Control of the Wnt pathways by nephrocystin-4 is required for morphogenesis of the zebrafish pronephros

Céline Burcklé1,2,3,4,†, Helori-Mael Gaudé1,3,†, Christine Vesque2,4, Flora Silbermann1,3, Rémi Salomon1,3,5, Cécile Jeanpierre1,3, Corinne Antignac1,3,6, Sophie Saunier1,3,*† and Sylvie Schneider-Maunoury2,4,*‡

1INSERM, U983, Tour Lavoisier, Hôpital Necker-Enfants Malades, 149, rue de Sévres, 75015 Paris, France, 2CNRS UMR 7622, INSERM U969, 9, Quai St Bernard, Université Pierre et Marie Curie Bâtiment C, 75005 Paris, France, 3Université Paris-Descartes, Paris, France, 4Université Pierre et Marie Curie, Paris, France, 5AP-HP, Département de Néphrologie Pédiatrique, Hôpital Necker-Enfants Malades, Paris, France and 6AP-HP, Département de Génétique, Hôpital Necker-Enfants Malades, Paris, France

Received February 1, 2011; Revised and Accepted April 12, 2011

Nephronophthisis is a hereditary nephropathy characterized by interstitial fibrosis and cyst formation. It is caused by mutations in NPHP genes encoding the ciliary proteins, nephrocystins. In this paper, we investigate the function of nephrocystin-4, the product of the nphp4 gene, in vivo by morpholino-mediated knockdown in zebrafish and in vitro in mammalian kidney cells. Depletion of nephrocystin-4 results in convergence and extension defects, impaired laterality, retinal anomalies and pronephric cysts associated with alterations in early cloacal morphogenesis. These defects are accompanied by abnormal ciliogenesis in the cloaca and in the laterality organ. We show that nephrocystin-4 is required for the elongation of the caudal pronephric primordium and for the regulation of cell rearrangements during cloaca morphogenesis. Moreover, depletion of either inversin, the product of the nphp2 gene, or of the Wnt-planar cell polarity (PCP) pathway component prickle2 increases the proportion of cyst formation in nphp4-depleted embryos. Nephrocystin-4 represses the Wnt-β-catenin pathway in the zebrafish cloaca and in mammalian kidney cells in culture. In these cells, nephrocystin-4 interacts with inversin and dishevelled, and regulates dishevelled stability and subcellular localization. Our data point to a function of nephrocystin-4 in a tight regulation of the Wnt-β-catenin and Wnt-PCP pathways, in particular during morphogenesis of the zebrafish pronephros. Moreover, they highlight common signalling functions for inversin and nephrocystin-4, suggesting that these two nephrocystins are involved in common physiopathological mechanisms.

INTRODUCTION

Nephronophthisis (NPH) is an autosomal recessive chronic interstitial nephropathy characterized by renal fibrosis and cyst formation and is the most common genetic cause of chronic renal failure in children and young adults. Three distinct forms of NPH have been described (juvenile, infantile and adolescent) according to the age of onset of end-stage renal disease (ESRD). Renal histology is characterized by tubular atrophy with thickened tubular basement membranes, diffuse interstitial fibrosis and cyst formation at the corticomedullary junction. NPH is a genetically heterogeneous disease and causal mutations in 13 genes (NPH1–NPH11, TTC21B, NPHP1L) have been identified to date (1,2). Most of these genes encode proteins of ubiquitous expression sharing a common, but not exclusive, location at

†To whom correspondence should be addressed. Tel: +33 144272154; Fax: +33 144273445; Email: sylvie.schneider-maunoury@snv.jussieu.fr (S.S.-M.); Tel: +33 144490099; Fax: +33 144490290; Email: sophie.saunier@inserm.fr (S.S.)
‡These authors jointly directed the project.
#The Author 2011. Published by Oxford University Press. All rights reserved.
For Permissions, please email: journals.permissions@oup.com
the base of the primary cilium. NPH belongs to a group of multifaceted syndromes associated with structural and/or functional abnormalities of cilia/centrosome, called ciliopathies, which display heterogeneous and partially overlapping phenotypes characterized by developmental and/or degenerative defects in multiple organ systems, namely kidney cysts, retinitis pigmentosa, cerebellar vermis hypoplasia or liver fibrosis (3,4). The critical importance of cilia in human cystic kidney disorders has been emphasized by the observation that virtually all proteins associated with cystic kidney diseases, including autosomal-dominant polycystic kidney disease, are localized to the cilia (4).

Cilia are microtubule-based organelles projecting out from the cell surface of most eukaryotic cells. Ciliogenesis and cilium function rely on intraflagellar transport (IFT), a polarized, bidirectional transport system involving IFT protein complexes and molecular motors tracking along the axoneme (5). In addition to the IFT machinery, several proteins encoded by ciliopathy genes, such as the BBS, MKS and NPH proteins, form complexes at the cilium base that regulate cilium assembly and function (6–8). We have previously identified the NPHP4 gene, whose mutations cause isolated juvenile NPH or NPH in association with retinitis pigmentosa (Senior-Løken syndrome) or ocular motor apraxia (Cogan syndrome) (9,10). We have shown that the product of this gene, nephrocystin-4, is enriched at the cilium base and at the cell junctions (11,12). Nephrocystin-4 interacts directly with cytoskeletal components and distinct protein complexes, such as other nephrocystins (NPHP1, RPGRIP1L), regulators of cell–cell/matrix adhesion and actin cytoskeleton (p130Cas and Pyk2), and the polarity complex proteins PALS1 and PATJ (10–12). Loss-of-function experiments performed in kidney-derived cell lines and in Caenorhabditis elegans have shown that nephrocystin-4 is necessary for correct ciliogenesis (8,11). Nephrocystin-4 is also required in vitro for tight junction formation and epithelial morphogenesis (11). Moreover, truncating mutations of nphp4 both in mice and dog lead to an early-onset retinal dystrophy without associated renal lesions (13,14).

Consistent with the frequent occurrence of kidney dysfunction in ciliopathies, several proteins involved in cilium assembly and/or function have an essential role in vertebrate kidney morphogenesis (15–18). In mouse models of ciliary kidney diseases, several cellular mechanisms have been put forward for cyst formation, such as increased proliferation, randomized cell division orientation or defects in convergence and extension (CE) in the elongating nephric tubules [for review (19)]. Loss-of-function of IFT or other ciliary genes in zebrafish embryos have been shown to result in pronephric cysts (20–25). The embryonic zebrafish pronephros is a rudimentary kidney formed by a vascularized glomerulus and two nephrons fused at the cloaca. The pronephric epithelium contains motile cilia and defects in cilium motility or beating coordination alter cilia-driven fluid flow and result in kidney cysts (20,26–28). Thus, the zebrafish pronephros provides a novel, simple and accessible model to study the role of ciliopathy genes in kidney morphogenesis.

Several lines of evidence suggest that the ciliary basal body could be a platform involved in regulating the balance between the Wnt-β-catenin (canonical) and Wnt-planar cell polarity (PCP) pathways and perturbations of the tight regulation of the Wnt canonical and PCP pathways have been proposed to cause kidney cysts (29–31). The Wnt-PCP pathway regulates nephric tubule morphogenesis in mice (29,30), and loss-of-function of various Wnt-PCP components in zebrafish embryos leads to pronephric cyst formation (32,33). Analysis of the mechanisms of Wnt pathway regulation has involved inversin, the product of the NPHP2 gene mutated in infantile NPH. Simons et al. (34) have shown that inversin represses the Wnt-β-catenin pathway in cell culture by targeting cytoplasmic dishevelled for degradation by the proteasome and that, during gastrulation in Xenopus laevis, inversin is required for CE movements, a process controlled by the Wnt-PCP pathway. In addition, the depletion of the other ciliary proteins, such as IFT88 and BBS1/4/6, in cultured cells results in an upregulation of the Wnt-β-catenin pathway and additional evidence for an interaction with the Wnt-PCP pathway comes from the functional analysis of ciliopathy genes in zebrafish (35).

