Loss of the Ciliary Kinase Nek8 Causes Left-Right Asymmetry Defects

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ABSTRACT

A missense mutation in mouse Nek8, which encodes a ciliary kinase, produces the juvenile cystic kidneys (jck) model of polycystic kidney disease, but the functions of Nek8 are incompletely understood. Here, we generated a Nek8-null allele and found that homozygous mutant mice die at birth and exhibit randomization of left-right asymmetry, cardiac anomalies, and glomerular kidney cysts. The requirement for Nek8 in left-right patterning is conserved, as knockdown of the zebrafish ortholog caused randomized heart looping. Ciliogenesis was intact in Nek8-deficient embryos and cells, but we observed misexpression of left-sided marker genes early in development, suggesting that nodal ciliary signaling was perturbed. We also generated jck/Nek8 compound heterozygotes; these mutants developed less severe cystic disease than jck homozygotes and provided genetic evidence that the jck allele may encode a gain-of-function protein. Notably, NEK8 and polycystin-2 (PC2) proteins interact, and we found that Nek8<sup>−/−</sup> and Pkd2<sup>−/−</sup> embryonic phenotypes are strikingly similar. Nek8-deficient embryos and cells did express PC2 normally, which localized properly to the cilia. However, similar to cells lacking PC2, NEK8-depleted inner medullary collecting duct cells exhibited a defective response to fluid shear, suggesting that NEK8 may play a role in mediating PC2-dependent signaling.


Cystic diseases of the kidney are prevalent in the human population and there is compelling interest in understanding the functions of the associated genes. The most common human cystic disease is autosomal dominant polycystic kidney disease (ADPKD) with an incidence of 1 in 750 individuals. ADPKD is caused by mutations in either PKD1 or PKD2, which encode the polycystin proteins PC1 and PC2, respectively. PC1 is a G-protein coupled receptor that partners with PC2, a transient receptor potential polycystic family ion channel that regulates both calcium entry into and intracellular calcium release within renal epithelial cells. The nephronophthisis (NPHP) types 1–11 and NPHPL1 comprise a less common group of autosomal recessive disorders, but they are collectively the leading genetic cause of ESRD within the first 3 decades of life. We previously reported that the mouse juvenile cystic kidneys (jck) model of recessive polycystic kidney disease (PKD) is due to a missense mutation in the Nek8 gene; jck animals develop cysts in distal nephron segments and collecting ducts within the first week of life and the disease rapidly progresses, causing renal failure and death by 6 months of age. Nek8 encodes a highly conserved member
of the Nek family of serine/threonine kinases, characterized by an N-terminal kinase domain homologous to that of the *Aspergillus nidulans* NIMA protein that controls cell cycle entry during mitosis. Mammalian Neks have divergent C termini, and the Nek8 C terminus contains multiple repeats that are homologous to RCC1, a guanine nucleotide exchange factor for Ran that is required for chromosome condensation, although Nek8 guanine nucleotide exchange factor activity has not been demonstrated. The jck mutation in Nek8 results in a glycine to valine change in the second RCC repeat of the protein. Subsequent studies have reported missense mutations in the RCC domain-encoding regions of both rat *Nek8* in the Lewis PKD model and human *NEK8*, in which three independent mutations were identified from 700 NPHP patients, making it a candidate for NPHP type 9. Together, these data demonstrate that the Nek8 C terminus is critical for its function and the protein plays an important role in the maintenance of renal tubule integrity in the postnatal kidney. Of note, all of the reported mutations of Nek8 are missense mutations. We describe here the generation of a mouse carrying a null mutation of *Nek8*; in this line, mutant pups die shortly after birth and exhibit a variety of laterality defects and associated cardiac anomalies.

Mutations in several PKD-associated genes cause laterality defects in the developing mouse, and dysfunction of primary cilia is the critical link between the phenotypes. Kidney tubular epithelial cells each express a primary cilium that collectively act as mechanosensors of fluid flow through the tubules, transducing calcium-dependent signals hypothesized to keep the cells in a differentiated state. Early in development, the establishment of appropriate asymmetry in mammals requires the functions of two populations of cilia within the node, a pit in the ventral surface of the embryo. Motile cilia rotate and generate a leftward fluid flow, and primary cilia subsequently produce a left-sided calcium gradient. Mutations in *Pkd2*, *Kif3A*, and *Tg737(Ift88)* illustrate how cilia are critical signaling centers in the kidney and the node. Loss of *Pkd2* from the adult kidney results in PKD, whereas *Pkd2*-null embryos exhibit left-right defects. Functionally, the PC1/PC2 complex mediates calcium influx in response to flow-induced shear stress in cultured renal cells, and loss of either protein abolishes the response; similarly, *Pkd2*-null embryos do not exhibit a left-sided calcium signal. Heterotaxy also occurs with loss of the *Pkd1*-related gene, *Pkd1II*; the gene product localizes to cilia and interacts with PC2, forming a node-specific complex required for proper asymmetry.

Alternatively, mutations that disrupt cilia assembly and/or structure cause both phenomena. PKD arises in the kidney-specific deletion of *Kif3A*, which encodes a kinesin subunit required for ciliogenesis, and in *Tg737 (Ift88/polaris)* hypomorphs that exhibit shorter cilia with bulbcd tips. Laterality defects occur upon targeted disruption of *Kif3A*, *Kif3B*, or *Tg737 (Ift88)*; each of these knockouts lack nodal cilia and die mid-gestation with randomized heart looping. Downstream of flow-induced calcium signals, left-sided determinants are expressed in the early embryo. The *Nodal* gene encodes a ligand of the TGF-β family and is expressed first in the node and subsequently in the left lateral plate mesoderm (LPM), and the transcription factor *Pitx2* is later expressed in the left LPM; mutant alleles of each of these genes are embryonic lethal with laterality defects.

A majority of proteins associated with human cystic diseases, including the polycystins and nephrocystins, localize to either the axoneme and/or the basal body of primary cilia. Similarly, Nek8 localizes to the base of renal cilia and is thus far the only kinase that has been localized to the ciliary axoneme. Cilia defects are indeed present in *jck* cystic renal epithelia and cells cultured from affected kidneys; mutant cilia are elongated and exhibit enhanced ciliary localization of PC1 and PC2 compared with wild-type cilia. In addition, PC2 is hyperphosphorylated in *jck* kidneys and both wild-type and mutant Nek8 communoprecipitate with PC2 from kidney lysates, suggesting that these proteins perform critical cooperative functions.

We originally hypothesized that the *jck* allele is hypomorphic because the only overt phenotype in homozygotes is PKD, mutant protein is expressed in the kidneys, and morpholino-mediated reduction of Nek8 in zebrafish causes pronephric cysts. Here, we characterize the Nek8-null mutant and determine that ciliogenesis is intact, but left-right patterning is perturbed. We revisit morpholino knockdown in zebrafish and demonstrate that Nek8 depletion causes laterality defects, confirming a conserved role for Nek8 in left-right patterning. Furthermore, we generated *jck/Nek8* compound heterozygotes; surprisingly, renal cystic disease in these mutants is much less severe than in *jck* homozygotes, suggesting the *jck* allele of Nek8 encodes a gain-of-function protein. Finally, there is marked similarity of Nek8–/– and Pkd2–/– embryonic phenotypes and we determine that PC2-associated activity is abrogated in cells lacking Nek8.

