Nephrocystin-4 is required for pronephric duct-dependent cloaca formation in zebrafish

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Received April 4, 2011; Revised and Accepted May 10, 2011

NPHP4 mutations cause nephronophthisis, an autosomal recessive cystic kidney disease associated with renal fibrosis and kidney failure. The NPHP4 gene product nephrocystin-4 interacts with other nephrocystins, cytoskeletal and ciliary proteins; however, the molecular and cellular functions of nephrocystin-4 have remained elusive. Here we demonstrate that nephrocystin-4 is required for normal cloaca formation during zebrafish embryogenesis. Time-lapse imaging of the developing zebrafish pronephros revealed that tubular epithelial cells at the distal pronephros actively migrate between the yolk sac extension and the blood island towards the ventral fin fold to join the proctodeum and to form the cloaca. Nphp4-deficient pronephric duct cells failed to connect with their ectodermal counterparts, and instead formed a vesicle at the obstructed end of the pronephric duct. Nephrocystin-4 interacts with nephrocystin-1 and Par6. Depletion of zebrafish NPHP1 (nphp1) increased the incidence of cyst formation and randomization of the normal body axis, but did not augment cloaca malformation in nphp4-deficient zebrafish embryos. However, simultaneous depletion of zebrafish Par6 (pard6) aggravated cloaca formation defects in nphp4-depleted embryos, suggesting that nphp4 orchestrates directed cell migration and cloaca formation through interaction with the Par protein complex.

INTRODUCTION

Nephronophthisis (NPHP), an autosomal recessive cystic kidney disease often presenting with polydipsia, polyuria, anemia and growth retardation, is one of the most frequent genetic causes of end-stage kidney failure in children and young adults. The most prominent histological alterations are abnormalities of the tubules with thickening of the basement membrane and interstitial fibrosis, progressing to cysts positioned at the corticomedullary junction in more advanced stages (1). Contrary to polycystic kidney disease, NPHP typically is associated with normal or diminished kidney size. Mutations in more than 10 genes (NPHP1–11, NPHPL1) have been identified as the cause of NPHP (1–4). In zebrafish, loss of NPHP genes function causes a stereotypical set of developmental changes, including an abnormal body curvature, heart edema, defects in cilia length or function and pronephric cyst formation (5–8). Primary cilia are sensory organelles that connect mechanosensory, visual, osmotic and other stimuli to signaling cascades controlling cell cycle, epithelial cell polarity and organ development (9). On the basis of their presence in the cilium and/or the basal body, the abnormalities caused by NPHP genes in vertebrates are attributed to defects in ciliary function.

NPHP4 mutations cause familial juvenile NPHP type 4. It accounts for about 2% of NPHP cases and can result in isolated cystic kidney disease or cystic kidney disease with oculomotor apraxia (Cogan syndrome), retinitis pigmentosa (Senior–Loken Syndrome, SLS) and liver fibrosis. The NPHP4 gene product nephrocystin-4 interacts with multiple adaptor and signaling components, but also with the gene products of NPHP1 (nephrocystin-1), NPHP8 (RPGRIP1L) and components of the Par polarity complex (10). Nephrocystin-4 localizes to cell–cell junctions, basal body and transition zone of primary cilium as well as to the cortical actin cytoskeleton of epithelial cells, implying variable functions at these different sites (6,10–13).
The functional kidney of the zebrafish larva, the pronephros, consists of a capillary tuft connected to two ducts, which run bilaterally along the body axis and fuse at the cloaca opening. The two pronephric ducts consist of highly polarized epithelial cells, carrying one or more motile cilia on the apical membrane. These directionally beating cilia generate a fluid-flow that contributes to lumen patency and urine excretion through the cloaca. Interference with urine flow by disruption of ciliary function or mechanical obstruction of the pronephric duct causes pronephric cyst formation (14–16). The cloaca in fish is the common opening of the urinary and the gastrointestinal tracts to the environment. In zebrafish, the cloaca forms around 24 h post-fertilization (hpf), when epidermal cells fuse with the distal pronephric duct. Markers such as prdm1, vox1, evx1, tbx2b and gata3 label the contributing epidermal and pronephric components during this stage (17,18).