In this paper, we took advantage of the zebrafish model and of cultured mammalian kidney cells to study the function of nephrocystin-4. Zebrafish embryos depleted in nephrocystin-4 display CE defects, impaired laterality, retinal defects and nephric cysts associated with cloacal abnormalities. We show that nephrocystin-4 is required for the elongation of the caudal pronephric primordium and for the regulation of cell rearrangements during cloaca morphogenesis. Nephrocystin-4 interacts with inversin and dishevelled and regulates subcellular amounts of dishevelled. Moreover, it represses the Wnt-β-catenin pathway and synergizes with inversin and the Wnt-PCP pathway for CE and pronephros formation. Our data point to a function of nephrocystin-4 in a tight regulation of the Wnt-β-catenin and Wnt-PCP pathways, particularly during morphogenesis of the zebrafish pronephros.

RESULTS

Nphp4 morphants develop a ciliary phenotype

In order to better understand the function of nephrocystins in kidney morphogenesis and their role in modulating Wnt signalling, we investigated nephrocystin-4 function during zebrafish embryogenesis. A single orthologue of the human NPHP4 gene was identified by gene database search in the zebrafish genome. The zebrafish nphp4 gene (Ensembl gene ENSDARG00000069014), located on chromosome 8, contains 29 exons and encodes a 1416 amino acid protein with 46% identity and 62% similarity with human nephrocystin-4. Nphp4 is expressed both maternally and zygotically at least until 48 h post-fertilization (hpf) (Supplementary Material, Fig. S1A). In situ hybridization analysis revealed a weak ubiquitous expression (data not shown). We then performed nphp4 loss-of-function experiments using antisense morpholinos (Mo). Two morpholinos were designed, targeting the ATG (N4ATG-Mo) and the splice acceptor site of exon 6 (N4sp-Mo). The N4sp-Mo efficiently suppressed the correct splicing of exon 6, as shown by reverse transcriptase–polymerase chain reaction (RT–PCR) and sequencing (Supplementary Material, Fig. S1B, C and E). Morphological examination of nphp4 morphants showed shortened body axis
at early somite stages, randomized heart looping, pronephric cysts and abnormal body curvature (Fig. 1A–G and data not shown).

*Nphp4* morphants usually died between 2.5 and 3 days post-fertilization (dpf). Both morpholinos gave a similar, dose-dependent phenotype, slightly more severe with N4ATG-Mo than with N4sp-Mo (Fig. 1D and Supplementary Material, Fig. S1D). The specificity of the knockdown was confirmed by the co-injection of human *NPHP4* mRNA with the N4ATG-Mo that led to a partial rescue of the phenotype (Fig. 1E and L).

A shortened and widened body axis is a strong indication of CE defects during gastrulation (36). At the 10–15 somite (s) stage (14–16.5 hpf), *nphp4* morphants displayed a characteristic CE phenotype encompassing a wide range of defects, from discrete notochord abnormalities (class I) to shortened body axis with somite compression and broader, misshapen notochord (classes II and III) (Fig. 1A). In the most severely affected embryos (class III), clusters of cells accumulated on the dorsal side of an extremely shortened trunk. At 2–3 dpf, surviving embryos were shorter and had a curved body (Fig. 1B and C). High doses of morpholino induced a CE phenotype in more than 50% of the embryos (*n* > 140 for each of the two morpholinos; Fig. 1D and Supplementary Material, Fig. S1D). The proportion of class III embryos was greater with the morpholino targeting the ATG. CE phenotype was partially rescued after co-injection of the *NPHP4* mRNA with the N4ATG-Mo (Fig. 1E), with a decrease in both the proportion and the severity of the affected embryos.

Abnormal heart looping [reversed (left) or no looping] was found in 45% (*n* = 147) of N4ATG-morphants and 23% (*n* = 144) of N4sp-morphants at 2.5 dpf, indicating defective left–right asymmetry. This correlated with randomized expression of the early laterality markers *spaw* (Supplementary Material, Fig. S2A and B) and *pitx2* (data not shown) in the lateral plate mesoderm of N4ATG and N4sp-morphants at the 20 s stage (19 hpf). Integrity of midline structures was preserved in these morphants, as shown by continuous expression of *notail* (ntl) in the notochord and of *sonic hedgehog* (shh) in the floor plate (data not shown).

Pronephric cysts, mostly bilateral, were conspicuous at 2.5 dpf in 35–65% of N4ATG-morphants (*n* = 500) and 22% of N4sp-morphants (*n* = 11; Fig. 1F–K). The proportion

**Figure 1.** Defects in axis elongation and pronephric morphogenesis in zebrafish *nphp4* morphants. (A) *nphp4* morphant embryos representative of the four CE phenotypic classes: unaffected (indistinguishable from controls), class I (mild), class II (severe) and class III (very severe). (B, C) 60 hpf control (B) and morphant (C) embryos showing the abnormal curvature of the trunk in *nphp4* morphants. (D) Number of embryos falling in each of the four phenotypic classes shown in (A) in different experimental conditions. LD, low dose of morpholino (1 nl of 0.2 mM Mo solution, corresponding to 0.2 pmole/embryo); HD, high dose of morpholino (1 nl of 0.75 mM Mo solution, corresponding to 0.75 pmole/embryo). (E) Rescue of CE defects by coinjection of human *NPHP4* mRNA. N4ATG-Mo was injected at a high dose (N4ATG HD) together with *Histone 2B* (H2B) mRNA used as a negative control or with *NPHP4* mRNA (90 pg). The different phenotypic classes are as indicated in (D). (F, G) Dorsal view of 3 dpf control (F) and morphant (G) embryos. In the morphant, the dilatation of the right pronephric tubule (arrowheads) and a cyst (arrow) are visible. (H–K) Transverse sections through the trunk of 3 dpf control (H, J) and morphant (I, K) embryos at the level of the glomerulus (H, I) or of the pronephric duct (J, K) showing dilatation of both structures in the morphant. gl, glomerulus; pct, proximal convoluted tubule; pd, pronephric duct. (L) Rescue of pronephric cysts by coinjection of human *NPHP4* mRNA. Morpholino and RNA concentrations are as indicated in (E).
Morpholino mRNA Pronephric cysts

