The Inv compartment of renal cilia is an intraciliary signal-activating center to phosphorylate ANKS6

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Connections between cilia and renal cystic diseases are well known, yet molecular mechanisms remain undefined. Cysto-proteins localized in the Inv compartment of cilia (INV, NPHP3, NEK8, and ANKS6) constitute a distinct group. Here we created and analyzed mutant mice (G2A mice) with a defective cilia localization signal in the *Nphp3* gene. Mutant NPHP3 was absent the binding capacity of UNC119, a carrier protein responsible for the delivery of myristoylated cargo to the cilium, so ciliary localization was reduced or lost in the kidney but not in the embryonic node. Mutant mice developed renal cysts but not *situs* abnormalities. Although ciliary localization of INV, NEK8, and ANKS6 did not change in the kidneys of *Nphp3* mutant mice, ANKS6 phosphorylation was impaired. In general, ANKS6 levels decrease with age in the kidneys of wild-type mice. However, cystic kidneys in G2A and *Inv* mice maintained high levels of a non-phosphorylated form of ANKS6. We found INV and NPHP3 cooperate and promote ANKS6 phosphorylation by NEK8 in renal cilia. Thus, there is a novel signaling path from cilia in which ANKS6 functions as a signal mediator and link between cilia and the cytoplasm to regulate kidney morphogenesis.

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Nephronophthisis (NPHP) is a rare autosomal recessive disease but is the most common cause of inheritable end-stage renal failure in children and young adults.[1] NPHP is frequently associated with extrarenal symptoms.[2] The disease is classified as Senior-Loken syndrome, Joubert syndrome, Meckel-Gruber syndrome, Sensenbrenner syndrome, and short-rib polydactyly syndromes/asphyxiating thoracic dystrophy (Jeune syndrome) depending on associated anomalies.[2,3] These disorders are collectively called NPHP-related ciliopathies.[4] Twenty causative genes for NPHP have been identified, and >25 genes have been identified for NPHP-related ciliopathies.[2] Most proteins encoded by genes found to be defective in NPHP cases, called nephrocystins, localize at primary cilia but are not uniformly distributed within the cilia.[5,6]

Primary cilia are complex structures protruding from most mammalian cells, including renal epithelial cells. In contrast to motile cilia, primary cilia are usually nonmotile except for cilia within the embryonic node. The renal axoneme comprises nine microtubule doublets at the proximal portion, whereas the distal portion comprises singlet microtubules and the circular arrangement is often not preserved.[7] INV/NPHP2, nephrocystin-3 (NPHP3), NEK8/NPHP9, and ankyrin repeat and sterile alpha motif domain containing 6 (ANKS6)/NPHP16 accumulate in the Inv compartment (IC),[8–14] which overlaps with doublet-region microtubules in the renal axoneme.[7] The cilia base comprises the basal body, which is identical to the mother centriole. The transition zone is thought to function as a barrier between the cytoplasm and cilia.[15]

To understand the function of the IC, it is important to know how IC nephrocystins (INV, NPHP3, NEK8, and ANKS6) interact and establish functional modules. INV acts as a molecular anchor for NPHP3 and NEK8 in the IC of mouse renal cilia.[11] NEK8 acts downstream of Inv during pronephros morphogenesis and left-right establishment in zebrafish.[16] A recent study reported that ANKS6 acts as both an NEK8 kinase activator and a phosphorylation substrate.[13] Although INV, NPHP3, NEK8, and ANKS6 all cause common phenotypes including renal cysts and *situs* anomalies, the phenotypes particularly relevant to extrarenal observations are not identical.[9,13,17,18] Possible causes include the diversity of the extraciliary localization of IC nephrocystins because most nephrocystins are not only localized to cilia but are also found outside the cilium. One of the difficulties with analyzing the ciliary function of nephrocystins is that null

mutants of a ciliary protein abrogate both ciliary and extraciliary functions; therefore, cilia-specific disruption of a ciliary protein is needed.

The function of NPHP3, compared with other IC nephrocystins, remains poorly understood. In humans, *NPHP3* mutations represent early embryonic patterning defects comprising *situs inversus*, polydactyly, central nervous system malformations, structural heart defects, preauricular fistulas, and a range of congenital anomalies of the kidney and urinary tract.[17] Mouse NPHP3 is a 1325-amino acid protein with 3 N-terminal coiled-coil domains and 8 tetratricopeptide repeats. NPHP3 has been suggested to be involved in the regulation of noncanonical Wnt-pathway regulation and to interact with INV.[17] NPHP3 also interacts with the NEK8–ANKS6 complex.[12,13] Ciliary localization of NPHP3 is regulated by myristoylation of the second glycine.[19,20]

In the current study, we created a knock-in mouse in which the second glycine of NPHP3 was mutated to prevent ciliary localization, which allowed us to investigate the cilia-specific functions of NPHP3. Although loss of ciliary NPHP3 in renal tubules did not affect the localization of other IC nephrocystins, the mutant developed renal cysts. We observed that NPHP3 together with INV assists ANKS6 phosphorylation by NEK8 in cilia and that ANKS6 phosphorylation is perturbed in the *Nphp3* mutant as well as *Inv* mutants.