Shaping of the cloaca requires extensive epithelial remodeling and orchestrated apoptosis of epidermal cells, which enables the duct to open to the environment. Bone morphogenetic proteins (Bmp) provide the main cell-patterning cues for the cloaca-forming events, and alterations in Bmp levels lead to defective cloaca formation (17,18).

Here, we describe the role of nephrocystin-4 in zebrafish pronephros development and function. We demonstrate that the morpholino oligonucleotide (MO)-mediated knockdown of zebrafish nphp4 affects cilia formation and function, leading to disturbed left–right asymmetry and pronephric cyst formation. Our analysis revealed an unexpected role of nphp4 in cloaca formation, implying a cross-talk between Bmp and nphp4 signaling.

In situ hybridization (ISH) with marker genes expressed in the cloaca region demonstrated that nphp4 depletion did not affect the specification of the ectodermal and pronephric components of the future cloaca. In vivo investigation of the cloaca development revealed that the directed migration of distal pronephric cells towards the site of fusion with ectodermal cells was abrogated in nphp4-deficient zebrafish embryos, preventing programmed cell death of ectodermal cells. This defect was aggravated by simultaneous depletion of the polarity protein Par6, suggesting that nphp4 orchestrate directed cell migration and cloaca formation through interaction with the Par protein complex.

RESULTS

Identification and cloning of nphp4

To identify full-length zebrafish nphp4 homolog, we used BLAST searches of the human NPHP4, comparing protein sequence against zebrafish Ensemble8 Genome sequences at the Sanger Institute. The best hit was located on chromosome 8, covering two partially overlapping predicted gene sequences, ENSDART00000100063 and ENSDART0000111181. Combining these two sequences, we obtained full-length zebrafish nphp4, which consists of 1357 amino acids and is 43.8% identical to its human ortholog (Fig. 1A).

As its human counterpart, zebrafish nphp4 does not contain known conserved protein domains. Interestingly, amplification of the sequence from mRNA isolated from embryos 2 days after fertilization yielded three different PCR products, which were identified as alternative splice variants of nphp4. The longest one, referred here as splice variant 1, contained exons 1–31 of the predicted sequence, giving rise to a full-length protein. In the second one (splice variant 2), exon 6 was directly spliced to the middle of exon 22, excluding exons 7–21. This shorter transcript contained a premature STOP codon, giving rise to a 390 amino acid-long protein. Based on the strength of the PCR bands, variants 1 and 2 were present at similar levels, but more abundant than variant 3 (data not shown).

Expression pattern of Zebrafish nphp4

To determine the expression pattern of zebrafish nphp4, we generated an antisense RNA probe and performed ISH.
Zebrafish nphp4 was expressed ubiquitously at low levels during the first 2 days of development with elevated expression levels in the Kupffer’s vesicle (KV) at the 6-somite stage (Fig. 1B–D, arrowhead) and the distal part of the pronephros at 24 h post-fertilization (hpf) (Fig. 1C, arrows).

Phenotypic changes after knockdown of Zebrafish nphp4

To study the function of nphp4 during zebrafish embryonic development, we designed antisense MO, targeting the translation initiation site of nphp4 mRNA (nphp4 ATG) and a splice MO targeting the splice donor site of exon1. Both MOs gave similar phenotypes; however, experiments with the translation blocking MO were more consistent, and subsequently used unless noted otherwise. To demonstrate the efficiency of the translational block, we injected mRNA, containing a fragment of zebrafish nphp4 with the MO binding region fused to green fluorescent protein (GFP) in combination with the nphp4 ATG MO. Subsequent fluorescent imaging of the injected embryos demonstrated that the MO efficiently prevented GFP translation (Fig. 2G, H). Injections of this MO into fertilized eggs produced embryonic defects resembling those observed after knockdown of other polycystic kidney disease (PKD) genes in zebrafish (8,16), including abnormal body curvature, hydrocephalus, pericardial edema and pronephric cysts (Fig. 2A–D). Using transgenic fish lines expressing GFP in the glomerulus (wt1b:gfp) (19) (Fig. 2D) and the pronephric duct (cldn2b:lyn-gfp) (20) (Fig. 2E, F, arrowheads), we detected cyst formation in the glomerular region (arrowheads in Fig. 2B–D) as well as a dilated pronephric duct (Fig. 2E and F, arrowheads). In addition, formation of a vesicle at the distal end of the pronephric duct was noted (arrows in Fig. 2B and F, quantified in Fig. 2I). While body curvature was observed in all embryos in an MO dose-dependent fashion, the number of embryos developing pronephric cysts strongly varied (between 25 and 80%), depending on the fish strain (e.g. the zebrafish TL strain developed more cysts than the zebrafish strain AB/TL). Over-expression of full-length human or zebrafish nphp4 caused concentration-dependent embryonic defects and failed to rescue the cloaca defects of nphp4 morphants, indicating that a precise balance between different nphp4 isoforms might be required to rescue the MO-mediated defects.