decreased by co-injection of

NPHP4 of pronephric cysts in N4ATG-morphants was drastically

correlation between pronephric cysts at 2.5 dpf and cloacal abnorm-

alities at 24 hpf after injection of N4ATG-Mo into the Tg(cldnb:gfp) line

<table>
<thead>
<tr>
<th>Morpholino mRNA</th>
<th>Pronephric cysts at 2.5 dpf (%)</th>
<th>Cloaca defects at 24 hpf</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2 mM N4ATG-Mo</td>
<td>– 23 (n = 22)</td>
<td>13% mild; 87% non-affected (n = 15)</td>
</tr>
<tr>
<td>0.4 mM N4ATG-Mo</td>
<td>– 75 (n = 16)</td>
<td>20% severe; 40% mild; 40% non-affected (n = 10)</td>
</tr>
<tr>
<td>≥0.6 mM N4ATG-Mo</td>
<td>– 100 (n = 15)</td>
<td>100% severe (n = 4)</td>
</tr>
<tr>
<td>0.4 mM N4ATG-Mo</td>
<td>H2B 70 (n = 13)</td>
<td>45% severe; 22% mild; 33% non-affected (n = 9)</td>
</tr>
<tr>
<td>0.4 mM N4ATG-Mo</td>
<td>NPHP4 18 (n = 33)</td>
<td>6% severe; 47% mild; 47% non-affected (n = 15)</td>
</tr>
<tr>
<td>0.3 mM N4ATG-Mo</td>
<td>– 17 (n = 24)</td>
<td>8% severe; 8% mild; 84% non-affected (n = 12)</td>
</tr>
<tr>
<td>0.1 mM Pk2-Mo</td>
<td>– 12 (n = 8)</td>
<td>100% non-affected (n = 11)</td>
</tr>
<tr>
<td>0.3 mM N4ATG-Mo + 0.1 mM Pk2-Mo</td>
<td>– 88 (n = 8)</td>
<td>11% severe; 22% mild; 67% non-affected (n = 9)</td>
</tr>
<tr>
<td>0.3 mM inv-Mo</td>
<td>– 12 (n = 5)</td>
<td>20% mild; 80% non-affected (n = 5)</td>
</tr>
<tr>
<td>0.3 mM N4ATG-Mo + 0.3 mM inv-Mo</td>
<td>– 100 (n = 14)</td>
<td>54% mild; 46% non-affected (n = 11)</td>
</tr>
</tbody>
</table>

Three independent experiments are presented in this table. In the first experiment (lines 1–3), increasing doses of N4ATG-Mo lead to progressively more severe cloacal defects and to a progressively higher number of morphants with cysts. In the second experiment (lines 4 and 5), coinjection of human NPHP4 mRNA rescues both cyst formation and cloacal defects in nphp4 morphants (H2B mRNA is used as a negative control). In the third experiment (lines 6–10), N4ATG-Mo was coinjected with of Pk2-Mo or inversin-Mo. Coinjection strongly enhances cyst formation. In contrast, coinjection of Pk2-Mo does not significantly enhance cloaca anomalies, whereas coinjection of inversin-Mo notably enhances mild but not severe cloacal defects. ‘n’ indicates the total number of embryos examined at each stage and for each experimental condition. Note that for a similar dose of N4ATG-Mo, the pronephric/cloacal phenotype was more severe in the Tg(cldhb:gfp) transgenic line than in the wild-type zebrafish lines.

Nephrocystin-4 is required for ciliogenesis in specific embryonic epithelia

Given the phenotype of nphp4 morphants, we investigated whether this gene was required for ciliogenesis in zebrafish embryos. For that purpose, we first examined the presence of cilia and their length on epithelia of the Kupffer’s vesicle (KV) and pronephros. The KV is a transient epithelial structure made of mononucleated cells, required for symmetry breaking in zebrafish. In N4ATG-morphants, KV formation was unaffected (Fig. 2A–D). A KV was present in all morphants and its diameter (61 ± 8 μm, n = 34) was similar to that of control embryos (61 ± 10 μm, n = 20) at the 11 s stage (14.5 hpf). However, the number of cilia was significantly reduced in the KV of N4ATG-morphants (41 ± 6 per KV, n = 19) when compared with controls (52.7 ± 5.4 per KV, n = 23) at the 8–11 s stage (13–14.5 hpf) (P < 0.001). Cilium length was also significantly reduced in the morphants (Fig. 2A–E), while cilium ultrastructure appeared unaffected (data not shown). Thus, this decrease in length and number of the KV cilia in nphp4 morphants will likely impair the KV flow and may explain the left–right asymmetry defects observed in these morphants.

The zebrafish embryonic pronephros contains two linear nephrons. Each nephron is subdivided into several proximal and distal tubule domains and ends with the pronephric duct. In the most distal part of the nephron, the pronephric ducts fuse and connect to the cloaca (38). The pronephric epithelium is made of mono- and multiciliated cells arranged in a mosaic pattern, with the exception of the pronephric duct lined by monociliated cells only (20). In N4ATG morphants, cilium length in multiciliated cells appeared unaffected at 1 dpf (Fig. 2F and G) and at 2.5 dpf (Fig. 2H and I). Moreover, cilium ultrastructure in multiciliated cells was preserved in the cystic epithelium of N4ATG morphants at 2.5 dpf (Fig. 2L and M). In contrast, monocilia of the pronephric duct and cloaca were significantly shorter in N4ATG morphants at 1 dpf (2.41 ± 1 versus 5.46 ± 1.7 μm in controls) (Fig. 2J, K and N). Cilia were sparse or absent in the cloaca region in 50% of the 2.5 dpf N4ATG-morphants with kidney cysts (n = 8) (data not shown). We further analysed cilia motility in the pronephros using high-speed mono-line confocal microscopy. We found that cilium beat frequency in multiciliated cells was similar in controls (64 ± 5.1 Hz, n = 3) and N4ATG morphants (70 ± 7.2 Hz, n = 3). In contrast, we observed slow-beating cilia in the cloaca of N4ATG morphants (41 ± 13 Hz, n = 7) when compared with controls (60.4 ± 7.3, P = 0.0052, n = 11) (Fig. 2O). Thus, in the pronephros of nphp4 morphants, cilia of multiciliated cells appear where photoreceptor cell bodies are located. Occasional lamination defects were also detected (Supplementary Material, Fig. S2E and F). The formation of the outer segment could not be examined because of early death of the nphp4 morphants.

In rare cases, severe lamination defects and a high number of pyknotic cells were observed, similar to what was described for RPGR morphants (37) (data not shown).

In conclusion, the phenotype of nphp4 morphants combines several features characteristic of a cilia dysfunction: laterality defects, impaired CE, retinal anomalies and kidney cysts.
normal in length, ultrastructure and motility, while monocilia of the most caudal region (cloaca and pronephric duct) are shorter and display reduced motility.

From these data, we conclude that nephrocystin-4 is required for cilium biogenesis in a subset of monociliated epithelia in zebrafish embryos: the KV and the distal part of the pronephros. Cilia of other epithelia such as the otic vesicle, the floor plate and the olfactory placode were of normal size in \textit{nphp4} morphants (data not shown).

Pronephric cysts in \textit{nphp4} morphants are associated with defective cloacal morphogenesis

Further examination of embryos injected with high doses of N4ATG-Mo and presenting with pronephric cysts revealed obvious cloaca morphology defects at 2.5 dpf in most cases (93\%, \(n = 100\)). Cystic dilatation of the cloaca was observed in the most severely affected embryos (Fig. 3A–C). In contrast, cloacal defects were never found in \textit{nphp4}-morphants without cysts. Mechanical obstruction of the cloaca in zebrafish larvae has been reported to lead to cyst formation in anterior pronephric segments (20). In order to test whether the cloacal defects led to urinary obstruction, we monitored fluorescent dextran excretion at the cloaca of 2.5 dpf control and N4ATG-morphants (injected with a high morpholino dose). In 100\% of the control embryos (\(n = 22\)), dye excretion was observed less than 5 min after injection. In contrast, in most (17/18) of the N4ATG morpholino-injected embryos with pronephric cysts, dye excretion was not detected (Fig. 3D and E). Thus, there is a tight association between morphologically abnormal cloaca at 2.5 dpf, cyst formation and pronephros obstruction in \textit{nphp4} morphants.