**RESULTS**

**Generation of Nek8-Null Mouse Model**

To generate a Nek8-null allele, we obtained a C57BL/6 (B6) bacterial artificial chromosome containing the Nek8 genomic locus. Nek8 exon 3 was targeted by recombinering using a cassette composed of an internal ribosomal entry sequence (IRES), the lacZ gene, and the neomycin resistance gene flanked by FLP recombinase recognition sites (Figure 1A). Nek8<lacZ was designed to encode a truncated protein lacking the kinase domain and a β-galactosidase reporter to recapitulate endogenous Nek8 expression. Nek8<lacZ-positive B6/129 hybrid embryonic stem cell clones were injected into B6 blastocysts, and the resulting chimeras were bred to obtain germline transmission of the Nek8<lacZ allele. Heterozygous Nek8<lacZ carriers are viable and fertile with no obvious phenotypes, whereas Nek8<lacZ homozygous pups die shortly after birth; carriers and homozygotes are obtained at expected 50% and 25% frequencies, respectively.
Heterozygous- and homozygous-Nek8:lacZ embryos were analyzed for β-galactosidase activity, but enzyme activity was not detected in PCR-positive samples (Figure 1B and data not shown). We derived mouse embryo fibroblasts (MEFs) from E14.5 wild-type and homozygous embryos, analyzed the cDNA from the cells, and discovered the targeted exon 3 is spliced out of the mutant transcript, rendering the reporter ineffective (Figure 1C and data not shown). However, the aberrant splicing of exons 2–4 causes a frameshift and a creates premature stop codon resulting in a true null allele of Nek8, which we herein refer to as Nek8−/−, because no protein is detected in homozygotes by Western blot analysis (Figure 1D).

**Nek8−/− Embryos Exhibit Randomization of Left-Right Asymmetry and Cardiac Defects**

Nek8−/− mice die shortly after birth and mid-gestation mutants frequently exhibit edema and focal hemorrhages that are suggestive of cardiac defects (Figure 2, A–D). Upon autopsy, we observe randomization of left-right asymmetry in the mutants. The characteristic asymmetry of mouse body plan is defined in the thorax by a four-lobed right lung and single left lung lobe with a left-oriented heart apex; in the abdomen, the stomach and adjacent spleen reside on the left side (Figure 2, E–G). Approximately 15% of Nek8−/− mutants have normal body asymmetry, whereas 24% display *situs inversus totalis*, in which the body plan is a mirror image to that of a normal fetus (Figure 2, E, F, and H). However, a majority of Nek8−/− mutants (61%) exhibit right pulmonary isomerism (RPI), a “duplication” of the four-lobed right lung on the left side (Figure 2I); one-third of these mutants have normal abdominal situs, whereas two-thirds have inverted abdominal situs (Table 1).

Mutants with laterality defects often have major structural cardiac anomalies. Therefore, magnetic resonance imaging (MRI) and histologic analyses were performed to assess the hearts of Nek8−/− embryos. In three mutants with RPI we identified double outlet right ventricle (DORV), a defect in which both the pulmonary artery and aorta exit the right ventricle of the heart, accompanied by atrial and ventricular septal defects (A/VSD) (Figure 2, J and K and data not shown). DORV is best exemplified in lateral MRI images: the pulmonary artery and aorta are captured in separate images in a wild-type embryo, but the structures run parallel to each other in the same image in the Nek8−/− mice. Although mice with *situs inversus totalis* can be viable, as in *inversus* mutant mice, no Nek8−/− mice survive the perinatal period and we found A/VSD in mutants with complete *situs inversus* (Figure 2, L and M).

To further examine the requirement for Nek8 in establishing normal patterning in a vertebrate system, we utilized morpholino knockdown of Nek8 in zebrafish. We previously used this approach to evaluate cystogenesis in adult fish, but laterality was not assessed. In this analysis, randomization of heart looping occurs in a dose-dependent manner in zebrafish embryos injected with a morpholino targeted to the initiator methionine codon of Nek8 (P<0.001; Figure 2N).

**Early Laterality Markers Are Misexpressed in Nek8−/− Embryos but Nodal Cilia Are Intact**

Left-right asymmetry mutants have defects in nodal signaling that are assessed via *in situ* hybridization of Nodal and Pitx2 between E8.0 and E8.5.43-45 In wild-type presomite embryos, *Nodal* expression is symmetrical in the node. By the 3–4 somite stage, it becomes asymmetrically expressed in the node, staining stronger on the left side than the right, and expression is restricted to the left LPM (Figure 3A). In contrast, in Nek8−/− embryos at the 3–4 somite stage, *Nodal* expression within the nodes was always symmetrical (n=9) and was absent from the LPM in seven of nine embryos, bilateral in one embryo, and expressed in the left LPM in one embryo (Figure 3, B–D and Supplemental Figure 1). We examined embryos at the 6–8 somite stage for *Pitx2* expression, which is normally apparent in the headfolds and a discrete region of the left LPM of wild-type embryos (Figure 3E). *Pitx2* was appropriately expressed in the headfolds of stage-matched Nek8−/− embryos (n=6), but was absent from the LPM in four of six mutants (Figure 3F), right-sided in one mutant, and correctly expressed in one mutant.

Several laterality mutants have short nodal cilia or lack them altogether, which disrupts asymmetric signaling downstream of nodal flow. Because Nek8 localizes to renal epithelial cilia, we examined the nodal cilia of E8.0 embryos using immunofluorescent detection of the ciliary marker acetylated α-tubulin and the anti-Nek8 antibody. Wild-type and
Nek8-/- nodes are indistinguishable (Figure 3, G and H) and NEK8 is expressed in the nodal cilia of wild-type E8.0 embryos (Figure 3I). Taken together, the in situ hybridization and immunofluorescence data suggest that Nek8 performs a critical function in laterality determination, but is not directly required for ciliogenesis.

Nek8-/- Kidneys Do Not Develop PKD
Nek8-/- pups die within hours of birth but they can produce urine, indicating that the kidney is functional. We examined renal development in Nek8-/- embryos and the mutant kidneys develop similarly to those of wild-type through E15.5; however, by E16.5 and through P0, glomerular cysts are apparent in periodic acid–Schiff (PAS)-stained samples (data not shown and Figure 4, A and B). In addition, P0 Nek8-/- proximal tubules appear thin-walled and slightly dilated compared with the wild-type proximal tubules, which are thicker due to intact brush borders on the apical surfaces. To further assess the tubular phenotype, we stained kidneys with the fluorescein-conjugated Lotus tetragonolobus lectin (LTL) and Dolichos biflorus agglutinin (DBA) to identify the proximal tubules and collecting ducts, respectively. LTL staining confirmed that the Nek8-/- proximal tubules are indeed dilated at P0 but not at E18.5, whereas the DBA staining showed that P0 mutant collecting ducts are largely unaffected (Figure 4, C–F and data not shown). Importantly, the abundance and appearance of cilia in wild-type and Nek8-/- tubes is similar (Figure 4, C–F, acetylated α-tubulin in red).