Morphant cilia phenotype

Defects in genes important for cilia development, e.g. intraflagellar transport (IFT) proteins, often lead to pronephric cyst formation in zebrafish (16,21). To examine the effect of nphp4 knockdown on ciliogenesis, we stained the KV and the pronephric duct of morphant and wild-type (WT) embryos for acetylated tubulin. Cilia of morphant zebrafish appeared curly (data not shown). Consistent with an abnormal body axis associated with defective KV fluid flow (22), heart looping was randomized in the nphp4 morphants (Fig. 3G–I, K). These findings suggest that zebrafish nphp4 plays a role in cilia formation and/or function.

Defective cloaca formation in nphp4-deficient Zebrafish

Investigating the defects of nphp4 MO-injected embryos, we noticed the presence of a vesicle at the distal end of the pronephric duct. Nevertheless, the size of the KV of the morphants was slightly smaller (Supplementary Material, Fig. S2); therefore, a more general developmental defect could account for the observed ciliary differences. Two days after fertilization, the cilia in the dilated pronephric ducts of nphp4 MO-injected embryos were disoriented and often appeared curly (data not shown). Consistent with an abnormal body axis associated with defective KV fluid flow (22), heart looping was randomized in the nphp4 morphants (Fig. 3G–I, K). These findings suggest that zebrafish nphp4 plays a role in cilia formation and/or function.
pronephric duct indicative of cloaca malformation (Fig. 2). Since mechanical obstruction of the pronephric ducts close to the cloaca causes cyst formation in the proximal pronephros (23), we hypothesized that a failure to generate patent distal pronephric ducts contributed to proximal cyst formation in nphp4-deficient zebrafish embryos. To determine whether cloaca malformation was the result of incorrectly specified tissue, we performed ISHs for gata3, tbx2b, prdm1, vox1 and evx1 (Fig. 4). The probes for gata3 and tbx2b mark the distal pronephros (17). The expression of prdm1 and vox1 in ectodermal cells of the fin ridges and the cloaca requires intact Bmp signaling (18,24), while evx1 is expressed in the distal pronephric as well as ectodermal cells (25). Although distorted by cloaca defects and obstruction of the terminal pronephros, expression of evx1, gata3 and tbx2b appeared largely intact (Fig. 4). The nphp4 MO-injected zebrafish embryos displayed two prdm1 positive domains. One domain marked the ectoderm, the other region localized to the distal pronephros partially displaced by the vesicle at the distal end of the pronephros (Fig. 4A and B). These findings suggest that both the distal pronephric duct and the ectodermal tissue forming the cloaca (proctodeum) were correctly specified even in the absence of nphp4 (Fig. 4A–L). When the ISHs were repeated for oval, an IFT88-deficient zebrafish mutant with defective ciliogenesis, the distribution of the markers, including their position in respect to the ectoderm did not significantly deviate from wild-type embryos (Fig. 4C, F, I, L). Since oval mutants did not display cloaca malformations despite an abnormal body curvature and severe cysts formation, these results indicate that the cloaca defects observed in nphp4 MO-injected embryos occur independently of cell fate specification and ciliogenesis.