We further investigated the origin of cloacal abnormalities in \textit{nphp4} morphants, focussing on early morphogenetic processes. The bilateral zebrafish pronephric nephrons epithelialize \textit{in situ} between the 12 s (15 hpf) and 24 hpf stages, from lateral primordia that join caudally in the future pronephric duct region. Formation of the zebrafish cloaca involves the fused pronephric ducts and ventral epidermis-derived proctodeum (39,40). Proctodeal cells form the distal portion of the cloaca, which opens to the exterior at around 21–24 hpf (39). In \textit{nphp4} morphants, the pronephric duct and proctodeum...
were correctly specified and were in contact with each other in the cloaca region (Supplementary Material, Fig. S3). We examined pronephros morphogenesis at 24 hpf in the Tg(cldnb:gfp) transgenic line, taking advantage of the green fluorescent protein (GFP) staining at the membrane of the pronephric cells and, at a lower level, of the proctodeal cells. Three-dimensional reconstruction of the pronephros showed a thickening of the pronephric duct in nphp4 morphants when compared with controls (Fig. 3F and G). Optical sections through the morphant pronephric ducts and cloaca revealed a striking increase in the number of cells per transverse section when compared with controls (Fig. 3H–K). In control embryo sections, we observed fusion of the two pronephric ducts and connexion of the fused kidney terminus to proctodeal cells to form the cloaca (Fig. 3H). A more ventral epithelial tubular structure presenting low levels of GFP expression, presumably the distal gut tube, also connected the cloaca at this stage (Fig. 3H). In nphp4 morphants, the two pronephric ducts and the gut fused but the cloaca was abnormal, had an enlarged lumen and, in most cases, did not open to the exterior (Fig. 3I and J). In addition, columnar organization of cloacal cells was lost. These cells had an altered shape, with an

![Figure 3](image-url)
increased height (Fig. 3I and J), although they did not present any obvious defect in apico-basal polarity as laminin, F-actin and ZO1 staining were preserved (data not shown).

In order to better analyse the association of pronephric cyst with early cloaca morphological defects, we injected various doses of N4ATG-Mo into the Tg(cldnb:gfp) transgenic line. We found a good correlation between the proportion of pronephric cysts at 2.5 dpf and cloacal abnormalities at 24 hpf (Table 1). High morpholino doses induced a severe phenotype, as described above. Low doses resulted in mild cloacal defects, characterized by higher cells along the apico-basal axis, a moderate increase in the number of cells per transverse section of the pronephric duct and cloaca, a normal columnar epithelial organization and a conserved aperture to the exterior (data not shown). Moreover, co-injection of NPHP4 mRNA with high doses of N4ATG-Mo led to a drastic decrease in the severity of cloaca defect (45 to 6% of nphp4 morphants with severe cloacal anomalies) concomitant with a reduction in the proportion of nphp4 morphants with pronephric cysts (70 to 18%) (Table 1).

The increased cell number in sections of the pronephric duct and cloaca could result from a local increase in proliferation, a reduction in apoptosis levels and/or a reduced elongation of the pronephric duct. We examined proliferation in the pronephros between 17 and 24 hpf using the mitosis marker phosphohistone-3, and apoptosis using the apoptosis marker caspase 3. The number of mitotic and apoptotic cells was very low during this period in the pronephros of both control and morphant embryos (Table 2). We did not detect any increase in the number of mitotic cells in the caudal third of the pronephros in morphant embryos when compared with controls (data not shown).

In order to investigate the role of pronephros elongation defects, we analysed the kinetics of pronephros extension along the AP axis. In control embryos, the pronephric primordium elongated with the body axis before the 12 s stage (15 hpf) (data not shown), while its length along the AP axis remained unchanged between 12 s and 24 hpf (time window of pronephric epithelialization) (Table 3). In nphp4 morphants, the pronephric primordium was already shorter and thinner than in the control embryos at 10 s stage as assessed by pax2 staining (Fig. 3L and M). Therefore, a reduction in the morphogenetic movements affecting early elongation of the pronephric duct primordium likely contributes to the thickening of the pronephric ducts in nphp4 morphants. Together, these results show that nephrocystin-4 controls both early pronephric duct elongation and cell rearrangements required for cloacal morphogenesis.

### Table 2. Number of mitotic and apoptotic cells

<table>
<thead>
<tr>
<th>Stage</th>
<th>Mitotic Cells</th>
<th>Apoptotic Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>17 hpf</td>
<td>4.33 ± 2.4</td>
<td>3 ± 1.5</td>
</tr>
<tr>
<td>21 hpf</td>
<td>3 ± 1.5</td>
<td>2 ± 1.2</td>
</tr>
<tr>
<td>24 hpf</td>
<td>0.4 ± 0.64</td>
<td>0.25 ± 0.37</td>
</tr>
</tbody>
</table>

### Table 3. Pronephros length (µm)

<table>
<thead>
<tr>
<th>Stage</th>
<th>Control</th>
<th>N4-Mo injected</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 hpf</td>
<td>791.5 ± 23.2</td>
<td>619.1 ± 20.2</td>
</tr>
<tr>
<td>30 hpf</td>
<td>773.3 ± 19.7</td>
<td>636.4 ± 46</td>
</tr>
</tbody>
</table>

### Nephrocystin-4 interacts with the Wnt pathways

Since CE movements are regulated by the Wnt-PCP pathway, we examined the functional interaction between nphp4 and several Wnt-PCP genes. We first performed depletion of nphp4 in zebrafish embryos with mutations in the core PCP gene vangl2 (trilobite, tri) which manifest severe CE defects during gastrulation at the homozygous state (41) (Fig. 4A). We confirmed that embryos issued from tri<sup>v<sup>−<sup>/− × wt</sup> outcrosses had no severe (class III) CE phenotype (n = 80 embryos), and embryos issued from tri<sup>v<sup>−<sup>/− × tri<sup>v<sup>−<sup>/−</sup> intercrosses displayed 30% class III phenotype. Injection of a low dose of N4ATG-Mo caused only mild CE defects in <30% of wild-type embryos. However, injection of the same dose of morpholino in embryos derived from a tri<sup>v<sup>−<sup>/− × wt</sup> outcrosses had no severe (class III) CE phenotype (n = 82) and >80% in tri<sup>v<sup>−<sup>/− × tri<sup>v<sup>−<sup>/−</sup> intercrosses (n = 29). These data indicate a genetic interaction between nphp4 and vangl2 genes for the control of axis elongation during gastrulation. We then confirm the interaction of nphp4 with the Wnt-PCP performing combined loss-of-function experiments using morpholinos targeting nphp4 and either the vertebrate PCP genes vangl2 or prickle1 (pk1). In both types of experiments, combined injections resulted in a dramatic increase in the proportion of severely affected embryos (Fig. 4B). To test whether CE defects in nphp4 morphants are caused by the downregulation of the Wnt-PCP pathway, we performed rescue experiments with ΔNDvl, a mutant form of Dishevelled that specifically activates the Wnt-PCP pathway without interfering with the canonical Wnt pathway (42). Consistent with this hypothesis, we found that coinjection of ΔNDvl with N4ATG-Mo partially rescues the CE phenotype: at 10–12 s (13–15 hpf), the proportion of unaffected and class I embryos was significantly higher in morphants coinjected with ΔNDvl and N4ATG-Mo (33%, n = 36) than in embryos injected with N4ATG-Mo only (0%, n = 32) (Fig. 4C). We conclude from these experiments that nphp4 controls axis elongation by positively regulating the Wnt-PCP pathway.