Although ciliogenesis appears normal in Nek8 mutant nodes and kidneys, it is difficult to identify overt length or structural defects via immunofluorescence in these tissues. Therefore, we analyzed cilia in control and Nek8-deficient mouse inner medullary collecting duct (IMCD) cells (Figure 4, G and H) as well as in wild-type and null MEFs (Figure 4, J and K). To generate Nek8-depleted IMCD cells, small hairpin RNA (shRNA) against Nek8 was introduced and individual clones were isolated and expanded; NEK8 is not detected via Western blot with N8KD10 compared with the vector control clonal (pLKO) line (Figure 4I). There are no morphologic or length differences between the cilia of wild-type and Nek8-deficient cells in culture: pLKO and N8KD10

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**Figure 2.** Nek8-/- mice exhibit laterality and cardiac defects. (A–D) Lateral views of littermates. E14.5 wild-type embryo (A) compared with Nek8-/- embryo (B) with edema (arrows) and focal hemorrhages. E16.5 wild-type embryo (C) is larger than the mutant (D). (E–H) E18.5 wild-type compared with a mutant with SI. (E) Lateral views of a wild-type embryo (left) and a null embryo (right) illustrate abdominal situs defect in the mutant. (F) Frontal views of the embryos in E, in which internal organs mirror each other. (G and H) Hearts and lung lobes dissected out of the thoracic cavities of embryos in E and F. (G) Wild-type lungs contain four right lobes and one left lobe (R4, L1). (H) The mutant with SI has one right lobe and four left lobes (R1, L4). (I) Heart and lungs dissected out of a mutant with RPI; right and left lungs are four-lobed (R4, L4). (J–M) Nek8-/- embryos exhibit cardiac defects. (J and K) MRI images through thoraces of a wild-type and a null littermate with RPI. Sagittal views show that wild-type pulmonary artery (J) and aorta (J’) are present in distinct images. (K) Mutant pulmonary artery and aorta run parallel in the same image. (L and M) H&E-stained transverse cardiac sections. (L) The wild-type heart has intact atrial (*) and ventricular septa. (M) The SI mutant heart exhibits atrial (*) and ventricular (arrow) septal defects. (N) Cardiac looping occurs in Nek8-ATG MO zebrafish embryos in a dose-dependent manner. Scale bars, 1.0 mm in A–I; 100 μm in J–M. SI, situs inversus; s, stomach; sp, spleen; R, right; L, left; h, heart; pa, pulmonary artery; a, aorta; vs, ventricular septa.
cilia lengths are $3.3 \pm 1 \mu m$ and $3.0 \pm 0.6 \mu m$, respectively; wild-type and $Nek8^{+/–}$ MEF cilia lengths are $2.9 \pm 0.7$ and $2.8 \pm 0.9 \mu m$, respectively. Statistical analysis using a nonparametric ANOVA test reveals that these differences are not significant, suggesting that ciliogenesis can occur normally in the absence of NEK8.

**jck/Nek8**—Compound Heterozygotes Have Less Severe Cystic Disease Than jck Homozygotes

We hypothesized that the jck allele of Nek8 is hypomorphic, and to test this we intercrossed jck/+ and Nek8+/- mice to generate compound heterozygous jck/Nek8- animals. Wild-type, jck/+ , jck/Nek8-, and jck/jck mice were sacrificed at 7 weeks of age, the kidneys were weighed and histology was performed to assess disease severity. Surprisingly, jck/Nek8- animals develop very mild renal cystic disease and the combined kidney weights are not much greater than those of wild-type mice (Figure 5, A–D).

Because Nek8-/– pups die at birth, we analyzed the cystogenic potential of null, jck, and compound mutant embryonic kidneys in an explant culture assay.46,47 Kidneys were removed from E14.5 embryos and cultured for 5 days with or without a cyst-inducing cAMP analog. In the presence of 8-Bromo-cAMP, jck/jck kidneys develop the greatest numbers of cysts, whereas Nek8-/– kidneys have the least cystogenic potential of all the genotypes (Figure 5, E and F). Taken together, the findings that jck/Nek8- adult and cultured embryonic kidneys develop less severe cystic disease than homozygous jck kidneys, and that the Nek8-null kidney explants do not develop tubular cysts, suggest the jck mutation results in a gain-of-function protein.

**Nek8-/– Phenotypes Resemble Those Caused by Loss of PC2 Function**

The Nek8-/– phenotypes are remarkably similar to those of Pkd2−/− embryos, including randomization of left-right asymmetry, RPI, cardiac malformations, and glomerular cysts in late-gestation kidneys,21,39 and NEK8 and PC2 have been shown to interact.37 Therefore, we sought to determine whether the Nek8−/− defects are due to loss of PC2 expression or a change in localization. PC2 localizes to all node monocilia18 and we observe its localization is identical in E7.75 wild-type and Nek8−/− embryo nodes (Figure 6, A and B). In addition, we do not detect gross differences in PC2 levels via Western blot between littermatched E18.5 wild-type and Nek8−/− kidney lysates (Figure 6C).

Because PC2 expression/localization is intact in Nek8-null tissues, we hypothesized that PC2 activity may be perturbed in NEK8-deficient cells. To investigate PC2 function, we subjected cultured wild-type and N8KD10 (knockdown) IMCD cells to fluid shear stress and measured changes in PC2-dependent intracellular Ca$^{2+}$ influx across the membrane using live cell microscopy; PC2 is properly localized in wild-type and N8KD10 cilia under the conditions used in the assay (Supplemental Figure 2). The cells stably express a genetically encoded calcium indicator, GCaMP3, used to monitor changes in intracellular Ca$^{2+}$ levels.48,49 Live fluorescence image time series were acquired while Ca$^{2+}$ depleted cells were exposed to fluid shear stress. As expected, wild-type IMCD cells exhibit a robust response to fluid shear stress (Figure 6, D and G). PC2-depleted IMCD cells50 were used as a negative control and show significantly reduced fluid flow response (Figure 6, E and G). The N8KD10 cells also have a reduced response to flow, suggesting that PC2 function may be perturbed in the absence of Nek8 (Figure 6, F and G).

**DISCUSSION**

The Nek8 gene, which is mutated in the jck mouse model of PKD, encodes the only kinase that to date has been localized to the ciliary axoneme. In this study we report that its targeted disruption in the mouse is lethal and causes heterogeneity defects, further demonstrating a link between cystogenesis and left-right asymmetry. Importantly, there is a conserved requirement for the gene in the specification of vertebrate left-right asymmetry as knockdown of Nek8 expression in zebrafish causes randomization of heart looping, a result described both here and by an independent group.31

A small number of genes implicated in human renal cystic diseases cause both PKD and laterality defects when disrupted in the mouse. Three such examples include Pkd2, inversin, and Nphp3; Pkd2 is mutated is 15% of human ADPKD cases and mutations in INVS (inversin) and NPHP3 cause NPHP types 2 and 3, respectively.1,52,53 NEK8 has been identified as an interacting partner of each of the mouse protein counterparts: NEK8 and PC2 reciprocally communoprecipitate from kidney and cell lysates,37 and NEK8 has been shown to colocalize with inversin and NPH3 in the base of the cilium in an inversin-dependent manner.35 The association of NEK8 with each of these proteins is intriguing due to the similarities and some of the differences we identified in the jck and Nek8-null mouse models compared with Pkd2, inversin, and Nphp3 mutants.