Defective Bmp signaling causes abnormal cloaca development (18). However, these studies did not investigate the relationship between cloaca formation and zebrafish pronephros development. To examine the relationship between cloaca malformation and pronephric cysts, we utilized a transgenic zebrafish line expressing noggin3 under the control of a
heat shock promoter (26). Transgenic embryos subjected to heat shock at the tail-bud stage developed pronephric cysts as well as cloaca vesicles similar to nphp4-depleted embryos (Supplementary Material, Fig. S1).

Bmp signaling defects prevent the programmed cell death of ectodermal cells forming the cloaca opening (17). Examination of cell death at 24 hpf using the vital dye acridine orange (AcrOrg) showed a dramatic reduction of cell death at the cloaca of nphp4 MO-injected embryos (Fig. 4P and O). Thus, despite intact Bmp-dependent specification of the ventro-ectodermal cells, these cells fail to undergo programmed cell death, suggesting that an inductive signal from the distal pronephric duct is required to induce apoptosis of ectodermal cells, leading to cloaca opening formation.

**nphp4 influences cloaca formation by affecting duct cells migration**

To investigate the role of zebrafish nphp4 at the cellular level, we performed time-lapse confocal imaging of nphp4 MO-injected and non-injected cldb:lyn-gfp transgenic embryos during the stages of cloaca formation (18–36 hpf). Pronephric duct cells, labeled with membrane-bound GFP, actively migrated between the yolk sac extension and the blood island to reach the epidermal cells of the proctodeum in WT embryos (Fig. 5A–C, Supplementary Material, Movie S1). Subsequently, the cells of the pronephric duct tip migrated over the ectodermal cells in a posterior-to-anterior direction, whereby both cell types formed protrusions towards each other until the pronephric duct cells reached the position where cloaca formation occurred. Following the re-organization of the epithelial structure between epidermis and pronephric duct, some of the epidermal cells underwent apoptosis to shape the opening of the cloaca (Supplementary Material, Movie S1). Finally, the cloaca channel, formed by polarized epithelial cells of mesodermal and ectodermal origins, opened to the environment at around 30 hpf (Fig. 5G). In nphp4 morphants, the tip cells of the distal pronephric duct failed to join the ectodermal cells committed to undergo apoptosis and to form the cloaca opening (Fig. 5D–F, G). As a consequence, the distal part of pronephric duct did not appear as a straight channel, but became distorted and formed a blunt-ended tube in nphp4-deficient embryos (Fig. 5D–F, G and Supplementary Material, Movie S2). Tracking of individual cells during the time of cloaca morphogenesis in WT embryos revealed that the pronephric duct cells were moving in a ventro-anterior direction, while the ectodermal cells followed a posterior direction (Fig. 5H, Supplementary Material, Movie S1). In nphp4 MO-injected embryos, the ectodermal cells continued to move posteriorly; however, the

![Figure 4](http://hmg.oxfordjournals.org/)

Figure 4. Pronephric and ectodermal components of the cloaca are correctly specified in nphp4-depleted embryos. (A–L) In situ hybridization of 2-day-old wild-type (WT), nphp4 MO-injected and ovl (ift88−/−) zebrafish embryos stained with cloaca and epidermis (prdm1 A–C, evx1 D–F and vox1 M, N) or pronephric duct (gata3 G–I, tsh2b J–L) markers. In all images, an arrow marks the end of the distal pronephric duct, and an arrowhead the cloaca. The expression patterns of the markers indicate that the components of the cloaca are correctly specified, but fail to converge and to form a functional cloaca opening. (O and P) Vital stain with acridin orange dye, detecting the extent of cell death at the cloaca region 24 h post-fertilization. WT embryos were strongly labeled, revealing extensive cell death at the duct opening (arrowhead in O). Only moderate staining was detected in nphp4 MO-injected embryos (arrowhead in P).
pronephric duct cells of *nphp4*-deficient embryos failed to display the coordinated directional movement that connects the pronephric duct tip cells with the ectodermal cells (Fig. 5I, Supplementary Material, Movie S2). Interestingly, although the leading pronephric duct cells in WT embryos exhibited typical features of migrating cells, e.g. formation of lamellipodia in the direction of migration (Supplementary Material, Movie S1), morphant cells remained stationary and failed to form cell protrusions (Supplementary Material, Movie S1). Thus, *nphp4* orchestrates the fusion of the pronephric duct with the ectoderm by supporting the directional migration of pronephric duct cells.