We next performed combined knockdown of nphp4 and of the vertebrate PCP genes scribble1 (scri) and prickle2 (pk2) using low doses of morpholinos to examine whether these genetic interactions also influence pronephric cyst formation. The ratio of morphant embryos with cysts was increased in nphp4, scrib double morphants (24%), as well as in nphp4, pk2 double morphants (74%) compared with simple nphp4 (8–16%), scrib (4%) or pk2 (0%) morphants (Fig. 4D). Further analysis in the Tg(cldnb:gfp) transgenic fish line confirmed a large increase of pronephric cyst in the nphp4, pk2 double morphants, without significant enhancement of cloaca morphological defects at 24 hpf (Fig. 4E and F and Table 1).
This indicates that PCP defects can influence cyst formation upon *nphp4* depletion independently of cloacal morphogenesis. Finally, we tested the effect of *nphp4* knockdown on the activity of the Wnt-β-catenin pathway using the *Tg(cldnB:gfp*) transgenic line (43) in the pronephros. We observed an enhanced reporter expression in N4ATG-morphants in the cloaca region of 24 hpf embryos (Fig. 4G–I), showing that nephrocystin-4 acts as a repressor of the canonical Wnt pathway in the developing zebrafish cloaca.

### Nephrocystin-4 controls dishevelled stability and subcellular localization

We further explored the regulatory mechanisms of nephrocystin-4 on β-catenin-dependent Wnt signalling in mammalian cells. Dishevelled is a critical cytoplasmic regulator that prevents β-catenin degradation. Using the β-catenin-responsive TCF/LEF-1-luciferase reporter (TOPflash) in HEK293T human kidney cells, we demonstrated that nephrocystin-4 overexpression blocked Dvl2-mediated β-catenin activation, leading to a 2.5-fold decrease in luciferase activity in cells expressing a V5-tagged NPHP4 (NPHP4-V5) construct (Fig. 5A, left panel). This repression was greater than that caused by overexpression of flag-tagged inversin (inv-Flag) (Fig. 5A, left panel). Dvl2-mediated activation of TCF-dependent transcription was also reduced in HEK293T cell lines stably overexpressing Dvl2-myc as assessed by Western blot analysis (Fig. 5A, right panel). Moreover, stable overexpression of NPHP4-V5 in HEK293T cells led to a significant decrease in the amount of the endogeneous Dvl2 and Dvl3 proteins (Fig. 5B). This suggested that
nephrocystin-4, like inversin, could favour dishevelled degradation by addressing it to the proteasome. Indeed, we found that treatment with clasto-lactacystin, a proteasome inhibitor, prevented the degradation of both endogenous Dvl2 and Dvl3 as well as Dvl2-myc in cells overexpressing either NPHP4-V5 or inversin-Flag (inv-Flag) (Fig. 5B and C).

We next examined the endogenous expression of dishevelled and β-catenin in Martin-Darby Canine kidney (MDCK) cells in which NPHP4 has been invalidated by shRNA (N4-KD-C1) and led to a residual NPHP4 expression of 15%, compared with empty vector control cell lines (Psi-C1) as assessed by quantitative RT–PCR (Supplementary Figure 5).
The expression of both endogenous Dvl2 and Dvl3 was enhanced in N4-KD-C1 cells compared with control cells (Fig. 5D). Moreover, the stable expression of NPHP4-V5 in N4-KD-C1 cells (N4-KD-C1 + N4V5) led to a decreased level of endogenous Dvl2 and Dvl3 compared with N4-KD-C1 and Psi-C1, indicating a specific effect of nephrocystin-4 on dishevelled amounts in these cells (Fig. 5D). Consistent with a repressive role of nephrocystin-4 on Wnt-β-catenin signalling, we found a significant increase of β-catenin level in cytoplasmic extracts of N4-KD-C1 compared with control cells. Moreover, stable re-expression of NPHP4-V5 in N4-KD-C1 cells resulted in a decrease in β-catenin amounts, both in the nucleus and in the cytoplasm extracts (Fig. 5E). These results show that the absence of nephrocystin-4 in renal epithelial MDCK cells leads to an increase in dishevelled amounts in the cytoplasm, resulting in the accumulation of cytoplasmic β-catenin, thereby favouring the activation of canonical Wnt target genes.

To confirm the effect of nephrocystin-4 on the stability of dishevelled proteins, and to analyse the dishevelled subcellular localization in more detail, we performed immunofluorescence experiments on MDCK cells depleted in NPHP4 (Fig. 6). In control MDCK cells, Dvl2 and Dvl3 localization was dependent on the state of epithelialization. After 1–2 days of MDCK culture (Fig. 6A), Dvl2 localized to the centrosome/basal body, as indicated by co-staining with γ-tubulin, and
at the plasma membrane (Supplementary Material, Fig. S5A). Dvl2 and Dvl3 localization to the apical pole of the cells overlapping with the basal body was still conspicuous at 8 days of culture (Fig. 6B). When cultured for 10 days on filters, MDCK cells were well-polarized and Dvl2 and Dvl3 were mainly localized at the membrane (Supplementary Material, Fig. S5B and C). Loss of NPHP4 (in N4-KD-C1 cells) led to a drastic enhancement of cytoplasmic Dvl2 and Dvl3 staining both during epithelialization and in well-polarized MDCK cells (Fig. 6A and B and Supplementary Material, Fig. S5C). In addition, dishevelled staining was weaker and spread in the basal body region in the NPHP4-depleted cells compared

Figure 7. Nephrocystin-4 is present in dishevelled/inversin complex and cooperates with inversin during phronephric morphogenesis. (A, B) Nephrocystin-4, dishevelled and inversin are in the same protein complex. (A) Dvl2-Myc and Edd-Myc as a negative control were coexpressed with V5-tagged NPHP4 (NPHP4-V5) in HEK293T cells (upper panel). After immunoprecipitation with anti-V5, Dvl2-Myc was specifically detected in NPHP4-V5 immunoprecipitates. In the same manner, inv-Flag was detected in NPHP4-V5 immunoprecipitates when these two proteins were coexpressed in HEK293T, whereas the negative control INF2-Flag was not (lower panel). Expression level of transfected proteins in cell lysates was confirmed by immunoblotting with appropriate antibodies. (B) Co-immunoprecipitation of endogenous Dvl2 with NPHP4-V5 and inv-Flag in HEK293T cells. Lysates were immunoprecipitated with either anti-Dvl2 antibodies or IgG as a control and immunoblotted with V5 and Flag antibodies. (C, D) Nephrocystin-4 stabilizes inversin. Levels of both transiently expressed (C, left) and endogenous inversin (C, right) were stabilized in HEK293T cells transiently (left) or stably (right) expressing NPHP4-V5. A similar result was obtained with transient co-expression of NPHP4-myc and inv-Flag in HEK293T cells (data not shown). Asterisk (*) denotes a non-specific band that appeared with Flag antibody in lysates not transfected with inv-Flag (last lanes). (D) Western blot analysis of HEK293T cells transfected with inv-Flag alone or co-transfected with NPHP4-V5 and treated 24 h after transfection with 40 μg/ml of cycloheximide (Cx) at 0, 2, 4 or 8 h to block protein synthesis. The amount of inversin and nephrocystin-4 was visualized by immunoblotting with V5 and Flag antibodies. (E, F) Nephrocystin-4 cooperates in vivo with inversin for cystogenesis. Diagram illustrating the ratio of Tg(cldnB:gfp) transgenic embryos with kidney cysts (E) and cloacal morphogenesis defects (F) after coinjection of N4ATG-Mo (0.3 μm) with inv-Mo (0.3 μm). Note that (E, F) correspond to the same injection experiment, thus allowing to compare the ratio of embryos with cysts to that of embryos with cloacal defects in each experimental condition.
with controls. This expression coincides with diffuse γ-tubulin staining that could reflect cytoskeletal disorganization in the pericentriolar region (Supplementary Material, Fig. S5D).

