Wdpcp, a PCP Protein Required for Ciliogenesis, Regulates Directional Cell Migration and Cell Polarity by Direct Modulation of the Actin Cytoskeleton

Cheng Cui1,2, Bishwanath Chatterjee1,2, Thomas P. Lozito3, Zhen Zhang1, Richard J. Francis1,2, Hisato Yagi1, Lisa M. Swanhart1a, Subramanian Sanker1, Deanne Francis2, Qing Yu2, Jovenal T. San Agustin1, Chandrakala Puligilla1b, Tania Chatterjee2, Terry Tansey2, Xiaolin Liu1, Matthew W. Kelley5, Elias T. Spiliotis6, Adam V. Kwiatkowski7, Rocky Tuan3, Gregory J. Pazour4, Neil A. Hukriede1,
Cecilia W. Lo1,2*

1 Department of Developmental Biology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, United States of America, 2 Laboratory of Developmental Biology, National Heart Lung and Blood Institute, National Institutes of Health, Bethesda, Maryland, United States of America, 3 Center for Cellular and Molecular Engineering, Department of Orthopaedic Surgery, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, United States of America, 4 Program in Molecular Medicine, University of Massachusetts Medical Center, Worcester, Massachusetts, United States of America, 5 Section on Developmental Neuroscience, National Institute on Deafness and Other Communication Disorders, National Institutes of Health, Bethesda, Maryland, United States of America, 6 Department of Biology, Drexel University, Philadelphia, Pennsylvania, United States of America, 7 Department of Cell Biology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, United States of America

Abstract

Planar cell polarity (PCP) regulates cell alignment required for collective cell movement during embryonic development. This requires PCP/PCP effector proteins, some of which also play essential roles in ciliogenesis, highlighting the long-standing question of the role of the cilium in PCP. Wdpcp, a PCP effector, was recently shown to regulate both ciliogenesis and collective cell movement, but the underlying mechanism is unknown. Here we show Wdpcp can regulate PCP by direct modulation of the actin cytoskeleton. These studies were made possible by recovery of a Wdpcp mutant mouse model. Wdpcp-deficient mice exhibit phenotypes reminiscent of Bardet–Biedl/Meckel–Gruber ciliopathy syndromes, including cardiac outflow tract and cochlea defects associated with PCP perturbation. We observed Wdpcp is localized to the transition zone, and in Wdpcp-deficient cells, Sept2, Nphp1, and Mks1 were lost from the transition zone, indicating Wdpcp is required for recruitment of proteins essential for ciliogenesis. Wdpcp is also found in the cytoplasm, where it is localized in the actin cytoskeleton and in focal adhesions. Wdpcp interacts with Sept2 and is colocalized with Sept2 in actin filaments, but in Wdpcp-deficient cells, Sept2 was lost from the actin cytoskeleton, suggesting Wdpcp is required for Sept2 recruitment to actin filaments. Significantly, organization of the actin filaments and focal contacts were markedly changed in Wdpcp-deficient cells. This was associated with decreased membrane ruffling, failure to establish cell polarity, and loss of directional cell migration. These results suggest the PCP defects in Wdpcp mutants are not caused by loss of cilia, but by direct disruption of the actin cytoskeleton. Consistent with this, Wdpcp mutant cochlea has normal kinocilia and yet exhibits PCP defects. Together, these findings provide the first evidence, to our knowledge, that a PCP component required for ciliogenesis can directly modulate the actin cytoskeleton to regulate cell polarity and directional cell migration.

Citation: Cui C, Chatterjee B, Lozito TP, Zhang Z, Francis RJ, et al. (2013) Wdpcp, a PCP Protein Required for Ciliogenesis, Regulates Directional Cell Migration and Cell Polarity by Direct Modulation of the Actin Cytoskeleton. PLoS Biol 11(11): e1001720. doi:10.1371/journal.pbio.1001720

Academic Editor: Matthew P. Scott, Stanford University, United States of America

Received August 9, 2013; Accepted October 18, 2013; Published November 26, 2013

This is an open-access article, free of all copyright, and may be freely reproduced, distributed, transmitted, modified, built upon, or otherwise used by anyone for any lawful purpose. The work is made available under the Creative Commons CC0 public domain dedication.

Funding: This work was supported by NIH grants HL098180 (CWL), GM060992 (GJP) and GM097664 (ETS) at http://www.nih.gov/. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

Abbreviations: AA or aa, amino acid; AER, apical ectodermal ridge (limb); AVSD, atrioventricular septal defects (heart); BBS, Bardet–Biedl Syndromes; Co-IP, coimmunoprecipitation; DBA, Dolichos biflorus agglutinin; DL, distal early tubule (kidney); EFIC, episcopic fluorescence image capture; EM, electronic microscopy; ENU, ethylnitrosourea; ES cells, embryonic stem cells; IHC, inner hair cell (cochlea); KO, knockout; LV, left ventricle (heart); MA, major axis; MEF, mouse embryonic fibroblast; MEF, membrane fluctuation period; MI, minor axis; MKS, Meckel–Gruber Syndromes; MO, morpholino; NG, normal goat serum; OFT, outer flow tract (heart); OHC, outer hair cell (cochlea); PAln, pulmonary alveoli (heart); PBT, phosphate buffered saline with Tween-20; PCP, planar cell polarity; PCR, polymerase chain reaction; PD, pronephric duct (kidney); PST, proximal straight (kidney); PTA, persistent truncus arteriosus (heart); Phal, Phalloidin; Ptch1, Patched1; SMA, smooth muscle actin; Sept2, septin 2; Shh, Sonic hedgehog; Smo, Smoothened; TEF, tracheoesophageal fistula.

* E-mail: ccl50@pitt.edu

† Current address: Department of Biology, Canisius College, Buffalo, New York, United States of America.
‡ Current address: Department of Pathology and Laboratory Medicine, Medical University of South Carolina, Charleston, South Carolina, United States of America.
Cilia are microscopic cell surface hair-like protrusions that can act as antennae to mediate cell signaling. Mutations disrupting ciliogenesis can cause many developmental anomalies associated with syndromes known as “ciliopathies.” Some developmental defects, such as limb polydactyly, arise from disruption of cilia-transduced sonic hedgehog signaling, while other defects, such as aberrant patterning of hair cells in the inner ear, arise from disrupted Wnt signaling resulting in modulation of planar cell polarity (PCP)—a process whereby cells are polarized and aligned. While ciliopathy phenotypes would suggest that cilia are involved in modulating PCP, the mechanistic link between cilia and PCP has been elusive. Our study using a mouse model carrying a mutation in \textit{Wdpcp}, a gene required for both ciliogenesis and PCP, suggest that Wdpcp modulation of PCP involves interactions with the actin cytoskeleton separate from its function in ciliogenesis. We observe Wdpcp localization in cilia, where it is required for recruitment of proteins essential for ciliogenesis and involves direct modulation of the actin cytoskeleton.

### Results

A mutant, named \textit{Wdpcp}^{Cys40}, was recovered from an ethynitrosourea mouse mutagenesis screen exhibiting a wide spectrum of developmental anomalies consistent with MKS/BBS. Particularly notable is their phenotypic similarity to \textit{Mks1} mutant mice that are models of MKS [27,28]. This included anophthalmia (Figure 1A), central polydactyly (Figure 1B,C), and cysts in the kidney and a variety of other organs (Figure 1D,E). \textit{Wdpcp}^{Cys40} mutants also exhibited complex congenital heart defects, usually consisting of persistent truncus arteriosus or pulmonary atresia (Figure 1F,G), and atroventricular septal defects (AVSDs; Figure 1L,M). Some mutants had duplex kidney (Figure 1E) and facial clefts and/or cleft palate (Figure 1A). Tracheoesophageal fistula (TEF) due to defects in septation of the oropharynx (unpublished data) and cloacal septation defects were also observed (Figure 1H,I).