*nphp1* synergistically enhances *nphp4* cilia-related phenotypes, but not cloaca defects

Several studies have demonstrated the physical interaction between the *NPHP1* and *NPHP4* gene products (10,27), and both *NPHP1* and *NPHP4* gene products localize to the primary cilium (12). To investigate whether these two proteins synergize in zebrafish during pronephros development and cloaca formation, we designed an ATG MO, which targets zebrafish *nphp1*. At 0.4 mM, the *nphp1* MO-injected embryos developed small pronephric cysts and mild duct dilatations (Supplementary Material, Fig. S3). Higher MO concentrations increased the number of malformed embryos and caused mild dorsalisation with absent ventral medial fin structures. Malformed embryos also displayed cloaca obstruction, although at a low percentage (Supplementary Material, Fig. S3). Cilia length, however, remained unaltered even in embryos injected with high doses of *nphp1* MO (Supplementary Material, Fig. S3). Embryos injected with low amounts of either *nphp1* or *nphp4* MO (0.2 mM) showed mild body curvature abnormalities, but formed neither cysts nor a vesicle within the cloaca region (Fig. 6A and B). Co-injection of both MO at 0.2 mM caused a dramatic increase of the abnormal body curvature, heart edema and approximately half of the double-injected embryos formed pronephric cysts (Fig. 6C–E). Immunofluorescent analysis of the double-injected morphants with pronephric cysts revealed duct dilations and misoriented cilia (Fig. 6G–I) as well as randomized left–right asymmetry (Fig. 6F), implying defective KV function. However, cloaca vesicles were not detected in any of the double morphants. These observations suggest that *nphp1* does not synergize with *nphp4* during cloaca formation.

*nphp4* genetically interacts with *pard6* during cloaca formation

Since nephrocystin-4 also interacts with Par6 (10), we tested whether Par6 participates in cloaca formation. The zebrafish *pard6-gammaB* matches the murine *Pard6gamma* (28). To examine the role of *pard6-gammaB* during cloaca formation, we designed an antisense MO targeting the splice donor site of exon 2 of *pard6-gammaB* (*pard6* MO). MO-induced aberrant splicing was verified by RT–PCR (Fig. 7G); the
sequence analysis revealed a deletion within the second exon of pard6-gammaB, leading to a frame shift and a premature STOP codon (data not shown). Depletion of pard6 in zebrafish embryos caused mild-to-strong body curvature abnormalities and notochord undulations in a concentration-dependent manner (Fig. 7A and B and data not shown). Pericardial edema occurred in all phenotypic classes, while heart beat and blood circulation were not impaired (data not shown).

The pard6-deficient zebrafish embryos also developed pronephric cysts, although at a low frequency (<10%) (Fig. 7A). However, expansion of the pronephric duct lumen was observed by regular light microscopy as well as in histological sections (Fig. 7C–F). Although the cilia in the ducts of morphant embryos had a normal length, they appeared disoriented (data not shown). Injections of either 0.2 mM nphp4 or 0.4 mM pard6 MO in combination with a control MO to adjust the total amount of injected MO to 0.6 mM had only moderate effects on cloaca development (Fig. 8A–D). In contrast, combined injections of 0.2 mM nphp4 and 0.4 mM pard6 MO caused a striking increase in the number of the embryos with obstructed cloaca (Fig. 8C and D). Time-lapse imaging of the migratory behavior of the distal pronephros showed that the cloaca forming pronephric duct cells of the double morphants failed to form protrusions directed towards the ectoderm, but only generated small blebs that quickly retracted (Fig. 8G, Supplementary Material, Movie S3). As a result, the pronephric duct failed to connect to the cloaca (Fig. 8E and F).

Co-expression of human NPHP4 mRNA ameliorated cloaca malformation caused by the nphp4/pard6 knockdown. While 33.9% of the nphp4-pard6 double morphants (n = 142) developed malformed cloaca, this defect was reduced to 5.9% (n = 125) after co-injection of human NPHP4 mRNA. These results indicate that nphp4 acts together with components of the Par complex to orchestrate cloaca morphogenesis.