The expression of NPHP4-V5 in N4-KD-C1 cells led to a reduction in cytoplasmic amounts of endogenous dishevelled and restored their preferential localization at the basal body (Fig. 6A and B). At days 1–2, the majority of overexpressed NPHP4-V5 localized to the cytoplasm but a fraction partially co-localized with Dvl2 to the basal body and centrosome (Fig. 6A). After 8 days of culture, NPHP4-V5 was mainly present at the basal bodies where it co-localized with Dvl2 and Dvl3 (Fig. 6B’ and Supplementary Material, Fig. S5E).

The co-localization of nephrocystin-4, Dvl2 and Dvl3 in MDCK cells suggests a physical interaction between these proteins. Through immunoprecipitation experiments in HEK293T cells, we demonstrated that nephrocystin-4 associated with dishevelled and inversin (Fig. 7A). We further showed that nephrocystin-4 co-precipitated with endogenous Dvl2 and that inversin was part of the complex (Fig. 7B).

The interaction of nephrocystin-4 with inversin suggests a cooperative function of these two proteins for dishevelled degradation. In this line, we observed that both inv-Flag and endogenous inversin protein expression were enhanced in the presence of nephrocystin-4 (Fig. 7C). We then demonstrated that the presence of nephrocystin-4 stabilized inv-Flag as assessed by cycloheximide treatment (Fig. 7D). In contrast, we did not observe nephrocystin-4 stabilization in the presence of inv-Flag (lysates in Fig. 7A and C).

To evaluate the relevance of the interaction between nephrocystin-4 and inversin in vivo, we performed combined nphp-2 (inv) and nphp-4 loss-of-function experiments in zebrafish embryos. Interestingly, knockdown experiments using low doses of inv-Mo and N4ATG-Mo led to a drastic enhancement of pronephric cyst formation at 2.5 dpf, whereas cloaca defects were mild and less penetrant at 24 hpf (Fig. 7E and F and Table 1). However, even if the cloaca was not obstructed in the double morphants, cloaca cells were increased in number and displayed a striking enhanced height along the apico basal axis. This demonstrates a functional interaction between nphp4 and inv for the control of pronephric/cloaca morphogenesis.

Together, these data show that nephrocystin-4, in cooperation with inversin, modulates Dvl sub-cellular amounts and localization and are required for proper pronephros morphogenesis. Moreover, nephrocystin-4 synergizes with inversin and the Wnt-PCP pathway in pronephros morphogenesis. In agreement with these observations in zebrafish, we demonstrate that nephrocystin-4 regulates subcellular amounts of cytoplasmic dishevelled in mammalian kidney cell cultures and represses the Wnt-β-catenin pathway. Our data suggest that nephrocystin-4, inversin and dishevelled act in concert for the regulation of the Wnt pathways required for pronephros morphogenesis.

What are the mechanisms leading to pronephric cyst formation in nphp4 morphants? Impaired fluid flow, resulting from cilium length/motility defects or from cloaca obstruction, is known to trigger cyst formation in the zebrafish pronephros. In nphp4 morphants however, it is unlikely that cilium defects directly affect fluid flow, as the cilia of multiciliated cells lining the anterior pronephros, known to drive urine flow (20), have a normal size and beat at the normal frequency. Defects in sensory cilia function in the distal part of the pronephros, where monocilia are shorter or absent, may also contribute to cystogenesis. Overall, the most likely explanation for cyst formation in nphp4 morphants is abnormal cloacal morphogenesis. Indeed, we demonstrated a tight association between cyst formation and abnormal morphogenesis of the pronephric duct and cloaca in these morphants, suggesting a causal relationship. Cloacal anomalies have also been associated with kidney cysts in other ciliary gene knockdown models, namely cep290/nphp6 (22) and pkk2 (21). While severe cloaca defects are obstructive as early as 24 hpf, we cannot ascertain that all mild cloacal abnormalities seen in low-dose nphp4 morphants lead to cloacal obstruction at 2.5 dpf. Moreover, in rescue experiments, co-injection of NPHP4 mRNA drastically decreased cyst formation and severe cloacal anomalies, but a large number of morphants with mild cloacal defects at 24 hpf were still observed. This indicates that additional mechanisms involved in pronephros morphogenesis between 24 hpf and 2.5 dpf are altered in nphp4 morphants. Mild cloacal defects could, for instance, cause pronephric cysts by impairing late events of collective cell migration required for anterior pronephros elongation (44). The involvement of additional mechanisms in cyst formation in nphp4 knockdown is further supported by our functional interaction experiments, in which deletion of Pk2 or inversin in nphp4 morphants dramatically enhances cyst formation but not cloacal defects.

Our results shed light on the early mechanisms leading to cloacal obstruction, which had so far remained elusive in ciliary gene knockdown (21,22). As early as 24 hpf, nphp4 morphants displayed a thickening of the pronephric ducts with an increase in cell number per transverse section, in the absence of increased proliferation. We propose that this enlargement is caused by impaired morphogenetic movements in the caudal region of the nphp4 morphant embryos. As we demonstrated, the absence of pronephros elongation between epithelialization and cloaca formation in both wild-type and morphant zebrafish embryos, CE movements required to lengthen the zebrafish nephron must occur before epithelialization of the pronephric anlage. Supporting this view, the number of cells per section is increased as early as the 10 s stage in the pronephric anlage of nphp4 morphants. In addition to these early CE defects, anomalies in epithelial cell shape
and arrangement are observed in the pronephric duct and forming cloaca during epithelialization in nphp4 morphants, notably a loss of columnar organization, higher cells and shorter cilia. These defects are evocative of alterations in epithelial morphogenesis. Epithelial morphogenesis anomalies have also been observed in three-dimensional cultures of NPHP4-depleted MDCK cells, in which cell junctions and single lumen formation are impaired during polarized epithelial cyst formation (11). Nephrocystin-4 has been shown to interact with different protein complexes involved in tight junction formation and apico-basal polarity (Par6 and Pals1/PATJ) (11) and in cell–matrix interaction (p130cas/pyk2) (12), and with the cytoskeleton (12). Thus, defects in cloaca morphogenesis in nphp4 morphants may possibly result from an alteration of cell adhesion and polarity and/or cytoskeleton dynamics.