With regard to left-right asymmetry, Pkd2−/− embryos die in utero and 90% of the mutants exhibit RPI,39,21,54 whereas a majority of inversin mutants exhibit situs inversus.55-57 The observation that Pkd2−/− early embryo nodes fail to elicit an asymmetric calcium signal in response to flow19 likely accounts for the absence of Nodal and Pitx2 expression in the LPM, causing the high percentage of RPI in mutant embryos.
Interestingly, inversin nodal cilia produce a weak net leftward flow much slower than wild type, which may cause right-sided Nodal expression, more frequently lead to inversion of the body plan, and preclude the development of RPI in the thorax. A majority of Nek8-null embryos exhibit RPI whereas only 24% have situs inversus, and both Nodal and Pitx2 are frequently absent from the left LPM of mutants; thus, Nek8−/− mutants are more similar to Pkd2−/− than to the inversin mutant mice. We determined that Nek8-null phenotypes are not due to loss of PC2, because the protein is expressed and properly localized in mutant embryos, tissues, and cells. Therefore, we utilized a fluid flow assay to measure PC2-dependent mechanosensitive activity in cells lacking Nek8. IMCD cells stably knocked down for Pkd2 or Nek8 exhibit decreased calcium influx in response to shear stress compared with wild-type cells, suggesting PC2 is not fully functional in the absence of NEK8. Given that PC2 is required for both the IMCD cell responses to flow and the calcium gradients detected in the mouse embryo node, we hypothesize that the ability of PC2 to “sense” nodal flow and elicit a left-sided signal is diminished in nodes lacking NEK8. Perhaps NEK8 is a critical component of the PC2 channel complex that is required to mediate the signaling processes downstream of nodal calcium flux that are indispensable for the establishment of appropriate left-right asymmetry.

Similarly to other heterotaxy mutants, Nek8−/− embryos exhibit severe cardiac malformations that include DORV coupled with A/VSD. It is not surprising to find such abnormalities in animals with RPI, because aberrant heart development accompanies the inappropriate symmetry in the thoracic cavity. However, we identified cardiac septal defects in mutants with complete situs inversus, a condition that is not lethal if the inverted body plan is appropriately established, suggesting that Nek8 may play a role in cardiac morphogenesis independent of the initial left-right asymmetry defect, as is the case for inversin mutants. It was originally reported that nearly 100% of inversin mutants display situs inversus totalis; therefore, the thoracic situs was “normal reversed” compared with wild-type littermates. However, a later study of inversin homozygotes found that 90% of the pups did have situs inversus, but 10% were identified with situs solitus; furthermore, cardiac malformations including outflow tract and septal defects were found in 37% of the pups, including those few with normal thoracic situs. Perhaps Nek8 and inversin are required in the cardiac cilia present in mid-gestation mouse embryos, a biologic question best addressed by conditional deletion of the gene.

Figure 3. Laterality marker genes are misexpressed in Nek8−/− embryos but nodal cilia are intact. (A–F) In situ hybridization of laterality markers confirms signaling defects in Nek8−/− embryos (arrows indicate LPM). (A–D) Nodal is expressed in the node proper in 4–5 somite wild-type and null embryos, as well as in the wild-type left LPM shown in A. Mutant embryos lack LPM staining (B) or have bilateral (C) or left LPM expression (D). Pitx2 is expressed in 6 somite wild-type and null headfolds and in the wild-type left LPM (E), whereas mutant embryos usually lack LPM expression (F). Anti-acetylated tubulin immunofluorescence of E8.0 embryos shows that wild-type nodal cilia (G) (n=10) and null nodal cilia (n=5) (H) appear similar. High-magnification view of wild-type nodal cilia (I) (arrows) stained with anti-acetylated tubulin (green) and anti-NEK8 (red); NEK8 localizes to all node monocilia (merge; inset, NEK8 at base of cilia) and is not detected in Nek8−/− nodal cilia (J). Scale bars, 200 μm in A–I. R, right; L, left.
Our original observations that PKD develops in jck homozygotes, affected animals express the mutant protein, and morpholino knockdown of zebrafish Nek8 causes pronephric cysts all suggested the jck allele represented a partial loss of function. This hypothesis was consistent with analysis of the Ift88 gene, which initially established the critical link between renal cysts and left-right asymmetry, because the hypomorphic Tg737 allele causes PKD, whereas the null mutation causes laterality defects.26,28 Similarly, the pcy mouse carries a missense mutation in the Nphp3 gene and develops slowly progressive PKD, whereas null mutants die mid-gestation with laterality and severe, complex cardiac defects.52,62

Of note, pcy/Nphp32 compound heterozygous mice are viable and develop severely cystic kidneys by week 12. This intermediate phenotype indicates that the pcy allele of the gene is hypomorphic compared with the null allele.62 We crossed the Nek8-null allele with jck carriers anticipating a similar result; that is, compound heterozygous mice would have a PKD phenotype more severe than that of jck homozygous jck mutants. The null pups die shortly after birth and the kidneys do have glomerular cysts and dilated proximal tubules, but the defect is likely due to impaired glomerular function rather than early signs of NEK8-dependent PKD. Supporting this notion is that jck mutant glomeruli and proximal tubules do not develop cysts; it is the distal DBA-positive collecting ducts that are affected,36,37,63 Therefore, we utilized embryonic kidney explant culture to further explore the cystic potential of the mutant kidneys.46 This in vitro system recapitulates what we observe in the mice: Nek82/2 kidneys do not develop tubular cysts, whereas jck/jck homozygous kidneys are dramatically affected and jck/2 kidneys have an intermediate phenotype.

The data therefore suggest that the jck allele encodes a gain-of-function protein, a hypothesis not initially considered because jck/+ adults do not exhibit PKD. However, Natoli et al. performed explant cultures with kidneys harvested a day earlier than in our study, and observed a significant difference between wild-type and jck/+ kidneys, reporting cyst percentages of 2.5% for wild-type and 7.5% for jck/+ compared with 15% for jck/jck kidneys.47 In the adult jck/+ kidney, perhaps the presence of wild-type NEK8 is protective against the deleterious effect of the mutant protein. It is currently unclear how the jck allele may result in a gain-of-function protein. A recent study demonstrated that wild-type NEK8 and the jck-equivalent NEK8G442V variant were similarly active in in vitro kinase assays toward β-casein and histone, but it remains unknown whether NEK8G442V exhibits altered protein function.

