody and mitotic nuclei with the fluorescent anti-phospho Histone H3 (pH3) antibody, using an approach similar to those previously published (Toyoshima and Nishida, 2007; Delaval et al., 2011). Immunofluorescence of whole-mount zebrafish larvae at 48 hpf revealed the spindle angle relative to the cell-substrate adhesion plane. In control embryos, the spindle angle in the entire pronephros showed mitotic spindle angles of 10-20 degrees (Fig. 6A,E,LM–O). In contrast, in wtip morphants, both in the anterior and the middle segments of the pronephros mitotic spindle angles ranged to up to 90 degrees (Fig. 6B,F,LM,N), clearly demonstrating a critical role for Wtip in spindle orientation. Interestingly, these defects in the mitotic spindle

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angles were not observed in the posterior pronephros of wtip morphants (Fig. 6J,O). Since the defects in the posterior pronephros of wtip morphants are linked to Wnt/PCP signaling, we decided to examine whether the mitotic spindle angles defects were also dependent on Wnt/PCP signaling. Like wtip morphants, vangl2 morphants demonstrated mitotic spindle defects that ranged to up to 90 degrees, and again those defects were observed only in the anterior and middle but not in the posterior pronephros (Fig. 6C,G,K,M–O). This effect of the wtip or vangl2 morphants was specific, since co-injection of either wtip or vangl2 mRNA could rescue the mitotic angle defects, respectively (data not shown). We next confirmed the possible interaction of Wtip and Vangl2 through combined loss-of-function experiments using morpholinos targeting both wtip and vangl2. Embryos injected with a low dose wtipMO (1 ng/embryo; n = 34) and vangl2MO (1 ng/embryo; n = 28) showed no obvious defects (data not shown). However, dual knockdown of wtip and vangl2 using low doses of morpholinos again perturbed the mitotic spindle angle. Indeed, the number of morphant embryos with mitotic spindle angle defects in the anterior (Fig. 6D,M) or middle pronephros (Fig. 6H,N) was increased for wtipMO and vangl2MO double morphants, but the posterior pronephros remained unaffected (Fig. 6L,O). Together, these data suggest that the two basal body proteins Wtip and Vangl2 interact and are required to establish the normal mitotic spindle angles in the anterior and middle pronephros.

**Discussion**

We showed in this study that, wtip is the first gene to be genetically linked to specific cell division phenotypes in the anterior and middle pronephros and simultaneously associated with cloaca malformation and loss of cilia motility due to their lack of rootlet structure. Moreover, we demonstrated that the non-canonical Wnt signaling pathway is required for cloacal morphogenesis, ciliogenesis and orientation of the mitotic spindles specifically in the anterior and middle pronephros. Dual knockdown of wtip and vangl2 acts synergistically on kidney ciliogenesis. Importantly, the specificity of the observed phenotypes is verified by its rescue with the injection of wtip and vangl2 mRNA (data not shown).

It has previously been reported that mechanical obstruction (Kramer-Zucker et al., 2005) or several zebrafish knockdown models of ciliary gene functions, including pdk2 (Obara et al., 2006), nphp6 (Sayer et al., 2006) and nphp4 (Burcklé et al., 2011; Slanchev et al., 2011) of cloaca malformation resulted in pronephric cyst formation. Overall, cyst formation accompanied