RESULTS

A G2A mutation of NPHP3 results in renal cyst formation but not situs inversus

To create a mutation in *Nphp3* that disrupts cilia targeting,[20] the second glycine of NPHP3 was replaced with alanine by homologous recombination of a neo cassette in embryonic stem cells (Supplementary Figure S1A). Two chimeric mouse lines (lines 35 and 88) were generated using a standard protocol. The chimeric mice were mated with C57BL/6 mice, and germline transmission was achieved. Southern blot analysis of knock-in mice showed appropriate targeting, and the mutation was verified using Sanger sequencing (Supplementary Figure S1B and C). The resultant mice were mated with CAG-Cre mice to remove the neo cassette (Supplementary Figure S1D). The neo cassette–deleted mice were named $Nphp3^{G2A}$ mice. From $Nphp3^{G2A}$ heterozygous crosses, we obtained homozygous $Nphp3^{G2A/G2A}$ mice (G2A mice) at the expected mendelian ratios.

At 3 weeks of age, severely enlarged kidneys with a cystic appearance developed in G2A mice (Figure 1a and b). Histologic examination confirmed multiple enlarged cysts (Figure 1c–e). We did not observe any *situs* abnormalities in the heart apex or stomach (Figure 1a). Both lines (lines 35 and 88) showed an identical phenotype (line 35 in Figure 1d; line 88 in Supplementary Figure S2A). Analysis was performed using line 35.

There was no difference in kidney-to-body weight ratios between wild-type (WT) and G2A mice at birth; however, the mutant kidneys were significantly enlarged by postnatal day 10 (Supplementary Figure S2B). No cystic changes in the kidneys of $Nphp3^{G2A/+}$ mice were observed until 10 months of age (Supplementary Figure S2C).

Histologically visible glomerular cysts in the G2A kidney were observed as early as embryonic day 16.5 (Figure 1c). At birth, the proximal tubule *Lotus tetragonolobus* lectin–positive and the *Lycopersicon esculentum* lectin–positive thick ascending limbs of the loop of Henle had expanded (Figure 1e), whereas no cysts were observed in *Dolichos biflorus* agglutinin (DBA)-positive collecting ducts. At 10 days of age, tubular expansion was prominent in *Lotus tetragonolobus* lectin–positive tubules and was also observed in *Dolichos biflorus* agglutinin–positive tubules in the G2A kidney (Figure 1e). These histologic features of a G2A kidney were the equivalent of human NPHP type 3.[17,21,22]

Impaired ciliary localization of NPHP3 in G2A knock-in mice kidney

We examined whether the G2A mutation abrogated NPHP3 cilia localization *in vivo*. NPHP3 immunoreactivity was observed in the proximal region of renal cilia in WT mice (Figure 2a). In WT mice, the NPHP3 signal was observed in 69.4% (109/157) of cilia and was absent in 30.6% (48/157) (Figure 2b). In G2A, the NPHP3 signal was observed in 35.5% (59/166) and was absent in 64.5% (107/166) of cilia (Figure 2b). The G2A kidney had fewer cilia with IC-localized NPHP3 than the WT kidney (G2A, 12.0%; WT: 51.6%). The intensity of the IC-localized NPHP3 signal was significantly decreased in G2A renal cilia (Supplementary Figure S3).

Northern blot analysis showed no differences in the size or amount of *Nphp3* mRNA between WT and G2A kidneys (Figure 2c). Protein levels of NPHP3 were almost identical between WT and G2A kidneys (Figure 2d). These results excluded the possibility that the reduced amount of *Nphp3* mRNA or NPHP3 protein caused a reduced intensity of the ciliary NPHP3 signal in G2A renal cilia.

Myristoyl-binding protein, uncoordinated (UNC) 119, is reported to be required for correct ciliary localization of NPHP3 *in vitro*.[19] Recent investigations have indicated that UNC119 forms a hydrophobic pocket to accommodate the myristoyl moiety of NPHP3.[19,23] We therefore examined whether G2A NPHP3 loses its ability to bind UNC119 *in vivo*. The UNC119 antibody was used for immunoprecipitation because the NPHP3 antibody is not suitable. UNC119 was detected binding to NPHP3 in WT mice but not G2A mice (Figure 2e), indicating that UNC119 can bind normal NPHP3 but not G2A mutant NPHP3. This supports the theory that UNC119 binding to NPHP3 is essential for cilia localization of NPHP3 *in vivo*. Our results are consistent with the development of renal cysts in G2A mice being caused by a decreased amount of cilia-localized NPHP3.

Null mutation of *Nphp3* results in *situs inversus*[17]; however, NPHP3 localization in node cilia has not been examined. In G2A, the percentage of NPHP3-positive node cilia was not significantly altered compared with that in WT (G2A: 34% [40/119], WT: 36% [(42/113]) (Supplementary Figure S4).