![Figure 4](https://www.jasn.org/article-figures/Figure4.jpg)

**Figure 4.** Nek8−/− kidneys exhibit glomerular cysts and proximal tubule dilation. (A and B) PAS-stained kidneys from P0 pups. (A and A') The wild-type kidney has normal glomeruli and proximal tubules stain pale purple (arrows). (B and B') The mutant kidney develops glomerular cysts and proximal tubules are distended. FITC-conjugated LTL lectin marks proximal tubules (arrows) and confirms that the wild-type tubules are intact (C), whereas the mutant tubules adjacent to glomerular cysts are distended (D). (E and F) FITC-conjugated DBA lectin marks the collecting ducts (arrows). Wild-type tubules (E) and mutant tubules (F) are similar in size and shape and cilia are present in both (red puncta, anti-acetylated tubulin immunofluorescence). Cilia (arrows; red, anti-acetylated tubulin) of control (pLKO) (G) and Nek8-knockdown (N8KD10) (H) IMCD cells are of similar morphology and length. (I) Western analysis of IMCD lysates illustrates N8KD10 cells lack NEK8 (arrow) compared with the larger nonspecific band recognized by the antibody. Cilia of wild-type (J) and Nek8−/− (K) MEFs are of similar morphology and length. Scale bars, 100 μm in A–F; 10 μm in G, H, J, and K. Original magnification, ×10 in A and B; ×40 images of boxed region in A' and B’. g, glomeruli; gc, glomerular cysts.
between wild-type (the severe PKD of "do not develop PKD. (A)

Figure 5. jck/Nek8-compound heterozygotes exhibit moderate PKD and Nek8–/– kidneys do not develop cysts. (A–C) H&E stained kidneys at 7 weeks. (A) jck/+ kidneys do not develop PKD. jck/Nek8– kidneys (B) develop moderate PKD compared with the severe PKD of jck/jck mutants (C). (D) Comparison of combined kidney weights between wild-type (n=7), jck/+ (n=8), jck/Nek8– (n=8), and jck/jck (n=15) animals. (E and F) Embryonic kidney explant culture cyst formation assay results. Nek8–/– kidneys do not develop many cysts, jck/Nek8– kidneys develop moderate cysts, and jck/jck cystogenic potential is the greatest when cultured with 8-Br-cAMP. Error bars in E represent SEM. Scale bars, 0.5 mm in A–C and F.

is upregulated and mislocalized in jck cystic tissues.37 Similar to loss of Pkd2, its transgenic overexpression in the kidney causes cysts65; perhaps the excess PC2 in jck kidneys underlies the progressive cystogenesis we observe in these mice. Overall, our data provide in vivo support for the importance of the biochemical interaction identified between NEK8 and PC2 and compel additional studies to determine the nature of the relationship between these two proteins, both of which are critical for the establishment of left-right asymmetry and are required later in life for the maintenance of renal epithelial integrity.

CONCISE METHODS

Generation of the Nek8:lacZ Allele, Mouse Husbandry, and Genotyping

EL350 E. coli were transformed with a C57BL/6 (B6) BAC (RP23–163A8) containing the Nek8 genomic locus and exon 3 was targeted with an IRES-lacZ cassette via gap repair.66 Nek8:lacZ was electroporated into B6/129 hybrid embryonic stem cells36,67 and a positive clone was injected at the Brigham and Women’s Hospital Transgenic Core. Chimeric males were crossed to B6 females and germline transmission of the Nek8:lacZ allele was confirmed by PCR. Male and female Nek8:lacZ carriers were intercrossed and homozygotes were not present at weaning. Matings were set, embryos were harvested at various gestational time points, and tails were used for genotyping to confirm homozygous offspring. A three-primer genotyping strategy was utilized as follows: a forward primer that

interactions and/or substrate specificity in vivo that would affect its function.64 Another potential consequence of the mutation is the mislocalization of jck-specific NEK8 proteins; both Smith et al. and Zalli et al. suggest the mutant NEK8 fails to localize to cilia and centrioles in cells cultured from jck/jck kidneys and RPE cells, respectively.36,64 However, Sohara et al. demonstrate that in sections of intact jck homozygous kidneys, ciliary expression of the mutant protein is detected along the entire axoneme, and is not confined to the proximal region in which it is normally localized.37 Further studies are therefore required to understand the consequences of the jck missense mutation.

The fact that jck homozygotes do not exhibit laterality defects suggests that the missense mutation does not abrogate PC2 activity. However, the PKD phenotype of jck mice may still be a consequence of PC2 dysregulation, because PC2

Zebrafish Morpholino Injection and Phenotype Analyses

For Nek8 morpholino knockdowns, wild-type strain TU-AB zebrafish embryos were injected at the one- to four-cell stage with 4.6 nl of a 0.125 mM, 0.25 mM, or 0.5 mM solution of a Nek8 ATG translation blocking oligo: 5’-CTTCTCATATTTCTCCATGTGGT-3’.


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Heart looping was determined at 29 hours postfertilization. Uninjected embryos or embryos injected with a scrambled control morpholino 5’-CCTCTTACCTCAGTTACAATTTATA-3’ exhibited normal development.

**Cell Lines, RT-PCR, and Western Blot Analyses**

MEFs were derived from E14.5 embryos using standard procedures and subsequently immortalized via retroviral infection of the large T antigen. Transcript analysis was performed on RNA isolated with Trizol (Invitrogen) from wild-type and mutant MEF lines; Superscript One-step RT-PCR (Invitrogen) was used to amplify transcripts with primers specific to Nek8 exon 2 (5’-CAACACCACCCACAGGTCAATC-3’) and exon 4 (5’-GCTACTCTCTGTGTTGATGGG-3’).

Nek8 knockdown was performed in IMCD cells by lentiviral infection of shRNA-expressing constructs that were generated by annealing complementary primers and cloning into the pLKO-puromycin vector (shRNA target sequences were obtained from the Broad Institute shRNA Consortium). A hairpin targeting nucleotide 1570 (sh1570) conferred the most robust knockdown when analyzed in a single infection; we subsequently isolated clonal lines and screened for the loss of NEK8 expression by Western blot and identified line N8KD10.

For fluid shear stress experiments, stable wild-type, N8KD10, and PC2-deficient IMCD cell lines expressing GCaMP3 were generated using a blasticidin-resistant retroviral vector as previously described. GCaMP3-positive cells were grown in MatTek chambers (35-mm petri dishes with a bottom coverslip insert) to 40%–50% confluency before serum starvation (0.5% FBS) (MatTek, No. 1.5). Cells were serum starved for 96 hours before fluid shear stress experiments in order to reach their highest frequency of ciliation.

For Western blot analyses, cell and tissue lysates were subjected to SDS-PAGE. Blots were incubated with primary antibodies overnight at 4°C at the following concentrations: anti-NEK8 (1:5000), anti-PC2 (1:1000), and anti-tubulin (1:500; Sigma).

**MRI**

A multi-channel 7.0-T MRI scanner (Varian Inc.) with a 6-cm inner bore diameter insert gradient set was used to acquire anatomic images of E18.5 mouse embryos. Before imaging, samples were immersed in 2 mM ProHance in PBS (gadoteridol; Bracco Diagnostics Inc.) for a week, and were then placed into 2 mM ProHance in low-melting-point agarose (Fisher). Parameters used in the scans were optimized for contrast within the mature mouse embryo as follows: a T2-weighted, three-dimensional fast spin-echo sequence, with repetition time/echo time (TR/TE) of 325/30 milliseconds, eight...
averages, field of view of 14×14×25 mm, and matrix size of 348×348×624, giving an image with 40 μm isotropic voxels.