The data presented here are consistent with a role of nephrocystin-4 in downregulating the Wnt-β-catenin pathway and promoting the Wnt-PCP pathway. The presence of axis-elongation defects in zebrafish nphp4 morphants, their rescue by ΔN-Dvl and the functional interaction between nphp4 and PCP genes for axis elongation, strongly suggest synergistic effects of nephrocystin-4 with the Wnt-PCP pathway. In the zebrafish pronephros, we show that nphp4 functionally interacts with pk2, a member of the Wnt-PCP pathway, for cyst formation through a mechanism independent of cloaca morphogenesis. Thus, similar to the mammalian metanephric nephron (30,45), morphogenesis of the zebrafish pronephros requires the synergistic activity of ciliary proteins and of the Wnt-PCP pathway. In addition, we observed that NPHP4 depletion or overexpression leads to modulations in Wnt-β-catenin-reporter activity, both in the zebrafish cloaca and in cultured renal epithelial cells. In mammals, both excessive and reduced canonical Wnt signalling lead to kidney cysts (46–50); for instance, increased canonical Wnt signalling has been proposed to be involved in cyst formation in Pkd1 mouse mutants (46). In nphp4 morphants, the role of this local increase in Wnt-β-catenin activity is unclear. Indeed, we did not detect any major defects in cell proliferation or cell specification in the formation of pronephros of the morphant embryos. The abnormal activation of the Wnt-β-catenin pathway observed locally in the cloacal cells of the nphp4 morphants at 24 hpf could participate in the destabilization of the cellular junctions, by alteration of the cellular subpopulations of β-catenin (51).

In this paper, we show that nephrocystin-4 binds dishevelled and regulates its protein expression level and subcellular localization in MDCK cells. We demonstrate that in the absence of nephrocystin-4, dishevelled amounts are higher in the cytoplasm of MDCK cells. Moreover, its localization to the basal bodies during epithelialization appears more diffuse. Our data point to a role of nephrocystin-4 in addressing dishevelled to proteasomal degradation, and in maintaining pools of dishevelled in specific subcellular localizations. A similar function in dishevelled degradation has been proposed for inversin, initially described as a molecular switch between the Wnt/PCP and Wnt-β-catenin pathways (34). Both inversin and nephrocystin-4 localize at cell–cell junctions and basal body and have several common interactors (12,52). We show that inversin, nephrocystin-4 and dishevelled are in the same protein complex in cultured renal epithelial cells and that nephrocystin-4 and inversin cooperate for pronephric and cloaca morphogenesis. Since nephrocystin-4 stabilizes inversin, its function in dishevelled degradation and in the modulation of the Wnt pathways might be mediated by its role in protecting inversin from degradation. In addition, nephrocystin-4 may also promote the association of inversin with specific interactors and/or its specific subcellular localization necessary to activate the PCP pathway. Indeed, it has been recently shown that the recruitment of dishevelled by inversin to the plasma membrane downstream of Frizzled-8 is required to promote proximal pronephros development in the embryos of Xenopus (53). Finally, nephrocystin-4 via the regulation of dishevelled may maintain apico-basal polarity in renal epithelial cells, modulating focal adhesion turn-over (54), E-cadherin-dependent cell adhesion (55), and/or apical docking and polarization of basal bodies (56).

Although the renal phenotype associated with mutations of NPHP4 and NPHP2/INVS in mice and humans varies in terms of severity and onset, notably causing juvenile and infantile NPH, respectively, in humans, the interaction of nephrocystin-4 and inversin suggests that they share common functions. Indeed, we have previously reported that patients with early-onset ESRD associated with mutations of INV may also present with a renal histology characteristic of juvenile NPH, such as thickening of the basement membrane and development of a massive interstitial fibrosis (57). Recently, a homozygous truncating mutation of inversin leading to the deletion of its dishevelled binding domain has been reported in a family with juvenile ESRD. In this patient’s kidney, abnormal expression of β-catenin and Dvl1 in the pre-epicystic renal tubules was detected (58). Thus, alteration of inversin and nephrocystin-4 in human kidneys may lead to a common physiopathological defect, possibly associated with a deregulation of the Wnt pathways.

MATERIALS AND METHODS

Zebrafish lines

The wild-type TL or TüAB strains, the vangl2 mutant trm209 mutant line (41) and the Tg(-8.0cldnb:lynEGFP) (hereafter named Tg(cldnB:GFP)) (59) and Tg(TOP.GFP) (43) transgenic lines were raised and maintained according to standard protocols (60). Embryos were staged as described previously (60).

Plasmids and antibodies

Inv-Flag was provided by I. Drummond (Harvard Medical School, Charlestown, MA, USA); Dvl2-Myc was provided by S. Sokol (Mt. Sinai School of Medicine, NY, USA). Edd Myc were provided by A. Smahi (Necker Hospital, Paris, France). INF2-Flag construct was given by G. Mollet (Necker Hospital, Paris, France). MDCK cells stably expressing a shRNA targeting NPHP4 have been described previously (11). Re-expression of NPHP4 in these cells was performed using the human NPHP4 cDNA sequence cloned in pRRL.SIN.cPPT.PGK/WPRE (61) after modification by insertion of a C-terminal V5 tag using site-directed
mutagenesis (NPHP4-V5). Lentiviral infections were performed as described (11). Antibodies used for immunofluorescence on zebrafish embryos were monoclonal anti-acetylated α-tubulin 6-11B-1 (Sigma), monoclonal α6F (DSHB), rabbit anti-E cadherin (a kind gift of J.A. Marrs), monoclonal anti-ZO1 (Zymed), monoclonal anti-phosphohistone H3(Ser10) (6G3) (Cell Signalling Technology), rabbit anti-phosphohistone H3(Ser10) (Upstate), rabbit anti anti-Pax2 (Covance), rabbit anti-caspase 3 (R&D systems). Antibodies used for immunofluorescence on MDCK cells were rabbit anti-Dvl2 (Biomol), rabbit anti-Dvl3 (Abcam), mouse anti-v5 (AbD Serotec), mouse anti-acetylated-γ-tubulin (Sigma). Nuclei were stained with DAPI (Invitrogen). Antibodies used for immunoprecipitation and western blot were mouse anti-α-tubulin (Sigma), mouse anti-β-catenin (Transduction Laboratories), mouse M2 anti-FLAG (Sigma), rabbit anti-Dvl2 and anti-Dvl3 (Cell Signalling) and rabbit anti-Histone Deacetylase (HDAC1) (Calbiochem). For immunofluorescence, the following secondary antibodies were used: Alexa 488-goat anti-mouse, Alexa 568-goat anti-mouse, Alexa 488-goat anti-rabbit, Alexa-568 goat-anti rabbit (Molecular Probes) and for western blot, HRP-anti Rabbit (GE Healthcare) or HRP-anti mouse.

Morpholino and RNA injections

Morpholinos and mRNAs were injected into one-cell stage embryos (1 nl per embryo) at the indicated concentrations. For nphp4-morpholino antisense oligonucleotides (Gene Tools, Philomath OR, USA) were designed to target either the translation start site (N4ATG-Mo; 5'-GCG CTT CTC CAC TCA GAC ATC AGA G-3') or the exon 6 splice-acceptor site (N4sp-Mo; 5'-TCT GGT TTC TCA AGA CGG ACT-3'). The knockdown efficacy of N4spMo was evaluated by RT–PCR with primers located in exon 5 (5'-TGA AAG ACC CTC TGC AAT G-3') and in exon 7 (5'-AGT TCC TCC AGA ATC AAG TTC-3'). Gapdh primers (5'-GG CAA AGG TCA TCA ATG ATA-3') and 5'-GCA GGT GTC TTA CCA AGA CGG ACT-3') were used as PCR controls. For functional interaction studies, we used previously described morpholinos targeting Inv5 (17), Vangl2 (62), Prickle1 (Pki) (63), Prickle2 (Pk2) and Scribble1 (Scrib) (33). Pk2 depletion was preferentially used to study the genetic interaction with nphp4 focusing on the kidney phenotype as it has been reported to generate pronephric cysts (33). For rescue experiments, mRNA was produced from X. laevis pCS2-myc-Dsh-ΔN plasmid (42) and from human pCS2-NPHP4 plasmid using the mMessage mMACHINE kit (Ambion). Human NPHP4 mRNA was injected at 90 pg/embryo with a high dose of N4ATG-Mo.