We mapped the mutation to a 6.36 Mb interval delimited by SNP rs26841005 and rs26856862 on mouse chromosome 11. RT-PCR analysis was carried out using RNA extracted from E12.5 hearts of \textit{Wdpcp}^{Cys40} mutant embryos to interrogate transcript expression from the 36 genes in the mapped interval. This analysis revealed an anomalous transcript from \textit{Wdpcp} (NM\_145425.3 and NP\_663400.2). Further sequencing analysis suggested this was derived from a splicing defect mutation, which was confirmed with genomic DNA sequencing. An A to G substitution was observed at nucleotide 224 of the mRNA (Figure 2A), corresponding to the 8th base before the splice donor site of exon 5 (Figure 2B). As a result, a premature stop codon (S5X4) is generated, causing protein truncation after amino acid 54 (Figure 2C). Quantitative PCR analysis with primers covering exons 5–6 showed only low trace amount (0.6%) of normal transcripts, suggesting \textit{Wdpcp}^{Cys40} is essentially a null or strong hypomorphic \textit{Wdpcp} mutant allele.

### Wdpcp Required for Recruitment of Proteins for Ciliogenesis

Analysis of \textit{Wdpcp}^{Cys40} mutant embryos revealed ciliogenesis defects. This was observed in the kidney-collecting duct (Figure 2N,O,R) and in the neuroepithelium (Figure 2P,Q). Mouse embryonic fibroblasts (MEFs) derived from \textit{Wdpcp}^{Cys40} mutant embryos, referred to as \textit{Wdpcp}^{Cys40} MEFs, confirmed a defect in ciliogenesis (Figure 2R; Figure S1A,B). Using an antibody raised to Wdpcp, we showed Wdpcp is localized to the ciliary axoneme and in a ring-like structure at the base of the cilium in IMCD3 cells (Figure 2D). A similar distribution was observed in NIH-3T3 cells transfected with Wdpcp-FLAG, a FLAG-tagged expression vector (Figure 2E–H). The Wdpcp ring-like structure in the cilium showed colocalization with Sept2,
which is known to be present as a ring in the ciliary transition zone ([Figure 2I–M; see Movie S2] [26]. In Wdpcp<sup>Cys40</sup> mutant MEFs, no specific Wdpcp immunostaining was observed, consistent with Wdpcp<sup>Cys40</sup> being a loss-of-function allele (see below). Even in rare Wdpcp<sup>Cys40</sup> mutant MEFs that are ciliated, Sept2 was usually not detected in the cilia ([Figure 3I–L; 11 of 12 with no Sept2 staining; one with very low Sept2 staining]). In contrast, wild-type MEFs typically showed strong Sept2 localization in the cilia (24 of 30 cilia).

To further interrogate the role of Wdpcp in ciliogenesis, we examined Wdpcp<sup>538-20</sup> mutant MEFs for the distribution of Nphp1, a protein also found in the ciliary transition zone [29], and Mks1, a transition zone protein expressed only in the mother centriole and required for basal body docking to the membrane [27]. Both proteins are known to be required for ciliogenesis. Analysis of the rare ciliated Wdpcp<sup>538-20</sup> mutant MEFs revealed Nphp1 was mislocalized to adjacent regions, such as in the basal body ([Figure 3E–H; five of eight cilia]), while Mks1 was absent ([Figure 3M–P; 9 of 12 cilia]). In comparison, Nphp1 (25 out of 27) and Mks1 (17 out of 21) were found in the cilia transition zone and basal body, respectively, of wild-type MEFs (p<0.01). These results show Wdpcp is required for recruitment of Mks1 and Nphp1 to the ciliary transition zone. However, Mks1 is not required for Wdpcp recruitment to the cilia, as Mks1 mutant MEFs showed normal distribution of Wdpcp in the ciliary transition zone ([Figure 3Q–T]). In comparison, Ift88 distribution in the ciliary axoneme and basal body was unchanged in Wdpcp<sup>538-20</sup> mutant MEFs, indicating IFT transport is not disrupted by Wdpcp deficiency ([Figure 3A–D]). Together, these observations suggest the ciliogenesis defect in Wdpcp<sup>538-20</sup> mutant MEFs arises from the combined loss of Sept2, Nphp1, and Mks1 from the ciliary transition zone.

**Wdpcp Required for Motile Cilia Function in Zebrafish But Not Mouse Embryos**

To examine whether Wdpcp also may play a role in motile cilia function, we examined cilia in the mouse embryonic node and in the trachea airway epithelium. The mouse embryonic node ([Figure S1C,D]) exhibited a normal pattern of ciliation with motile
cilia (Movie S1) that generated effective flow (unpublished data). This is consistent with the absence of laterality defects in the WdpcpCys40 mutants. Similarly, the trachea airway epithelia from E17.5 WdpcpCys40 mutant embryos were ciliated, and the cilia were motile and exhibited normal cilia motility (Movie S3).

In zebrafish, wdpcp is expressed starting from 10 somite stage (Figure S2A–O), and surprisingly, wdpcp morpholino (MO) knockdown resulted in a constellation of defects indicative of motile cilia defects. This included curved body axis (Figure 4B), pericardial effusion and kidney cysts (Figure 4B), hydrocephalus (Figure 4D), and increased number of otoliths (Figure 4D). Consistent with motile cilia defects, wdpcp morphants exhibited a 20% incidence of heterotaxy (Figure S3). Wdpcp morphants also exhibited kidney cysts (20%) and cilia disarray in the pronephric tubule, which were more pronounced proximally (Figure 4G–L). Videomicroscopy showed abnormal cilia motility throughout the pronephric tubule (Movie S4). As observed in the Wdpcp mouse mutants, some wdpcp zebrafish morphants (37%; N = 208) exhibited pericardial effusion and kidney cysts (Figure 4B), hydrocephalus (Figure 4D), and increased number of otoliths (Figure 4D).
obstructed cloaca (Figure S3K–P). This was not correlated with the pronephric cilia disarray, as cilia disarray was observed in 38% of morphants with cloaca obstruction (n = 63) and 50% without cloaca obstruction (n = 82). Wdpcp antibody staining showed punctate Wdpcp localization in the ciliary axoneme in the pronephric tubule (Figure 4F), which was lost with \textit{wdpcp} MO knockdown (Figure 4E). Western blotting confirmed Wdpcp protein expression is reduced with \textit{wdpcp} MO knockdown (Figure S2P,Q). Specificity of the MO knockdown effects was confirmed with injection of \textit{wdpcp} mRNA, which showed complete rescue of the defect phenotypes seen with \textit{wdpcp} MO knockdown (Figure S4E,F).

These findings suggest that unlike mouse embryos, Wdpcp is required for motile cilia function in zebrafish embryos. This raises the question of whether the \textit{WdpcpCys40} allele is a null mutation or if it might have residual function that could account for the differences between the mouse and fish phenotypes. To examine this question, we generated a \textit{Wdpcp} knockout mouse model by gene targeting (Figure S5A). Breeding of mice carrying the knockout allele showed homozygous \textit{Wdpcp} knockout mice died at birth with the same spectrum of developmental anomalies seen in \textit{WdpcpCys40} mutants, including anopthalmia, cleft palate, heart outflow tract (OFT) septation defects, duplex kidney, limb polydactyly (Figure S5B–E), and multiple organ cysts. As in \textit{WdpcpCys40} mutants, no laterality defects were noted. These findings demonstrate that the phenotypes observed in \textit{WdpcpCys40} mutants reflect the loss of Wdpcp function.

Disruption of Shh Signaling in \textit{WdpcpCys40} Mutants

Many of the \textit{WdpcpCys40} mutant phenotypes, such as the limb polydactyly, TEF, and cleft palate, are consistent with the
disruption of Shh signaling, which is known to be cilia transduced. Consistent with this, analysis of \textit{WdpcpCys40} mutant MEFs showed little response to stimulation with SAG, a Shh agonist (Figure S6B). In situ hybridization analysis of the developing limb buds in \textit{WdpcpCys40} mutants showed expansion of \textit{Fgf4} and \textit{Gremlin} expression in the apical ectodermal ridge and digit forming mesenchyme, respectively, while \textit{Ptch1} expression was diminished (Figure 5A–F). These results confirmed the disruption of Shh signaling in the limb bud, consistent with the polydactyly phenotype. We also observed disruption of Shh signaling in the neural tube, which was indicated by dorsalization of the neural tube (Figure S6A). Further examination of \textit{WdpcpCys40/Cys40; Smo}^{-/-} (\textit{n} = 3) and \textit{WdpcpCys40/Cys40; Ptc1}^{-/-} (\textit{n} = 4) double mutant embryos showed rescue of the severe \textit{Smo} and \textit{Ptc1}
knockout phenotypes (Figure 5G–J). This indicated Wdpcp functions downstream of Smo and Ptch1. Abnormal Gli transcription factor processing was revealed with Western blotting analysis of isolated limb bud and whole embryo extracts. This is indicated by alterations in the ratio of full-length activator versus shorter cleaved inhibitor Gli3 protein (Figure 5K; Figure S6C). We also observed more full-length Gli2 in the WdpcpCys40 mutant embryos (Figure S6D).