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**Fig. 6. wtip knockdown leads to mitotic cell division defects in the pronephros.** Whole-mount immunofluorescence of mitotic spindles for the 48 hpf pronephros were stained with anti-acetylated α-tubulin (cilia, green), anti-α-tubulin (spindle, green) and anti-phosphorylated histone H3 (pH3, red) for the anterior (A–D), middle (E–H) and posterior (I–L) pronephros. In control embryos, cells with mitotic spindle fibers oriented in the longitudinal plane of the anterior (A), middle (E) and posterior (I) pronephros. In the anterior (A–D) and middle (E–H) pronephros, wtip morphants (B,F,J), vangl2 morphants (C,G,K) and wtip + vangl2 morphants (D,H,L) resulted in mitotic spindle orientation defects. The posterior pronephros (I–K) showed mitotic spindles oriented normally in the longitudinal plan (I–L). Mitotic spindle angles were photographed by confocal imaging in the three segments of the anterior (A–D,M), middle (E–H,N) and posterior (I–L,O) pronephros at 48 hpf. The angle between the long axis of the pronephros and the spindle fibers was measured; angles were grouped into 10 degree bins. Scale bars are 100 µm in A–L.
by cloaca malformation is usually explained as a result of impaired fluid excretion from the cloaca. However, the details of morphogenesis and ciliogenesis in the caudal pronephros, such as number of ciliated cells, cilia motility, and cilia structure defects leading to pronephric cysts, differ in each case. In pkd2 (Obara et al., 2006; unpublished) and npgph6 (Sayer et al., 2006) knockdown, no defects were observed in cilia length and cilia motility in cloaca. In npgph4 knockdown, pronephric ducts displayed increased cell number, shorter cilia length, slower cilia motility in cloaca and programmed cell death of ectodermal cells forming the cloaca opening failed to occur (Burcklé et al., 2011; Pyati et al., 2006). In the case of Wtip, our results show that it is localized to the basal body and that its contribution to cloacal ciliogenesis is different from that reported in previous research (Obara et al., 2006; Sayer et al., 2006; Burcklé et al., 2011; Slanchev et al., 2011). We demonstrated that cloaca defects by morpholino targeting ATG site (wtipMO) and also wtip exon2 for deletion via a splice blocking morpholino (wtipex2MO) which lacks part of the first LIM domain, suggesting that the Wtip LIM1 domain is essential to maintain the number of ciliated cells in the posterior pronephros and cloaca. Prior to the gross cloaca morphogenesis defects demonstrated at 34 hpf, the pattern of cloaca-specific gene (evx1) expression was altered from 24 hpf in wtip knockdown zebrafish. The basal body structure was not affected in wtip knockdown. However, the rootlet structure normally connected to the basal body was never formed in ciliated cells of cloaca and the 9+2 cilia were non-motile. By contrast, the number of ciliated cells and cilia motility were unaffected in the anterior and middle segment of pronephros, which indicated that the role of Wtip is specific to the pronephros segments. Furthermore, other cystic genes such as ifi88 knockdown and mutants (unpublished data) display no cloaca malformation despite glomerulus cysts, body curvature, hydrocephalus and pericardial edema. This suggests that cystogenesis may proceed independently from defects in caudal pronephros (Kramer-Zucker et al., 2005; Burcklé et al., 2011; Slanchev et al., 2011).

Recent studies indicate that PCP is regulated by the non-canonical Wnt pathway and that some of the proteins involved in human kidney cystic diseases localized to the basal body are known to contribute to Wnt/PCP signaling (May-Simera et al., 2010; Burcklé et al., 2011; Jonassen et al., 2008). PCP core protein Vangl2 is known to localize to the basal body (Ross et al., 2005) and our results demonstrated that Wtip localized to the basal body is a maternal gene product. Therefore, we examined whether specific defects caused by wtip knockdown in the caudal pronephros are related to Wnt/PCP signaling. Interestingly, vangl2 morphants do develop cyst formation accompanied by cloaca malformation and generate fewer ciliated cells, a phenotype strikingly similar to that of wtip morphants. The involvement of additional mechanisms in cyst formation in wtip knockdown is further supported by functional interaction experiments, in which vangl2 knockdown dramatically enhances pronephric cyst formation accompanied by cloaca malformation.

Our results showed that the basal body protein Wtip plays segment-specific roles in the pronephros. Our working hypothesis regarding cystic kidneys is that the plane of cell division may be altered in wtip knockdown. Recently, it has been noted that in cystic kidneys, mitotic spindles along the tubules fail to form a proper orientation; however, the contribution of misaligned cell division to cystic kidneys remains unclear (Jonassen et al., 2008; Jonassen et al., 2012; Fischer and Pontoglio, 2009). Due to the simple structure of the zebrafish pronephros, in contrast to the mouse, we were able to analyze the mitotic spindle formation in the same nephron in a segment-specific manner, from the anterior to posterior pronephros, during the different embryonic developmental stages which had thus far remained elusive in zebrafish ciliary knockdown. In the anterior and middle pronephros, wtip knockdown failed to properly orient the mitotic spindles along the tubules. Interestingly, vangl2MO synergistically enhanced wtipMO mitotic spindle defects only in the anterior and middle pronephros and resulted in fewer ciliated cells in the cloaca. Therefore, we demonstrated for the first time that the signaling of the core PCP protein Vangl2 is required for proper mitotic orientation along the tubules in the anterior and middle segment of the pronephros and for the number of ciliated cells in the posterior pronephros. The exact relationship of Vangl2 and Wtip is unclear at present but they may govern apical actin assembly and migration of the centrosome during early cell polarization which is a crucial step for cilia formation. Our data suggest that it will be interesting to determine whether this phenotype may be extrapolated to other cystic genes that are studied in zebrafish, other animal models, and human ciliopathies.

Another possible model of Wtip function is that its cell-type specific actions may specifically affect fluid flow in the middle of pronephros, where we speculate that Wtip is affecting cell fate (unpublished). One main advantage of the zebrafish model is its optical translucency, which facilitates live imaging of the cellular processes underlying morphogenesis. In particular, it will be interesting to verify the Wtip protein localization in a cell type specific manner during the different developmental stages and conduct the noninvasive observation of morphogenetic processes in real time to develop early detection markers for human disease.

The present study introduces Wtip as a new player in kidney development and ciliopathy. Furthermore, this work will help to determine, whether we can utilize zebrafish as a novel target for therapeutic intervention in ciliopathy, diseases for which there are currently no approved treatments beyond supportive care.

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Competing Interests
The authors declare that there are no competing interests.

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