In situ hybridization and immunochemistry on zebrafish embryos

Whole-mount in situ hybridization was performed as previously described (64). The expression pattern of spaw, pitx2, notail, shh, gata 3, ret-1, pax2-1, prdm1, evx1, cdh17 were previously reported (20,65). Whole-mount immunohistochemistry was performed on embryos fixed either in 4% paraformaldehyde (PFA) or methanol:dimethyl sulfoxide (80:20) as described (20). Imaging was performed using a Leica SP5 confocal microscope. For cilia length measurement, cilia were stained with anti-acetylated tubulin and Z-stack images of flat-mounted embryos were acquired for three-dimensional reconstruction with Velocity software (Perkin-Elmer). Confocal images where individual cilia bases and ends could be discerned (minimum 30 measures per embryo) were outlined and cilia length was calculated.

Histological and ultrastructural analysis of zebrafish embryos

For histology, embryos were fixed in 4% PFA, embedded in glycemethacrylate (JB-4 resin, EMS) and sectioned at 5 μm. Sections were stained with 1% toluidine blue or haematoxylin & eosin and photographed with a Nikon eclipse E800 microscope. For electron microscopy, embryos were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer pH 7.2, and processed as previously described (66). Thin sections were cut on a Reichert Ultracut S microtome, and stained with uranyl acetate and lead before examination in a JEOL 1011 TEM at 80 kV.

Cilium beat frequency measurements and dextran excretion assay on live zebrafish embryos

For cilium beat frequency measurements, anaesthetised embryos were embedded in 1% low-melting agarose, and observed with the Leica SP5 confocal microscope. The x-t imaging option of Leica LASAF software enables the acquisition of one line at high frequency (8192 lines/s). Beating cilia in the pronephros were detected in live view mode, and the focussed line was positioned. Signals generated by cilium movements in and out of the focused line were captured on the x-t mode for 0.5 s. Beat frequency was deduced from recurrence of similar signals in this time frame. Urine excretion assays were performed as described (67) using a solution of 5% tetramethylrhodamine-conjugated 70 kDa dextran (Molecular Probes). After vascular injection, dye excretion was detected at cloaca under a Nikon fluorescence microscope. The time required for dye excretion was recorded.

Luciferase assays

HEK293T cells were seeded at 7.5 x 10^5 cells in 6-well plates and transiently transfected with a TOPFlash luciferase reporter construct (Upstate Biotechnology), pGL4.74[hRLuc/TK] Renilla luciferase expression vector (Promega) and vectors directing the expression of Dvl2-Myc, inv-Flag, NPHP4-V5 or empty vector. The total amount of transfected DNA was 2.0 μg per well. Cells were lysed in reporter lysis buffer (Promega), and luciferase activity was assessed using a dual-luciferase reporter assay system (Promega) and a luminometer (Berthold) according to the manufacturers’ protocols. The relative Firefly luciferase activity was normalized to Renilla luciferase expression to adjust for variation in the transfection efficiency, and all experiments were performed in triplicate.
Co-immunoprecipitation

Plasmids were transfected into HEK293T cells by the calcium phosphate method. Co-immunoprecipitation assays were performed as described previously (11). V5-tagged proteins and endogenous Dvl2 were immunoprecipitated using, respectively, mouse anti-V5 (AbD Serotec) and rabbit anti-Dvl2 (Cell Signalling) antibodies. After washing and denaturation, samples were immunoblotted with the indicated antibodies. Images were captured with a Fusion Fx7 system and quantified with Bio-ID software (Vilbert-Lourmat).

Immunofluorescence studies in MDCK cells

MDCK cells were either plated at 2.5 × 10^5 cells on cover slips and fixed after 36 h (days 1–2) or plated at 10^5 cells on 12 mm Transwell filters (Costar) and grown for 8 or 11 days post-confluence. Cells were fixed in 4% PFA in phosphate-buffered saline (PBS) for 20 min followed by treatment with 50 mM ammonium chloride for 10 min. For detection of Dvl2 at the membrane, cells were fixed in cold methanol for 10 min. Cells were then treated with PBS, 0.1% Tween-20, 3% bovine serum albumin, 10% donkey serum for 30 min, before incubating with primary antibody solution for 2 h followed by the appropriate fluorescent secondary antibody. Confocal images were captured as described above (11).

Subcellular fractionation

To analyse nuclear levels of β-catenin, MDCK cells were plated at 8 × 10^6 cells per 100 mm culture dishes (BD Biosciences) and harvested after 36 h (days 1–2) with trypsin and then centrifuged at 500g for 5 min. The cell pellets were washed in PBS and 5–10 × 10^6 cells were lysed using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific) according to the manufacturers’ instructions.

Protein half-life assay

HEK293T cells were co-transfected in 6-well plates by the calcium phosphate method with Dvl2-Myc, inv-Flag or NPHP4-V5. Cells were treated with 40 μg/ml cycloheximide for 0, 2, 4 or 8 h to determine the half-life of inv-Flag in the presence or absence of NPHP4-V5. To inhibit proteasome-dependent degradation, cells were treated with 10 μM of clasto-lactacystin β-lactone (Sigma) for 7 h before collection. Cells were harvested and lysed for 10 min with lysis buffer containing 1% Triton-X100, 50 mM Tris–HCl, pH 7.5, 150 mM NaCl and 1 mM sodium orthovanadate with complete protease inhibitor cocktail (Roche Applied Science). Insoluble debris were removed by centrifugation at 14000g for 10 min. Equal amounts of protein were resolved by 7.5% sodium dodecylsulphate–polyacrylamide gel electrophoresis and then blotted with appropriate antibodies.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

ACKNOWLEDGEMENTS

We are grateful to D. Gilmour, R. Moon and L. Solnica-Krezel for providing the Tg(cldnb:GFP), Tg(TOP:GFP) and triplezebrafish lines, respectively, and to I. Drummond and S. Sokol for providing the inv-Flag and the Dvl2-Myc plasmids, respectively. We thank S. Harvey for critical reading of the manuscript. We thank the zebrafish and Imaging facilities of the IFR 83 for technical assistance and particularly Virginie Georget for help in establishing the protocol of cilia beat frequency measurements. We also thank Nicolas Goudin and Meriem Garfa-Traroré for their technical assistance with confocal microscopy (Imaging facilities of the IFR 94) and Alain Schmitt for his assistance with transmission electron microscopy (Imaging facilities of the Cochin Institute).

Conflict of Interest statement. None declared.

FUNDING

This work was supported by the Centre National de la Recherche Scientifique (CNRS), the Institut National de la Santé et de la Recherche Médicale (INSERM), the Ministère de l’Éducation Nationale de la Recherche et de la Technologie (MRT), the Fondation pour la Recherche Médicale (S.S. is laureate of the ‘Équipe FRM’ grant DEQ20071210558), the Association pour l’Utilisation du Rein Artificiel (AURA) and the Agence National de la Recherche (ANR, S.S.M.). C.B. was the recipient of a grant from the Société d’Néphrologie.

REFERENCES