**PCP Defects in WdpcpCys40 Mutants**

We investigated WdpcpCys40 mutants for PCP defects, given Wdpcp plays an important role in PCP regulated convergent extension cell movement in the *Xenopus* embryo [26]. We examined patterning of hair cells in the cochlea, as it is well described to be PCP dependent. The hair cells are normally arrayed in repeating rows, all exhibiting the same polarized orientation as defined by the actin-based stereocilia bundles. These are organized in a stereotypical “chevron” configuration, each with a single microtubule-based kinocilium protruding at the tip of the chevron (for review see [30]) (Figure 6A,C,E). Examination of the cochlea of WdpcpCys40 mutants showed the hair cells were disarrayed, with some of the chevrons misaligned (Figure 6B,F).

Although the kinocilia were present, they were mislocalized (Figure 6D). We also observed expression of Vangl2, a membrane-localized PCP core component normally asymmetrically expressed in the hair cells, was nearly extinguished in the WdpcpCys40 mutant cochlea (Figure 6G,H). WdpcpCys40 mutants also exhibit outflow tract septation defects, another PCP-dependent developmental process. PCP is thought to regulate outflow tract septation via its role in modulating myocardialization of the outflow tract—a process in which cardiomyocytes invade and migrate into the conotruncal region of the heart to form the muscular outlet septum [31,32]. In E13.5 control hearts, cardiomyocytes can be seen invading into the outflow tract at the base of the aorta and pulmonary trunk (Figure 6I), with the invading cells exhibiting an elongate morphology with long cell processes projecting into the outflow cushion aligned with the direction of cell migration (Figure 6K). However, in the WdpcpCys40 mutant heart, cardiomyocytes failed to invade the OFT cushion (Figure 6J), and they did not exhibit the polarized cell projections seen in the wild-type heart (Figure 6L). These observations suggest polarized cell migration required for formation of the outflow septum in the embryonic heart is compromised in the WdpcpCys40 mutant.

**Figure 5. Sonic hedgehog signaling defect in WdpcpCys40 mutant.** (A–F) In-situ hybridization of E10.5 forelimbs shows WdpcpCys40 mutants with expanded expression of Fgf4 in the AER (apical ectodermal ridge) (A, B) and Gremlin in the limb mesenchyme (C, D), but reduced expression of Ptch1 (E, F and asterisk in F). In (A) and (B), black arrowheads indicate the span of the AER, and white arrowheads are the anterior and posterior bases of the limb bud. (G–J) Wdpcp deficiency rescued the severe defect phenotypes of Smo<sup>−/−</sup> (G) and Ptch1<sup>−/−</sup> (I) mutant embryos at E10.5 dpc. The Wdpcp<sup>Cys40/Cys40</sup>Smo<sup>−/−</sup> (H) and Wdpcp<sup>Cys40/Cys40</sup>Ptch1<sup>−/−</sup> (J) double homozygous mutant embryos collected at E10.5 dpc show more robust growth with better axial development and also more normal head and heart development. (K) Western blotting of Gli3 in tissue extracts obtained from the limb and neural tube shows a decrease of Gli3-R/Gli3-FL ratio in WdpcpCys40 mutant embryos. Scale bars, 200 μm in (A), 1 mm in (G). Scales are the same in (A–F) and (G–J).

doi:10.1371/journal.pbio.1001720.g005
Canonical Versus Noncanonical Wnt Signaling in WdpcpCys40 Mutants

Using real-time PCR analysis, we further examined the expression of transcripts for components of the noncanonical Wnt signaling pathway known to regulate PCP using RNA obtained from the base of the OFT where the outlet septum forms (Table S1). This analysis showed a reduction in the expression of Wnt5a, a noncanonical Wnt ligand (Figure 6O). We also examined expression of the canonical Wnt signaling components, as opposing changes in noncanonical versus canonical Wnt signaling have been observed in ciliopathy mutants [24]. Indeed, real-time PCR analysis showed an increase in Axin2 and Dishevelled (Dsh1/2/
transcripts, while transcripts for the canonical Wnt inhibitors Dkk1/2/3 were reduced (Figure 6O). Consistent with these real-time PCR results, analysis of WdpcpCys40 mutants carrying the BAT–lacZ canonical Wnt reporter [33] also showed marked increase in lacZ expression at the base of the outflow tract (Figure 6M,N). Together, these observations suggest that while noncanonical Wnt signaling is reduced, canonical Wnt signaling is upregulated in the WdpcpCys40 mutants.

Wdpcp Modulation of Actin Stress Fibers

To investigate the mechanism by which Wdpcp may regulate PCP, we further examined Wdpcp distribution in the cytoplasm, in particular its interaction with Sept2, which is known to associate with the actin cytoskeleton [34,35]. Interestingly, in wild-type MEFs, Wdpcp showed extensive colocalization with actin filament bundles or stress fibers delineated by phalloidin staining (Figure 7A–H). Given the known role of Sept2 in associating with and stabilizing actin filaments, we investigated whether Wdpcp and Sept2 may be colocalized in actin filaments. Phalloidin staining to visualize actin filaments together with double immunostaining with Sept2 and Wdpcp antibodies indeed showed regions of Wdpcp and Sept2 colocalization in actin stress fibers in wild-type MEFs (Figure 7A–H). Similar analysis of the WdpcpCys40 mutant MEFs revealed the loss of Wdpcp immunostaining, and interestingly, the actin cytoskeleton was markedly changed. None of the aligned stress fibers comprising of thick actin filament bundles were observed, but instead only thin actin filaments were seen (Figure 7J,N). Sept2 exhibited a beaded arrangement that were loosely aligned with but not colocalized with the actin filaments (Figure 7M,N,P). A magnified view showed these beaded structures were comprised of ‘o’ and ‘c’ shaped structures similar to those previously reported in cells treated with latrunculin to disrupt the actin cytoskeleton [36]. Quantitation showed phalloidin staining was reduced by 28% in the mutant (n = 144) versus control (n = 111) MEFs (p = 0.011), consistent with the observed reduction in actin stress fibers. These observations suggest Wdpcp, through interactions with Sept2, may play an essential role in modulating actin filaments and the formation of stress fibers.

Wdpcp and Sept2 Interaction

To interrogate Wdpcp interaction with Sept2, we carried out coimmunoprecipitation experiments to determine if Wdpcp and Sept2 may be found in the same protein complex. For these

Figure 7. Wdpcp colocalizes with Sept2 and actin filaments. (A–D) Confocal imaging of control MEFs stained with phalloidin, and antibodies to Wdpcp and Sept2 showed Sept2 (red) and Wdpcp (green) are colocalized in actin stress fiber (phalloidin stained, blue) (examples denoted by arrow). This is better visualized in the magnified image shown in (E–H). (E–H) Magnified view of the region indicated by the asterisk-denoted arrow from (A–D) show colocalization of Wdpcp (green) and Sept2 (red) with actin filaments (blue). (I–L) Confocal imaging of WdpcpCys40 mutant MEFs showed only background fluorescence (green, panel J) with the Wdpcp antibody. However, Sept2 immunostaining remained robust (red, panel I), but there was no colocalization with actin filament visualized with phalloidin staining (blue, panel K). The region indicated by the asterisk-denoted arrow is magnified in (M–P). Inset shown is magnified view of a region from (M), showing ‘c’ and ‘o’ shaped Sept2 immunostained structures. (M–P) Magnified view of the region marked by the asterisk-denoted arrow in (I–L). While actin (blue, panel O) and Sept2 filaments (red, panel M) can be observed, Sept2 is not colocalized with actin (P). Sept2 immunostaining delineated ‘c’ and ‘o’ shaped structures (M), which are better visualized in the further magnified view shown in the inset in (I). Scale bars, 20 μm in (A), 10 μm in (E), and 5 μm in inset image in (I). Scales are the same in (A–D), (I–L); (E–H), (M–P).

doi:10.1371/journal.pbio.1001720.g007
with FLAG immunoprecipitation, which showed FLAG–Wdpcp was present in much lower abundance in the transiently transfected versus stably transfected cells (lane 7 versus 8 in Figure 8A).