Histology and Immunofluorescence
Embryos and kidneys were fixed in Bouin’s solution and were embedded in paraffin, and 6-μm sections were cut for hematoxylin and eosin (H&E) staining. Kidneys were fixed in 4% paraformaldehyde and were embedded in paraffin, and 8-μm sections were cut for PAS staining and immunofluorescence/lectin procedures. PAS staining (Sigma) was performed according to the manufacturer’s instructions. For immunofluorescence and lectin staining, slides were subjected to citrate buffer antigen retrieval and incubated with anti-acetylated tubulin antibody (Sigma) at a 1:10,000 dilution combined with either FITC-conjugated LTL or DBA (Vector Labs) at 4°C overnight. Goat anti-mouse Alexa Fluor 594 (Invitrogen) was used to visualize the tubulin and slides were mounted with Vectashield (Vector Labs).

Whole-mount immunofluorescence was performed on E7.75–E8.0 embryos with anti-NEK8 (1:500) or anti-PC2 (1:1000) antibodies overnight at 4°C. Embryos were then incubated with antiacetylated tubulin for 1 hour at room temperature followed by goat anti-rabbit Alexa Fluor 488 and anti-mouse Alexa Fluor 647 secondary antibodies (Invitrogen). Embryos were mounted under individual coverslips with Vectashield (Vector Labs) and processed for genotyping after imaging.

For cilia length analysis, IMCD cells and MEFs were plated on glass coverslips (VWR Scientific); at approximately 30% confluence, the cells were serum-deprived for 48 hours before incubation with antiacetylated tubulin and Alexa Fluor 647. A nonparametric ANOVA test was carried out to compare the cilia length distributions obtained.

Whole-Mount In Situ Hybridization and Fixed Tissue Genotyping
E8.0–E8.5 embryos were harvested and fixed overnight in 4% paraformaldehyde at 4°C, and were then dehydrated through a graded methanol series. For hybridization, embryos were rehydrated and taken through the whole-mount *in situ* procedure described by Wilkinson.22 Embryos were hybridized with digoxigenin-labeled mPitx2c23 and *nodal*44 probes overnight at 65°C, incubated with antidigoxigenin antibody (Roche) conjugated with alkaline phosphatase and probes were visualized with BM purple substrate (Roche). Whole litters were processed simultaneously; after images were obtained, DNA was prepared from the embryos for genotyping. The forward primer is the same as described above, whereas the reverse primers wild-type 5’-CAGAGGCCAGAGATCTGCAC-3’ and mutant 5’-CAGCTTCGCGACGTAACGTTAG-3’ efficiently amplify DNA target sequences from fixed tissue.

Renal Explant Culture
Renal explant culture was performed as described by Natoli et al.57 Kidneys were harvested from E14.5 embryos and transferred to Corning transwell filters suspended in DMEM/F12/10% FBS. At 24 hours, media were supplemented with either DMSO or 100 μM 8-bromo-cAMP (Sigma) and changed daily for 4 days. Cyst percentage was calculated from images by obtaining the total area of the cysts divided by the area of the kidneys, as determined using ImageJ software and data were analyzed with Prism software. The SEM was calculated from the cyst percentages of pooled data from at least four independent experiments (n=10 kidneys per genotype), and the *P* values were determined using the Bonferroni multiple comparison test.

Microscopy and Imaging
Whole-mount embryos and renal explants were analyzed on a Leica DM125 dissecting microscope and images were captured with Leica FireCam software. Histologic and immunofluorescent experiments were analyzed on a Zeiss Imager.Z1 microscope and images were captured with AxioVision software; immunofluorescence slides were imaged with the ApoTome engaged for better resolution of cilia and nuclei.

Fluid Shear Stress Experiments and Statistical Analyses
GCaMP3-positive IMCD cells were introduced to CO2 independent Hanks balanced salt solution media supplemented with HEPES (25 mM final concentration), nonessential amino acids, sodium pyruvate, glucose and GlutaMAX. Intracellular calcium stores were purged by challenging cells with 2 mM EGTA and 100 μM ATP before Ca2+ influx measurements. Cells were introduced back to EGTA-free Hanks balanced salt solution–based media and the cell chambers were mounted on the stage of a Nikon Eclipse Ti inverted microscope equipped with a Nikon Plan Apo 20×A 0.75 NA objective lens and a CoolSnap-HQ (Photometrics). The Nikon FITC cube was used to efficiently reflect 488 nm wavelength and pass the emission wavelengths into the charge-coupled device camera detection channel. While fluorescence image time series were acquired, cells were challenged with fluid shear stress via the controlled addition of Ca2+ media. The acquisition settings were kept constant for all samples so that valid comparisons could be made between measurements from different data sets. Acquisition parameters were set within the linear range of the charge-coupled device camera detection.

A custom MATLAB (The MathWorks Inc) subroutine was written to analyze acquired image time series. The mean intensity of the background noise was calculated from empty dark regions in the images and each frame was individually corrected. Regions of interest (ROIs) from individual cells were chosen for the subsequent analysis. The mean intensity values measured from the first 30 frames (before exposing cells to fluid shear stress) of acquired image time series defined ROIs and used to normalize the values of fluorescence intensity to unity. The SDs of the recovered mean values were obtained from the analysis of multiple ROIs in multiple independent experiments.

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DISCLOSURES
None.

REFERENCES


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Loss of the ciliary kinase Nek8 (NPHP9) causes randomization of left-right asymmetry, cardiac defects, and impaired kidney development

Manning, et al.

Complete Methods

Generation of the Nek8: lacZ allele, ES cell culture, PCR genotyping, and mutants

To generate the Nek8: lacZ targeting construct, EL350 E. coli (6) were transformed with a C57BL/6 BAC containing the Nek8 genomic locus and we subsequently performed a two-step gap-repair process (BAC RP23-163A8, CHORI: http://bacpac.chori.org). First, a 9.5kb fragment encompassing exons 2 through 15 of Nek8 was recombined with the pPNT plasmid backbone (13). Exon three was then targeted with a cassette that encoded an internal ribosomal entry sequence-lacZ gene (IRES-lacZ) followed by a neomycin resistance gene flanked by Flip recombinase recognition sequences. Nek8: lacZ was electroporated into ES cells derived from B6/129 F1 mice (3) and G418 (neomycin, Sigma) resistant clones were isolated, expanded, and subjected to Southern blotting and PCR to confirm the targeting event (5). Three of 148 clones screened were positive for Nek8: lacZ, but two of the lines had abnormal karyotypes. Therefore, one positive clone was injected in the Brigham and Women’s Hospital Transgenic Mouse facility. The resulting chimeric males were crossed to B6 females and progeny were analyzed by PCR to confirm germline transmission of the Nek8: lacZ allele. A 3-primer strategy was utilized as follows: a forward primer 5’-GACACCATTAGCGCCTTCC-3’ that anneals to both WT and mutant loci, a WT-specific reverse primer 5’-CTTCTCAAGGCGCTTGG-3’, and a mutant-specific reverse primer 5’-GGGGATCCATATTATCATCG-3’.