Figure 6. Depletion of nphp1 does not augment cloaca malformation in nphp4-deficient zebrafish embryos. (A–D) Images of the wt1b: gfp transgenic zebrafish embryos at 2 days post-fertilization injected with 0.2 mM solution of nphp1 (A), nphp4 (B) or a combination of both morpholinos (C, D). The insets in every picture show a fluorescent image of the glomerulus region of the same embryo. The combined injection increased the level of body curvature and promoted cyst formation in double morphants to about 50% (C and E), but did not cause cloaca obstruction. The number of embryos with abnormal body symmetry, detected by a heart-looping defect, was also increased (F). (G–I) Confocal images of the pronephric duct of single and double MO-injected cldb:lyn-gfp transgenic embryos stained for GFP and acetylated tubulin. Double morphants show an increased duct diameter and disoriented cilia. These experiments suggest that cilia-dependent functions were more severely impaired in double than in single morphants.
Knockdown of zebrafish nphp4 leads to developmental defects commonly attributed to ciliary dysfunction, including an abnormal body curvature, situs inversus and formation of pronephric cysts. The reduction of ciliary length in the KV and pronephric duct of nphp4 MO-injected embryos are consistent with the hypothesis that ciliary defects cause these morphological changes. Nephrocystin-4 localizes to the basal body of the primary cilium, and is required for normal ciliogenesis in MDCK cells. Nephrocystin-4 also interacts with components of the focal adhesion and polarity complex; however, the functional implication of these various interactions is currently poorly understood (10,12).

We now report that nphp4-depletion in zebrafish embryos is associated with defects in cloaca formation. We attribute this abnormality to a migratory defect of the most distal pronephric duct cells. In wild-type embryos, these cells migrate towards the prospective cloaca position and fuse with the ectodermal cells to form the cloaca opening. Our time-lapse movies revealed that the leading pronephric duct cells in nphp4-deficient zebrafish embryos fail to form cell protrusions and do not exhibit directional migration towards the ectodermal cells of the prospective cloaca opening.

Nephrocystin-4 forms a complex with the tight junction proteins PALS1/PATJ and Par6 proteins (10). Par6 also participates in TGF-β signaling, a key regulator of renal fibrosis (29). Since TGF-β-dependent phosphorylation of Par6 facilitates tight junction dissolution and epithelial-to-mesenchymal transition in mammary gland epithelial cells (30), it is conceivable that nephrocystin-4 mediates the migration towards ectodermal cells through interaction with Par6. Cloaca obstruction is also observed in zebrafish embryos with defective Bmp signaling in the ventral mesoderm/proctodeum designated to form the cloaca (17,18,31). Similar to nphp4 morphants, Bmp-defective embryos exhibit abnormal programmed cell death required for cloaca opening. However, in contrast to Bmp mutants (17,18), prdm1 and voxl staining demonstrate that the ectoderm of the prospective cloaca is properly specified in nphp4 morphants. Thus, nphp4 appears to instruct the distal pronephric duct cells to migrate towards the ectodermal cells that subsequently undergo...
apotosis to form the cloaca opening. Our findings suggest that the programmed cell death of ectodermal cells required for cloaca formation is a non-cell autonomous, nphp4-dependent event, initiated by distal pronephric duct cells.

Previous studies have demonstrated the physical interaction between nephrocystin-1 and -4 (27). Our studies confirm the genetic interaction between nphp1 and nphp4 during zebrafish pronephros development and specification of a normal body axis; simultaneous depletion of the two proteins leads to an increase of cilia-related phenotypes, including pronephric cyst formation and laterality defects. However, nphp1 depletion does not promote cloaca obstruction, suggesting that nephrocystin-4 acts independently of nephrocystin-1 to control the migration and fusion of distal pronephric duct cells. Our data, demonstrating that nephrocystins can autonomously engage in cellular functions may explain differences in disease manifestations observed in patients with nephropathies.

MATERIALS AND METHODS

Zebrafish lines

Zebrafish of wild-type AB/TL and transgenic strains were maintained and raised as described (32). The following transgenic and mutant zebrafish strains were used: wtlb:gfp (19), Cldn2b:lum-gfp (20), Oval, HsP:noggin3 (26). Staging was performed according to Kimmel (35).