When the same cell lysates were immunoblotted with anti-GFP antibodies, a 66 kDa Sept2–GFP fusion protein band (361 AA for Sept2 and 230 AA for GFP) was observed (lanes 2 and 3 in Figure 8B). Significantly, this band was also detected in the anti-FLAG immunoprecipitates (lanes 7 and 8, Figure 8B), indicating that Sept2–GFP and FLAG–Wdpcp are in the same protein complex. When the same cell lysates were immunoblotted with anti-Sept2 antibodies, the endogenous Sept2 band (~40 kDa) was observed in all whole cell lysates (Sept2 band in lanes 1, 2, and 3 in Figure 8C), and in the anti-FLAG immunoprecipitates from cells stably transfected with FLAG–Wdpcp (Sept2 band in lane 7 in Figure 8C). These results indicate Sept2–GFP and endogenous Sept2 are both incorporated into a FLAG–Wdpcp-containing protein complex. The specificity of the FLAG immunoprecipitations were confirmed with positive control using FLAG-tagged bacterial alkaline phosphatase (FLAG–BAP band in lane 6 of Figure 8A), and negative control with nontransfected cells (lane 5 in Figure 8B) or blank immunoprecipitation elution control (lane 4 in Figure 8B). Together these results confirm that Wdpcp and Sept2 are recruited in the same protein complex.

**Wdpcp Modulation of Cell Motility and Focal Adhesion Contacts**

To examine the role of Wdpcp in actin dynamics and motile cell behavior, we carried out time-lapse videomicroscopy to assess cell motility in the WdpcpCys40 mutant and wild-type MEFs. Time-lapse imaging showed dynamic membrane ruffling with lamellipodial and/or filopodial cytoplasmic extensions in wild-type MEFs (Figure 9A–D). In contrast, WdpcpCys40 mutant MEFs exhibited minimal membrane ruffling activity (Figure 9E–H, arrowheads) and often with unusually long and thin filopodial extensions that appeared unable to disengage from the substratum (Movie S5) (see arrows in Figure 9E–H). This was confirmed with quantitative analysis of the time-lapse videos, which showed the mutant MEFs were more likely to have filopodia (46.03% versus 19.99% in wild-type MEFs; \( p < 0.0001 \)), and the filopodia persisted longer (3.0 h versus 1.12 h in wild-type MEFs; \( p < 0.0001 \)). To assess membrane ruffling, we examined the frequency of brightness change at the cells’ leading edge and calculated the fluctuation period as a measure of membrane dynamics (see Materials and Methods). This analysis showed significantly less membrane ruffling in the mutant MEF, which had a fluctuation period of 272 s versus 167 s for wild-type MEFs (\( p < 0.0002 \)).

As cell motility requires actin remodeling coordinated with the assembly/disassembly of focal contact, we further examined the role of Wdpcp in the modulation of focal adhesion contacts. Phalloidin, anti-Wdpcp, and anti-vinculin triple staining showed Wdpcp is enriched in the cell cortex, where it is extensively colocalized with vinculin at points of actin filament insertion into focal adhesions (Figure 9I–L). While wild-type MEFs exhibited elongated focal contacts, focal contacts in mutant MEFs were more likely to have filopodia (46.03% versus 19.99% in wild-type MEFs; \( p < 0.0001 \)), and/or filopodial cytoplasmic extensions in wild-type MEFs (\( p < 0.0001 \)). To assess membrane ruffling, we examined the frequency of brightness change at the cells’ leading edge and calculated the fluctuation period as a measure of membrane dynamics (see Materials and Methods). This analysis showed significantly less membrane ruffling in the mutant MEF, which had a fluctuation period of 272 s versus 167 s for wild-type MEFs (\( p < 0.0002 \)).
increase in roundness (58.9 in \(Wdpcp^{Cys40}\) mutant versus 37.7 in the wild-type MEFs, \(p<0.0001\); Figure 9P). These marked changes in the organization of the focal adhesion contacts may contribute to the defects in cell motility and directional cell migration observed in \(Wdpcp^{Cys40}\) mutant MEFs.

Wdpcp Modulates Polarized Cell Migration

To investigate the role of Wdpcp in polarized cell migration, we carried out a wound-scratch assay to examine the ability of the \(Wdpcp^{Cys40}\) mutant MEFs to engage in directional cell migration to fill the wound gap. For this analysis, wild-type and \(Wdpcp^{Cys40}\) mutant MEFs were grown to confluence, and then a wound gap was created to assess directional cell migration required for wound closure. Wild-type MEFs migrated into the wound gap in a highly organized manner with cells aligned with the direction of wound closure (Figure 10A). In contrast, mutant MEFs migrated in a haphazard manner with no consistent cell alignment (Figure 10B). Immunostaining with a Golgi marker showed the Golgi apparatus in wild-type MEFs were localized at the cells’ leading edge and were forward facing relative to the direction of wound closure (0–60° in Figure 10C,E), consistent with polarized cell alignment and directional cell migration. In contrast, in the mutant MEFs, the

Figure 9. Perturbation of membrane ruffling and focal adhesion contacts in \(Wdpcp\)-deficient cells. (A–H) Time-lapse imaging of wild-type (A–D) and \(Wdpcp^{Cys40}\) mutant (E–H) MEFs shown in 40 s intervals revealed the mutant MEFs have less membrane ruffling (arrowheads) and little or no membrane protrusive activity. In (E–H), the bottom-left arrow points to a long filopodial extension from a cell out of the field of view and top-left arrow points to the filopodial extension of the cell in the field of view that were immobile for the entire duration of the 320 s time-lapse sequence. (I–L) Immunostaining showed Wdpcp (green, J) is enriched at the cell cortex where actin filaments (phalloidin, K) insert into vinculin-containing focal adhesions (arrow and arrowheads in L). In wild-type MEFs (N), the vinculin-containing focal adhesions were smaller and more rounded compared to wild-type MEFs (M). This was demonstrated by quantitative measurements of the major axis and roundness of vinculin-containing focal adhesions (O, P). In wild-type MEFs (\(n=279\)), the vinculin-containing focal contacts were more elongated (O) and less round (P), than \(Wdpcp^{Cys40}\) mutant MEFs (\(n=206\)). The \(p\) values were calculated with student’s t test. Scale bars, 10 \(\mu\)m in (A), 2 \(\mu\)m in (L), and 10 \(\mu\)m in (N). Scales are the same in (A–H); (I–L); and (M, N).

doi:10.1371/journal.pbio.1001720.g009
direction of Golgi orientation was randomized (Figure 10D,E). These observations show Wdpcp is required for establishing the planar cell polarity needed to engage in directional cell migration.

Discussion

We showed WdpcpCys40, a mouse mutant with a wide spectrum of developmental defects consistent with MKS/BBS ciliopathy syndromes, harbors a Wdpcp loss of function mutation. We generated a Wdpcp knockout mouse model, which exhibited identical phenotypes to those seen in the WdpcpCys40 mutants. A role for Wdpcp in human disease is suggested by a previous finding of a homozygous WDPCP mutation in a screen of MKS/BBS patients [26]. We observed Wdpcp deficiency disrupted ciliogenesis and this was associated with the disruption of Shh signaling, accounting for many of the defect phenotypes observed in the WdpcpCys40 and Wdpcp knockout mice.