Male and female Nek8: lacZ carriers were intercrossed and litters were first analyzed at weaning for the presence of homozygous pups, but none were identified. Therefore, timed
matings were set and embryos were harvested at various gestational timepoints; examination of females for copulatory plugs was performed in the morning and if detected, noon was considered to be E0.5. Embryo tails were used for genotyping and Nek8: lacZ homozygotes survive gestation (E18.5), but die upon or shortly after birth and we refer to the Nek8: lacZ allele as Nek8- as the reporter is spliced out of the resulting transcript. To determine if the splicing defect is due to the neomycin cassette in the targeting construct, we crossed β-actin-FLPe deleter mice (11) with Nek8: lacZ carriers and generated Nek8: lacZΔneo homozygotes; the neomycin-deficient transcript also lacks exon 3. Maintenance and genotyping for the jck mouse line has been described previously (7), and intercrosses were performed between jck/+ females and Nek8+/− males to obtain compound heterozygotes. All animals were housed in accordance with Harvard Medical School ARCM regulations.

Zebrafish morpholino injection and phenotype analysis

For Nek8 morpholino knockdowns, WT strain TU-AB zebrafish embryos were injected at the one- to four-cell stage with 4.6 nl of a 0.125-mM, 0.25mM, or 0.5mM solution of a Nek8 ATG translation blocking oligo: 5'-CTTCTCATACTTCTCATGTTTTCG-3'. Heart looping was determined at 29 hours post-fertilization (hpf). Uninjected embryos or embryos injected with a scrambled control morpholino 5'-CCTCTTACCTCAGTTACATTATA-3' exhibited normal development. A chi square analysis was performed and the occurrence of cardiac looping defects in morpholino-injected embryos was significant, with a p value < 0.001.
Cell lines, tissue culture and Western Blot analysis

Mouse embryo fibroblasts (MEFs) were derived from E14.5 embryos using standard procedures (5). Briefly, littersmates were harvested, tails were removed for genotyping, and each embryo was processed to establish independent MEF lines. The cell lines were subsequently immortalized via retroviral infection of the large T antigen (4) and selection for neomycin resistance was carried out for 14 days. MEF lines were expanded and characterized by Western blot to confirm genotyping results with respect to wild-type and null status.

Nek8 knockdown was performed in IMCD cells (1) by lentiviral infection of shRNA-expressing constructs that were generated by annealing complementary primers, the sequences of which were obtained from the Broad Institute shRNA Consortium, and cloning into the pLKO-puromycin vector. The construct expressing a small hairpin against nucleotide 1570 (sh1570) conferred the most robust knockdown when analyzed in a single infection and 48-hour drug selection, so we subsequently isolated clonal lines and screened by Western blot for the loss of NEK8 expression. 5/12 lines lacked detectable levels of NEK8 and line N8KD10 maintained the loss of NEK8 after the cells were expanded. MEFs and IMCD cell lines were maintained in DMEM/10% FBS with 100 units/mL penicillin/0.1 mg/mL streptomycin, and incubated in a humidified, 5.0% CO₂ atmosphere at 37°C.

For shear stress experiments, IMCD cell lines were cultured in DMEM/10% FBS supplemented with GlutaMAX, 100 units/mL penicillin, 0.1 mg/mL streptomycin, and 0.1 mM nonessential amino acids. Wild-type, N8KD10 and PC-depleted IMCD cell lines stably lines expressing GCaMP3 were generated using a blasticidin-resistant retroviral vector as previously described (2). GCaMP3-positive cells were transferred from a culture flask after detachment
using Trypsin-EGTA into 35 mm petri dishes with a bottom coverslip insert (No. 1.5; MatTek). The cells were grown in MatTek chambers to 40-50 % confluency prior to serum starvation (0.5 % FBS). Cells were serum starved for 96 hours prior to fluid shear stress experiments in order to reach their highest frequency of ciliation.

For Western blot analyses, cells and tissues were lysed in Tris buffer (20mM Tris pH7.5, 150mM NaCl, 1mM MgCl, 0.5% NP40) and subjected to SDS-PAGE on a 10% acrylamide gel. Imobilon (Millipore) membranes were blocked in 5% milk/TBST and incubated with primary antibodies overnight at 4°C in the following concentrations: NEK8 (1:5000, reference 7), PC2 (1:1000, reference 10), anti-tubulin (1:500, Sigma). Membranes were rinsed and incubated with HRP-conjugated secondary antibodies and detected with the SuperSignal West Femto substrate (Thermo Scientific).

**Magnetic Resonance Imaging of mouse embryos**

A multi-channel 7.0-T MRI scanner (Varian Inc.) with a 6-cm inner bore diameter insert gradient set was used to acquire anatomical images of E18.5 mouse embryos. Prior to imaging, the samples were immersed in phosphate-buffered saline (PBS) and 2 mM ProHance® (gadoteridol, Bracco Diagnostics Inc.) for a week. The samples were then placed into 13-mm-diameter plastic tubes filled with low-melting-point (LMP) agarose (Fisher) and 2 mM ProHance. Three custom built, 14-mm-diameter solenoid coils with a length of 18.3 cm and over wound ends were used to image three embryos in parallel. Parameters used in the scans were optimized for contrast within the mature mouse embryo: a T2-weighted, 3D fast spin-echo sequence, with TR/TE = 325/30 ms, eight averages, field-of-view 14 x 14 x 25 mm and matrix
size=348 x 348 x 624 giving an image with 40 µm isotropic voxels. Total imaging time was 14.5 h.

**Histology and immunofluorescence**

Embryos and kidneys were fixed for 7-14 days in Bouin’s solution and subjected to vacuum embedding in paraffin. E18.5 WT and Nek8/- thoraces were submitted to the Harvard Medical School Rodent Histopathology Core and 6 micron transverse serial sections were obtained, stained with hematoxylin and eosin (H&E) and analyzed for structural cardiac defects. Atrial and ventricular septal defects were identified in mutants by this method. Embryonic kidneys fixed in Bouin’s were sectioned at each day of development from E15.5 through E18.5 and subjected to H&E to identify developmental defects. For periodic acid Schiff (PAS) staining and immunofluorescence(IF)/lectin procedures, kidneys at E18.5 and P0 were fixed overnight in 4% paraformaldehyde at 4°C, vacuum embedded in paraffin, and 8 micron frontal sections were obtained. Slides were deparaffinized and PAS staining (Sigma) was performed according to the manufacturer’s instructions. After deparaffinizing, IF and lectin staining was carried out as follows: slides were rinsed in PBS, subjected to citrate buffer antigen retrieval, incubated with blocking solution (10% goat serum, 1% BSA, 0.1% triton in PBS) for one hour at room temperature, and incubated with anti-acetylated tubulin antibody (Sigma) at a 1:10,000 dilution combined with either FITC-conjugated LTL or DBA (Vector Labs) at 4°C overnight. Slides were rinsed in PBS and incubated with goat anti-mouse AlexaFluor 594 (Invitrogen) for one hour at room temperature, rinsed in PBS and mounted with Vectashield (Vector Labs).