Zebrafish embryo manipulations

Fertilized eggs were microinjected with 4 nl of injection solution at the one-to-two-cell stage with morpholino (MO; Genetools LLC, Philomath, OR, USA) diluted in 200 mM KCl, 0.1% Phenol Red and 10 mM HEPES. For ISHs and antibody staining, embryos were anesthetized and fixed at the desired stage in 4% PFA overnight, transferred in methanol and stored at -20°C until stained. Two separate MOs targeting zebrafish nphp4 were designed (Genetools), a translation-blocking MO with the sequence GCCTTCTCCACCTGAGCATCAGAG (nphp4 ATG) and an MO targeted against the splice donor site of exon1 with the sequence ATTTATCCCATCCACTGGTC (nphp4 Ex1). The MO against nphp1, targeted to the ATG region, had the sequence CCCCCTCTCTTTGGAGGCATTTG (nphp1 ATG). Pard6 MO targeting splice donor site of exon 2, GAACGCAGTGAGCTGACCTTGTC (pard6 Ex2). All MOs were co-injected with p53 MO to reduce the toxicity of these reagents (34). Phenotype rescue experiments were attempted using in vitro transcribed full-length human and MO-resistant zebrafish nphp4 mRNA, which were co-injected with nphp4 ATG MO at concentrations 100, 200 and 500 ng/μl.

Imaging

Embryos were analyzed at the stage of interest under a Leica MZ16 stereo microscope (Leica, Solms, Germany). DIC and non-confocal fluorescent images were taken with a SPOT Insight Fire Wire System (Diagnostic Instruments, Sterling Heights, MI, USA). Confocal images and confocal time-lapse movies were taken with Zeiss LSM510 microscope and processed with LSM (Zeiss) and Imaris (Bitplain) software.

Immunohistochemistry and ISH

ISH was performed as described previously (35). Antisense probes for nphp4, prdm1, tbx2b and gata3 were amplified from zebrafish embryonic cDNA, cloned in TOPO (Invitrogen) and linearized with corresponding restriction enzymes. Dig-labeled antisense RNA was synthesized using Roche Dig labeling kit (Roche). Primary antibodies rabbit polyclonal anti-GFP (Biozol), rabbit anti-PKC zeta (Santa Cruz) and mouse anti-acetylated tubulin (Sigma) were used in a 1:500 dilution. Secondary anti-mouse and anti-rabbit antibodies labeled with Alexa488, Alexa546 or Cy3 antibodies were obtained from Jackson Immuno Research Laboratories. For imaging the cilia of the KV, 6-somite-stage embryos were fixed in Dent’s fixative and stained for acetylated tubulin. The KV region was dissected, embedded in fluoromount and imaged using confocal microscopy.

Paraffin sections and eosin/hematoxylin staining

For histological studies, embryos were anesthetized and fixed O/N in 4% PFA. Further, embryos were dehydrated in ethanol series, cleared in toluol and embedded in paraffin wax. Six-micrometer-thick sections were cut using a microtome (Leica), stained for Hematoxylin and Eosin and imaged with an Axioplan2 microscope and AxioVision software (Zeiss, Germany).

Time-lapse imaging of cell migration

For time-lapse movies, we used un-injected and MO-injected Cldb-Lyn-gfp zebrafish embryos (20). The embryos were embedded in 1.5% low-melting agarose, and imaged over time at 40x, using a Zeiss LSM510 confocal microscope. Z-stacks were acquired every 3 min and processed with Imaris software.

Acridine orange staining

To assay the levels of cell death in the cloaca region, 30 hpf embryos were dechorionated and incubated in 2 μg/ml acridine orange (Sigma) solution in E3 medium for 30 min. The embryos were then washed repeatedly, embedded in low-melting agarose and imaged using a Leica MZ16 stereo microscope.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

Conflict of Interest statement. None declared.

FUNDING

This work was supported by the German Research Council (DFG) (to A.K.-Z., G.W.) and by the European Community’s
Seventh Framework Program (grant agreement number 241955, SYSCILIA) (to G.W.).

REFERENCES