The WdpcpCys40 mutant mice exhibited phenotypes that are remarkably similar to those observed in a MKS mutant mouse model, Mks1<sup>Miks335</sup> [27]. While heterotaxy was observed in the Mks1 mutant, no laterality defects were found in the WdpcpCys40 mutants. Consistent with this, nodal cilia motility and nodal flow were unaffected in WdpcpCys40 mutant embryos. Surprisingly, wdpch MO knockdown in zebrafish embryos did not disrupt ciliogenesis, but perturbed motile cilia function. This was associated with a low incidence of heterotaxy. It is interesting to note that wdpch MO knockdown in Xenopus embryos also perturbed motile cilia function [26]. These species differences may reflect evolutionary divergence in the function of Wdpcp and other cilia-related proteins.

Wdpcp Regulates Ciliogenesis via Recruitment of Ciliary Proteins

Our findings indicate the ciliogenesis defect in WdpcpCys40 mutants arises from the failure of Wdpcp-deficient cells to recruit proteins required for ciliogenesis to the ciliary transition zone, including Nphp1, Sept2, and Mks1. Wdpcp is observed to form a ring structure in the ciliary transition zone overlapping with Sept2. Septins are well described to form ring structures both in vitro and in vivo [37]. In the ciliary transition zone, the septin ring forms a diffusion barrier regulating protein trafficking into the ciliary compartment [38]. Thus, failure to recruit Sept2 to the transition zone in Wdpcp-deficient cells is expected to disrupt ciliogenesis. Wdpcp-deficient cells also failed to recruit Mks1, a Meckel syndrome–associated protein localized to the mother centriole and required for basal body docking to the membrane [27,28].
Analysis of Mks1-deficient cells showed Wdpcp acts upstream of Mks1. We also observed Wdpcp-deficient cells with mislocalization of Nphp1, a cilium transition zone protein required for ciliogenesis and associated with cystic kidney defects in Joubert syndrome and other ciliopathies [39]. Together these findings indicate Wdpcp may play an important role in recruiting proteins essential for ciliogenesis. Consistent with this is the presence of two WD repeats in the Wdpcp protein. The WD40 domain has been identified in many protein interaction pairs [40], and many WD40 repeat-containing proteins have been shown to serve as scaffolds for assembly of multiprotein complexes. Together these findings suggest Wdpcp may serve as a scaffold in the cilium transition zone to facilitate the assembly of multiprotein complexes required for ciliogenesis.

**Wdpcp Constrains Hedgehog and Canonical Wnt Signaling**

While our studies with Wdpcp mutant MEFs showed Wdpcp deficiency disrupted Shh signaling, surprisingly the loss of Wdpcp function partially rescued the severe defect phenotypes of the Ptch1 or Smo knockout embryos. This suggests Wdpcp may constrain Shh signaling downstream of Smo/Ptch1. As ciliogenesis is disrupted with Wdpcp deficiency, how Gli processing required for Shh activation is regulated in the Wdpcp−/− (Ptch1 or Wdpcp−/−)Smo−/− mutant OFT is unknown. However, it is worth noting Drosophila hedgehog signaling occurs in the absence of the cilium [41].

Our studies also suggested Wdpcp may have a role in constraining canonical Wnt signaling, as BAT–lacZ and the expression of canonical Wnt transducers (Dkk1/2/3) are upregulated, while canonical Wnt inhibitors were down-regulated (Dkk1/2/3) in the Wdpcp−/− mutant OFT. While a role for the cilium in constraining canonical Wnt signaling is well described, the mechanism remains unclear [18]. We note Chibby, a basal body protein that negatively regulates canonical Wnt signaling, can bind β-catenin and prevent its entry into the nucleus [42]. One possibility to consider is whether Wdpcp may function in the same multiprotein complex with Chibby to regulate β-catenin trafficking.

**Wdpcp Modulates PCP and the Actin Cytoskeleton**

We showed Wdpcp−/− mutants exhibited PCP defects, such as malpatterning of stereocilia in the cochlea and abnormal myocardialization of the outflow tract in the heart. The myocardialization defect was characterized by failure of the invading cardiomyocytes to elongate and align their actin cytoskeleton with the direction of cell migration. We note the Loop-tail (Lp) mouse mutant harboring a mutation in the PCP component, Vangl2, also exhibits outflow tract defects [31]. Similar to our findings, in the Lp mutant cell protrusions into the outflow cushion were absent, and the organization of the actin cytoskeleton was disrupted. Perturbation of the actin cytoskeleton also may contribute to defects in the cochlea, as the stereocilia are actin-based structures and in Wdpcp−/− mutants, formation of the kinocilia was not affected. While cochlea expression of Vangl2 was reduced in Wdpcp−/− mutants, we did not observe mislocalization of PCP core components that would suggest a disruption in PCP signaling.

Using Wdpcp-deficient MEFs, we investigated the role of Wdpcp in establishing planar cell polarity. Our studies indicate Wdpcp plays an essential role in PCP via regulation of the actin cytoskeleton. We showed Wdpcp modulates the actin cytoskeleton by mediating Sept2 interaction with actin. In wild-type cells, Wdpcp is colocalized with Sept2 in actin filaments, but in Wdpcp-deficient cells, Sept2 is no longer actin localized and actin stress fibers are largely absent. Instead, Sept2 is observed in “o” and “c” configurations, structures also observed with inhibition of actin polymerization [36]. These results suggest formation of a Wdpcp–Sept2 complex may be required for Sept2 binding to actin filaments and the stabilization of actin filaments. Coimmunoprecipitation and Western immunoblotting confirmed Wdpcp and Sept2 localization in the same protein complex, but further experiments are needed to examine Wdpcp and Sept2 interaction with actin filaments.

A further indication of a role for Wdpcp in the modulation of actin dynamics was the reduction in membrane ruffling in Wdpcp mutant MEFs. We noted Wdpcp is enriched in the cell cortex where actin filaments insert into vinculin-containing focal adhesions. As focal contacts are also sites of actin polymerization, the abundance of Wdpcp in the vicinity of focal contacts may facilitate recruitment of Sept2 to enhance actin filament stabilization and stress fiber formation. We observed Wdpcp-deficient cells had smaller focal contacts, but with significantly higher concentration of vinculin. This could account for the failure of cell processes to disengage from the substratum in Wdpcp-deficient cells.

Most significantly, Wdpcp-deficient cells showed defects in motile cell behavior that was associated with defects in planar cell polarization. Thus, Wdpcp-deficient cells were unable to establish cell polarity in a wound scratch assay. This is indicated by failure of the Golgi to reorient to the cell’s leading edge and align with the direction of cell migration. As a result, migrating cells were unable to engage in directional cell motility. It is significant to note that Golgi orientation is specified by the microtubule-organizing center or centrosome, which also templates formation of the cilium. This defect in establishing cell polarity together with perturbation in membrane and actin dynamics and the modulation of focal adhesion contacts may underlie the PCP defects associated with Wdpcp deficiency. Together, these findings suggest Wdpcp may regulate planar cell polarity by modulating both the microfilament and microtubule cytoskeleton.

**Dual Functionality of Wdpcp in Ciliogenesis and PCP**

We showed the PCP effector Wdpcp plays an essential role in both ciliogenesis and PCP. This appears to involve separable functions of Wdpcp in recruitment of proteins to the ciliary transition zone required for ciliogenesis, and in the modulation of actin dynamics in the cytoplasm. Our findings provide the first evidence, to our knowledge, that a PCP component required for ciliogenesis can directly modulate the actin cytoskeleton to regulate planar cell polarity and directional cell migration. These observations suggest Wdpcp regulation of PCP is independent of its role in ciliogenesis. It is interesting to consider whether such dual functionality may have evolved as a means to functionally integrate pathways regulating ciliogenesis with those regulating PCP and the cytoskeleton. Whether such dual functionality may account for other PCP proteins known to be required for ciliogenesis is an important question that needs to be addressed in future studies.

**Materials and Methods**

**Institutional Approval for Animal Studies**

All mouse experiments were carried out using protocols approved by the Institutional Animal Care and Use Committee at the National Heart Lung Blood Institute and at the University of Pittsburgh. The zebrafish experiments were carried out with approved protocols at the University of Pittsburgh.
Mapping and Recovery of the Wdpcp Mutation and Mouse Breeding

C57BL/6j/B6/C3H hybrid mutant offspring were used to map the mutation using 48 microsatellite markers polymorphic between B6/C3H [43]. Once the chromosome interval was identified, additional polymorphic DNA markers were used to narrow the interval to a 5–10 Mb region. Then cDNA sequencing was carried out for each gene in the interval. 

Construction of FLAG-Tagged Wdpcp Construct

To make the mouse FLAG–Wdpcp construct, we isolated RNA from mutant heart and amplified the full-length cDNA using one step RT-PCR using Superscript III/HS Platinum Taq DNA polymerase and cloned cDNA into pCR2.1 TOPO vector. We then confirmed the clones by Sanger sequencing. We amplified TOPO vector containing Wdpcp (NM_145425.3) cDNA using primers (Table S1) with restriction site at the 5′ end (Sigma-Aldrich). The subsequent experiments were performed by using the FLAG at the 5′ end of Wdpcp.

Zebrafish wdp cp Morpholino Knockdown and Rescue

Zebrafish embryos were obtained from in crossing wild-type adults and following manipulation, maintained at 28.5°C, and staged according to [44]. A complementary MO (5′-CTGCTGCGACAGGACACATCT-3′) targeting the initiation codon of zebrafish wdp cp and standard control (5′-CCTCTTTACCTCAGTTACAATTTATA-3′) morpholino (MO) were designed and obtained from GeneTools LLC. Embryos at the one-cell stage were injected with 5 nl of morpholino at 2.0 ng/nl in phenol red. Control embryos were either injected with the standard control (2.0 ng/nl) MO or un.injected. Capped mRNAs for rescue experiments were synthesized in vitro from linearized pCS2+ mouse Wdpcp construct (NM_145425.3) using SP6 mMessage mMachine kit (Ambion). We microinjected 200 pg of Wdpcp mRNA at 200 pg/1 nl into the blastomere at the one-cell stage. After injections, the embryos were incubated in 1× E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4, 0.01% methylene blue) at 28°C. Control embryos were either injected with the standard control (2.0 ng/nl) MO or un injected. Capped mRNAs for rescue experiments were synthesized in vitro from linearized pCS2+ mouse Wdpcp construct (NM_145425.3) using SP6 mMessage mMachine kit (Ambion). We microinjected 200 pg of Wdpcp mRNA at 200 pg/1 nl into the blastomere at the one-cell stage. After injections, the embryos were incubated in 1× E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4, 0.01% methylene blue) at 28°C. Control embryos were either injected with the standard control (2.0 ng/nl) MO or un injected. Capped mRNAs for rescue experiments were synthesized in vitro from linearized pCS2+ mouse Wdpcp construct (NM_145425.3) using SP6 mMessage mMachine kit (Ambion). We microinjected 200 pg of Wdpcp mRNA at 200 pg/1 nl into the blastomere at the one-cell stage. After injections, the embryos were incubated in 1× E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4, 0.01% methylene blue) at 28°C until the desired stage and imaged using Leica MZ16 microscope fitted with Retiga 1300 camera. Statistical distribution of morphants and morphologically normal embryos from three separate rescue experiments were analyzed using Graphpad prism version 6.

Zebrafish in Situ Hybridization and Immunocytochemistry

For in-situ hybridization, zebrafish embryos were fixed in 4% paraformaldehyde and processed as described [45]. Partial zebrafish wdp cp cDNA clone obtained from OpenBiosystems (#EDR1052-579515134, clone ID 7911360) was used to generate sense and antisense in-situ probes for wdp cp. Plasmids for cmic2 and fabp10a in-situ probes were obtained from members of the zebrafish community and were described previously [46,47]. For immunocytochemistry, zebrafish embryos were fixed in Dent’s fixative (80% Methanol and 20% DMSO) for 4 h at room temperature, washed with methanol, and stored at −20°C. Antibody staining for acetylated α-tubulin was performed as described [46]. Wdpcp and acetylated α-tubulin double immunostaining, embryos were rehydrated in a series of methanol/PBT washes and permeabilized with 0.02% trypsin for 5 min. Following blocking with 10% normal goat serum (NGS, Sigma), embryos were incubated overnight at 4°C in PBT/10% NGS with Wdpcp (1:100; Aves, Inc) and acetylated α-tubulin (1:1,000; Sigma) antibodies. Goat anti-mouse Alexa Fluor 488 and goat anti-chicken Alexa Fluor 555 (both 1:1,000; Invitrogen) secondary antibodies were used. Embryos were de-yolked, mounted in Aqua Poly/Mount (Polysciences, Inc.), and visualized with a Zeiss LSM 510 Meta inverted laser scanning confocal microscope.

Immunocytochemistry and Immunohistology

MEFs derived from E11.5–E12.5 embryos were serum starved for 24 to 48 h to grow out the cilia, with staining of ciliary proteins carried out using various antibodies including acetylated α-tubulin antibody (Sigma T7451, 1:1,000), γ-tubulin antibody (Sigma T6557, 1:1,000), Wdcp chicken polyclonal antibody (Aves, Inc.) made against synthetic peptide (DTTILEYREPVSKYARR) corresponding to Wdcp amino residues 529-545, Wdcp goat antibody (Santa Cruz), Sept2 antibody (Millipore, 1:1,000), Mks1 antibody (1:2,000) [27], and antibodies to Ift88 and Nphp1 as previously described [49,50]. FLAG antibody (Sigma F7452, 1:1,000) was used for detection of Wdpcp–FLAG fusion protein.

Production of Wdpcp KO Mice

The Wdpcp target or null allele was generated by gene targeting in 129 ES cells, as shown in Figure S3A. Exon 5 was flanked by two loxP/Flox sites and an FRT-flanked PGKneo cassette (neomycin-resistant gene driven by the PGK promoter) was inserted in intron 5 of Wdpcp by homologous recombination. ES cell clones with correct homologous recombination were screened by long-range PCR. Two independent ES cell clones were injected into C57BL/6j blastocysts, and germ line transmission was achieved with both clones. The PGKNeo cassette was removed by FLP-FRT recombination to generate Wdpcp flox allele. The Wdpcp global knockout allele was generated by CMV-Cre/Lox recombination.

Analysis of Shh Signaling

DIG-labeled probes were made with DIG RNA reaction mixture (Roche), and procedures for whole mount in-situ hybridization were described previously [51] and gene expression patterns were visualized with BM purple AP substrate (Roche). RNA in-situ probe plasmids for Fgf4 (Lee Niswander lab), Gremln (Richard Harland lab), and Ptch1 were gifts from Dr. Susan Mackem (NCI). A 600 bp nucleotide Gli3 in-situ probe was generated by amplifying cDNA specific to Gli3 encompassing exons 12–14. For limb Gli3 Western blotting, left fore- and hindlimbs at E10.5 (32–37 somite stages) were harvested and bisected into anterior and posterior halves, whereas the right fore- and hindlimbs were harvested intact. The remaining tissues were
further removed until the neural tube was left, which was then used for Gli2 and Gli3 Western blotting. In addition, whole mutant and wild-type E10.5 embryos were lysed and processed for Gli3 Western blotting. The Western blotting procedures for Gli2 and Gli3 were carried out as described previously in [27]. Gli2 and Gli3 antibodies were gifts from Dr. Baolin Wang at Cornell University and Dr. Susan Mackem at NCI, respectively.

Quantitative Analysis of Vinculin-Containing Focal Adhesions

Focal adhesions were visualized using a vinculin antibody (Sigma V9264, 1:1,000). The area of vinculin immunostaining was traced using the image editor Gimp (www.gimp.org), and the area and intensity of staining were measured in 8-bit images. To quantify the length and roundness of vinculin-containing focal adhesion sites, we measured the major axis (MA) and minor axes (MI) of each focal adhesion. The mean MA length was determined and was plotted as a histogram with bin size of 10 pixels (equivalent to 0.57 μm). The roundness factor for each focal adhesion contact was measured using MI/MA^2*100, with 100 being focal adhesions that were perfectly round with major and minor axes of equal length.

Scanning EM and Videomicroscopy of Nodal Cilia and Pronephric Duct

Scanning EM of the nodal cilia and neural tube epithelium was carried out as described previously [27]. Analysis of nodal cilia motility in E8.0 mouse embryos and pronephric duct of zebrafish embryos were carried as described previously [32]. The videos were converted into Quicktime movies.

Time-Lapse Videomicroscopy for Assessing Cell Motile Behavior

Time-lapse videomicroscopy was carried out to quantify the motile behavior of wild-type and Wdpcp mutant MEFs using an inverted microscope (Leica, DMIRE2) and a Hamamatsu ORCA-ER camera. Video sequences encompassing an 8 hr recording period with images captured every 10 min were used to measure the number of cells having one or more filopodia/total number of cells (percentage of cells with filopodia), and we also measured the number of frames in which the same filopodia was observed (persistence of filopodia). To quantify membrane ruffling activity, we recorded cultured cells at a shorter time-lapse interval comprising 10 s for a total of 30 min. These time-lapse images were then used to measure the frequency of brightness change at the cells’ leading edge to quanitate the membrane ruffling activity. This entailed selecting boxed regions from the leading edge of a migrating cell, summing the brightness for each frame, and performing Fourier transform to obtain the frequency of brightness changes. The inverse of this frequency provided an index of the “period” of membrane fluctuation (MFP, in seconds), which is a measure of membrane dynamics, with greater membrane ruffling indicated by lower MFP.

Wound Scratch Assay for Assaying Polarized Cell Migration

Confluent MEF cultures were scratched using a 10 μl micropipette tip to generate a gap. Then time-lapse imaging was carried out using a 40× objective on an inverted microscope (Leica, DMIRE2) with images captured every 10 s over a 30 min interval using a Hamamatsu ORCA-ER camera. To examine cell polarity, cells were immunostained with a Golgi antibody (Sigma HPA021799, 1:1,000) followed by DAPI staining. Cells that are polarized and aligned with the direction of migration have Golgi situated in front of the nucleus (forward facing) and aligned with the migration direction. Polarity was scored by overlaying a clock face on each cell, and polarized cells are defined as those with Golgi situated within a 60° sector centered along the direction of wound closure [53,54].

Supporting Information

Figure S1 Ciliogenesis defect in WdpcpCys40 mutant. (A, B) Immunostaining of cilium with acetylated α-tubulin, a-tub (red), and γ-tubulin, (g-tub, green) antibodies showing a shorter cilium in WdpcpCys40 mutant MEF (B). Scanning EM images of embryonic nodes of control (C) and mutant (D) embryos at E8.0 showing the node cells are ciliated normally and cilia in mutant embryonic node are of normal shape and length. Scale bars, 2 μm in (A) and (C). Scales are the same in (A, B) and (C, D).

Figure S2 Wdpcp zebrafish in situ hybridization and morphant at 48 h, Western blotting with Wdpcp chicken antibody. (A–O) Embryonic wldpcp mRNA localization (purple) by whole mount in-situ hybridization with wldpcp antisense riboprobe at the four-cell stage (A, B), eight-cell stage (C, D), 1,000-cell stage (E, F), and shield stage (G) showed absence of maternal wldpcp transcripts. Embryonic wldpcp expression is observed at the 10-somite stage (H, I) and at 24 hpf (J, K). At 48 hpf (L–O) wldpcp staining appears less specific, since faint staining is observed with both the sense and antisense probes. (P) Immunoblot using wldpcp antibody (green) with 24 hpf zebrafish embryo lysate showed effective knockdown of wldpcp protein expression. α-Tubulin (red) was used as a sample loading control. Lane 1, protein molecular weight markers; lane 2, lysate from embryos injected with 10 ng control morpholino (MO); and lane 3, lysate from embryos injected with 10 ng wldpcp morpholino. (Q) The ratio of wldpcp (green) to α-tubulin (red) in the immunoblot was quantified using Image studio version 2.0 from LI-COR Biosciences (Lincoln, NE), which showed significant reduction in the wldpcp protein with wldpcp MO knockdown. Scale bars, 200 μm in (A), (G), (I), (J), and (L) and 150 μm in (M). Scales are the same in (A–F), (H, I), (J, K), (L, N), and (M, O).

Figure S3 Laterality defects in Wdpcp zebrafish morphants. (A–D) Ventral view of RNA in-situ hybridization staining with cmlc2 probe delineating the heart tube in 54 hpf embryos in wdp morphants revealed normal right-sided looping (B), no looping (C), or reversed heart looping (D) orientation. (E–H) Dorsal view of gut orientation as observed with LEAP in-situ hybridization analysis delineating liver position in 54 hpf embryo. Three types of gut orientation were observed: normal left-sided (F), duplicated (G), and right-sided (H). (I, J) Distribution of heart (I) and gut (J) looping orientation in Wdpcp morphants, with asterisk indicating statistically significant differences between control versus wdp morphants. (K–P) In-situ hybridization with an evx1 probe on 24 hpf embryos (K–M) delineated the normal cloaca in uninjected (K) and control MO (L) injected embryos, while in the wdp morphant (M), the cloaca is abnormally formed. Comparison of the corresponding brightfield images (N–P) suggests the cloaca in the wdp morphant may be obstructed. The arrowhead denotes the obstructed cloaca, which was seen in 37% of the wdp morphants (n = 208).

Figure S4 Rescue of wdp morpholino (MO)-induced phenotype. (A) Representative images of 48 hpf embryos...
injected at one-cell stage with 10 ng of scrambled control MO. (B) Representative images of 48 hpf embryos injected with 10 ng of *wdpcp* MO at one-cell stage showing pericardial edema (black arrows) and a curved tail. (C) Representative images of 48 hpf embryos injected at one-cell stage with 200 pg synthetic mouse *wdpcp* mRNA. (D) Representative images of 48 hpf embryos coinjected at one-cell stage with 10 ng of *wdpcp* MO and 200 pg synthetic mouse *wdpcp* mRNA showing rescue of morphant phenotype. (E, F) Morphant phenotypes (normal, mild, and severe) obtained in the experiments examining *wdpcp* mRNA rescue of *wdpcp*-MO-injected embryos are summarized in the graph shown in (F) and the table in (G).

**References**

1. Baraban JL, Minuma N, Beales PL, Katsanis N (2006) The ciliopathies: an emerging class of human genetic disorders. Annu Rev Genomics Hum Genet 7: 125-148.
2. Gaspar T, Larkins CE, Anderson KV (2007) The graded response to Sonic Hedgehog depends on cilia architecture. Dev Cell 12: 767-778.
3. Huangfu D, Anderson KV (2005) Cilia and Hedgehog responsiveness in the mouse. Proc Natl Acad Sci U S A 102: 11325-11330.
4. Huangfu D, Liu A, Rakeman AS, Murcia NS, Niswander L, et al. (2003) Gli3 localize to cilia and require the intraflagellar transport protein polaris for processing and function. PLoS Genet 1(4): e53. doi:10.1371/journal.pgen.0010053
5. Rohatgi R, Milenkovic L, Scott MP (2007) Patched1 regulates hedgehog signaling at the primary cilium. Science 317: 372–376.
6. Corbit KC, Aanstad P, Singhala V, Norman AR, Stainier DY, et al. (2005) Vangl2 and Gli3 localize to cilia and require the intraflagellar transport protein polaris for making FLAG–Cys40 mutant MEFs. J Cell Sci 118: 2915-2922.
7. Hildebrandt F, Attanasio M, Otto E (2009) Nephronophthisis: disease mechanisms of a cilio-pathy. J Am Soc Nephrol 20: 23–33.
8. Wallingford JB, Harland RM (2002) Neural tube closure requires Dishevelled-dependent convergent extension of the midline. Development 129: 5815–5825.
9. Wallingford JB, Harland RM (2002) Neural tube closure requires Dishevelled-dependent convergent extension of the midline. Development 129: 5815–5825.
10. Wallingford JB, Harland RM (2002) Neural tube closure requires Dishevelled-dependent convergent extension of the midline. Development 129: 5815–5825.
11. Wang J, Hamblet NS, Mark S, Dickinson ME, Brinkman B, et al. (2006) Dishevelled genes mediate a conserved mammalian PCP pathway to regulate convergent extension during neurulation. Development 133: 1767–1778.
12. Montcuquet M, Sans N, Hose D, Kach J, Dickman JD, et al. (2006) Asymmetric localization of Vangl2 and Fzd14 regulate cell polarity and planar cell polarity in mammals. J Neurosci 26: 5265–5275.
13. Wang J, Mark S, Zhang X, Qian D, Yao SJ, et al. (2005) Regulation of polarized extension and planar cell polarity in the cochlea by the vertebrate PCP pathway. Nat Genet 37: 980–985.
Wdpcp Regulates PCP via Cytoskeleton Modulation

14. Wang Y, Guo N, Nathans J (2006) The role of Frizzled3 and Frizzled6 in neural tube closure and in the planar polarity of inner-ear sensory hair cells. J Neurosci 26: 2147–2156.

15. Jones C, Chen P (2008) Primary cilia in planar cell polarity regulation of the inner ear. Curr Top Dev Biol 85: 197–224.

16. Jones C, Roper VC, Foucher I, Qian D, Banizs B, et al. (2008) Ciliary proteins link basal body polarization to planar cell polarity regulation. Nat Genet 40: 69–77.

17. Kiefer CW, Lu X (2011) Kif3a regulates planar polarization of auditory hair cells through both ciliary and non-ciliary mechanisms. Development 138: 3441–3449.

18. Wallingford JB, Mitchell B (2011) Strange as it may seem: the many links between Wnt signaling, planar cell polarity, and cilia. Gene Dev 25: 201–215.

19. Vlader EE, Antic D, Axelrod JD (2009) Planar cell polarity signaling: the developing cell’s compass. Cold Spr Harb Perspect Biol 1: a002964.

20. Saburi S, Hester I, Fischer E, Pontoglio M, Eremina V, et al. (2008) Loss of Fat4 disrupts PCP signaling and oriented cell division and leads to cystic kidney disease. Nat Genet 40: 1010–1015.

21. Ross AJ, May-Simera H, Eichers ER, Kai M, Hill J, et al. (2005) Disruption of planar cell polarity signaling results in congenital heart defects and cardiomyopathy attributable to early cardiomyocyte disorganization. Circ Res 96: 292–299.

22. Philips HM, Leibovich JC, Henderson DJ (2005) VangII acts via RhoA signaling to regulate polarized cell movements during development of the proximal outflow tract. Curr Res 96: 535–547.

23. Phillips HM, Rhee HJ, Murdoch JR, Hildreth V, Pearl JD, et al. (2007) Disruption of planar cell polarity signaling results in congenital heart defects and cardiomyopathy attributable to early cardiomyocyte disorganization. Curr Res 101: 137–145.

24. Simons M, Gloy J, Ganner A, Bullerkotte A, Bashkurov M, et al. (2005) Inversin, a novel membrane protein of the teneurin family, is required for planar cell polarity in vertebrates. Nat Genet 37: 1135–1140.

25. Collier S, Lee H, Burgess R, Adler P (2005) The WD40 repeat protein fritz links ciliary mechanisms. Nat Cell Biol 10: 70–76.

26. Kim SK, Shindo A, Park TJ, Oh EC, Ghosh S, et al. (2010) Planar cell polarity in vertebrates. Nat Genet 37: 303–311.

27. Corbit KC, Shyer AE, Dowdle WE, Gaulden J, Singla V, et al. (2008) Kif3a constrains beta-catenin-dependent Wnt signaling through dual ciliary and non-ciliary mechanisms. Nat Cell Biol 10: 70–76.

28. Simon M, Gloy J, Ganner A, Bashkurov M, et al. (2006) Inversin acts via RhoA signaling to regulate polarized cell movements during development of the proximal outflow tract. Curr Res 96: 292–299.

29. Simons M, Gloy J, Ganner A, Bullerkotte A, Bashkurov M, et al. (2005) Inversin, a novel membrane protein of the teneurin family, is required for planar cell polarity in vertebrates. Nat Genet 37: 303–311.

30. Schermer B, Hopker K, Omran H, Ghenoiu C, Fliegauf M, et al. (2005) WD40 repeat protein fritz links cytoskeletal planar polarity to frizzled subcellular localization in the Drosophila epidermis. Genetics 169: 2035–2045.

31. Wallingford JB, Mitchell B (2011) Strange as it may seem: the many links between Wnt signaling, planar cell polarity, and cilia. Gene Dev 25: 201–215.

32. Schermer B, Hopker K, Omran H, Ghenoiu C, Fliegauf M, et al. (2005) WD40 repeat protein fritz links cytoskeletal planar polarity to frizzled subcellular localization in the Drosophila epidermis. Genetics 169: 2035–2045.

33. Castori M, Valente EM, Donati MA, Salvi S, Fazzi E, et al. (2003) NPHP1 gene deletion is a rare cause of Joubert syndrome related disorders. J Med Genet 42: e9.

34. Schermer B, Hopker K, Omran H, Ghenoiu C, Fliegauf M, et al. (2005) WD40 repeat protein fritz links cytoskeletal planar polarity to frizzled subcellular localization in the Drosophila epidermis. Genetics 169: 2035–2045.

35. Schermer B, Hopker K, Omran H, Ghenoiu C, Fliegauf M, et al. (2005) WD40 repeat protein fritz links cytoskeletal planar polarity to frizzled subcellular localization in the Drosophila epidermis. Genetics 169: 2035–2045.

36. Wallingford JB, Mitchell B (2011) Strange as it may seem: the many links between Wnt signaling, planar cell polarity, and cilia. Gene Dev 25: 201–215.

37. Schermer B, Hopker K, Omran H, Ghenoiu C, Fliegauf M, et al. (2005) WD40 repeat protein fritz links cytoskeletal planar polarity to frizzled subcellular localization in the Drosophila epidermis. Genetics 169: 2035–2045.

38. Schermer B, Hopker K, Omran H, Ghenoiu C, Fliegauf M, et al. (2005) WD40 repeat protein fritz links cytoskeletal planar polarity to frizzled subcellular localization in the Drosophila epidermis. Genetics 169: 2035–2045.

39. Schermer B, Hopker K, Omran H, Ghenoiu C, Fliegauf M, et al. (2005) WD40 repeat protein fritz links cytoskeletal planar polarity to frizzled subcellular localization in the Drosophila epidermis. Genetics 169: 2035–2045.

40. Schermer B, Hopker K, Omran H, Ghenoiu C, Fliegauf M, et al. (2005) WD40 repeat protein fritz links cytoskeletal planar polarity to frizzled subcellular localization in the Drosophila epidermis. Genetics 169: 2035–2045.

41. Schermer B, Hopker K, Omran H, Ghenoiu C, Fliegauf M, et al. (2005) WD40 repeat protein fritz links cytoskeletal planar polarity to frizzled subcellular localization in the Drosophila epidermis. Genetics 169: 2035–2045.

42. Schermer B, Hopker K, Omran H, Ghenoiu C, Fliegauf M, et al. (2005) WD40 repeat protein fritz links cytoskeletal planar polarity to frizzled subcellular localization in the Drosophila epidermis. Genetics 169: 2035–2045.

43. Schermer B, Hopker K, Omran H, Ghenoiu C, Fliegauf M, et al. (2005) WD40 repeat protein fritz links cytoskeletal planar polarity to frizzled subcellular localization in the Drosophila epidermis. Genetics 169: 2035–2045.

44. Schermer B, Hopker K, Omran H, Ghenoiu C, Fliegauf M, et al. (2005) WD40 repeat protein fritz links cytoskeletal planar polarity to frizzled subcellular localization in the Drosophila epidermis. Genetics 169: 2035–2045.

45. Schermer B, Hopker K, Omran H, Ghenoiu C, Fliegauf M, et al. (2005) WD40 repeat protein fritz links cytoskeletal planar polarity to frizzled subcellular localization in the Drosophila epidermis. Genetics 169: 2035–2045.

46. Schermer B, Hopker K, Omran H, Ghenoiu C, Fliegauf M, et al. (2005) WD40 repeat protein fritz links cytoskeletal planar polarity to frizzled subcellular localization in the Drosophila epidermis. Genetics 169: 2035–2045.