Whole mount IF was performed on E7.75-E8.0 embryos as follows: embryos were fixed in 4% PFA for 30 minutes at room temperature, rinsed in PBS, permeablized in PBT, and
incubated in blocking solution for one hour followed by anti-NEK8 (1:500) or anti-PC2 (1:1000, reference 12) antibodies overnight at 4°C. Embryos were subsequently rinsed in PBT and incubated with anti-acetylated tubulin for one hour at room temperature followed by a combination of goat anti-rabbit AlexaFluor 488 and anti-mouse AlexaFluor 647 secondary antibodies (Invitrogen). Embryos were rinsed in PBT and mounted under individual coverslips with Vectashield (Vector Labs), photographed, removed from the coverslips, and processed for genotyping (described below).

For cilia length analysis, IMCD cells and MEFs were plated on glass coverslips (VWR Scientific) and at approximately 30% confluence the cells were serum-deprived in DMEM/0.2% FBS for 48-hours; cells were then rinsed in PBS, fixed in 4% paraformaldehyde at room temperature for 10 minutes, rinsed in PBS and permeabilized in PBS/0.4% triton. Cells were incubated in blocking solution, mouse anti-acetylated tubulin antibody and goat anti-mouse AlexaFluor 647. Coverslips were subsequently rinsed in PBS and briefly dipped in 100% ethanol, air dried, and mounted with Vectashield (Vector Labs).

To analyze PC2 expression in IMCD cells under shear stress plating conditions (Supplemental Figure S2), cells were incubated with rabbit anti-PC2 antibody 1:100 (sc-25749; Santa Cruz Biotechnology) and mouse anti-acetylated tubulin antibody. Primary antibodies were detected with anti-rabbit Alexa 647 and anti-mouse Alexa 405 secondary antibodies (Invitrogen).

**Whole-mount in situ hybridization and fixed tissue genotyping**

E8.0-E8.5 embryos were harvested and fixed overnight in 4% paraformaldehyde at 4°C, then dehydrated through a graded methanol series (25% MeOH in PBS-tween (PBT) through 100% MeOH) and stored in 100% methanol at -20°C. For hybridization, embryos were
rehydrated through PBT, and taken through the whole-mount in situ procedure described by Wilkinson (14). Briefly, embryos were incubated in 10 ug/ml proteinase K for 5-10 minutes, washes and hybridization solutions were made as described, and hybridization was carried out overnight at 65°C. \textit{mPitx2c} (15) and \textit{nodal} (8) probes were synthesized with T3 and T7 polymerases, respectively, using the MAXIscript T7/T3 (Ambion) \textit{in vitro} transcription kit supplemented with digoxygenin (DIG) RNA labeling mix (Roche) and probes were purified with NucAway (Ambion) spin columns. After hybridization, embryos were washed and incubated overnight with anti-DIG antibody (Roche) conjugated with alkaline phosphatase at 4°C and BM purple substrate (Roche) was utilized to visualize the probes. Whole litters were processed simultaneously and after images were obtained embryos were digested with 100ug/ml proteinase K solution overnight, isopropanol-precipitated with glycogen, washed in 70% ethanol, resuspended in water, and genotyping was performed. The forward primer is the same as described above, while reverse primers were designed to efficiently amplify DNA target sequences from fixed tissue (WT 5’-CAGAGCCAGCAGGATCTGCAC-3’ and mutant 5’-CGGCTTCGGCCAGTAACGTTAG-3’).

\textbf{Renal explant culture}

Renal explant culture was performed as described by Natoli et al. (9). Briefly, kidneys were harvested from E14.5 embryos, transferred to 1.0mm Corning transwell filters in 6-well plates with DMEM/F12/10% FBS, and incubated overnight. At 24-hours media was supplemented with either DMSO or 100uM 8-bromo-cAMP (Sigma). Media was changed daily and images were captured four days after treatment began. Cyst percentage was calculated by obtaining the total surface area of the cysts divided by the surface area of the kidneys, as
determined using ImageJ (http://rsbweb.nih.gov/ij/). Data was analyzed in Prism software. The standard error of the mean (SEM) was calculated from the cyst percentages of pooled data from at least four independent experiments and n=10 kidneys per genotype, and the p values were determined using Bonferroni’s multiple comparison test.

Microscopy and imaging

Whole-mount embryos and renal explants were analyzed on a Leica DM12, dissecting microscope and images were captured with Leica FireCam software. Histological and immunofluorescent experiments were analyzed on a Zeiss Imager.Z1 microscope and images were captured with AxioVision software; IF slides were imaged with the ApoTome engaged for better resolution of cilia and nuclei.

Fluid shear stress experiments and data analysis

IMCD cells stably expressing GCaMP3 were introduced to CO₂ independent HBSS media supplemented with HEPES (25 mM final concentration), non-essential amino acids, sodium pyruvate, glucose and GlutaMAX. Intracellular calcium stores were purged by challenging cells with 2 mM EGTA and 100 µM ATP prior to Ca²⁺ influx measurements. Cells were introduced back to EGTA free HBSS based media and the cell chambers were mounted on the stage of a Nikon Eclipse Ti inverted microscope equipped with a Nikon Plan Fluor 20x A 0.75 NA objective lens and a CoolSnap-HQ (Photometrics). The Nikon FITC cube was used to efficiently reflect 488 nm wavelength and pass the emission wavelengths into the CCD camera detection channel. While fluorescence image time series were acquired, cells were challenged with fluid shear stress via the controlled addition of Ca²⁺ media. IMCD3 cells stably knocked
down for PC2 (m351.2 clone, reference 1) and stably expressing GCaMP3 were utilized as a negative control for PC2-dependant intracellular Ca\textsuperscript{2+} influx measurements. The acquisition settings were kept constant for all samples so that valid comparisons could be made between measurements from different data sets. Acquisition parameters were set within the linear range of the CCD camera detection.

A custom MATLAB (The MathWorks Inc.) subroutine was written to analyze acquired image time series. The mean intensity of the background noise was calculated from empty dark regions in the images and each frame was individually corrected. Regions of interest (ROI) from individual cells were chosen for the subsequent analysis. The mean intensity values measured from the first 30 frames (prior to exposing cells to fluid shear stress) of acquired image time series defined ROIs and used to normalize the values of fluorescence intensity to unity. The standard deviations of the recovered mean values were obtained from the analysis of multiple ROIs in multiple independent experiments.
References


Supplemental Figure Legends

Supplemental Figure S1. Nek8-/- embryos maintain symmetrical Nodal expression in the node proper

(A-D) Nodal is expressed in the node proper in 4-5 somite WT and null embryos. Expression in the (A) WT node is stronger on the left side (arrow; R, right; L, left), but somite-matched mutant embryos (B-D) exhibit symmetrical nodal expression (arrows). Scale bar (A-D) 200µm.

Supplemental Figure S2. PC2 localizes to cilia in WT and NEK8-knockdown IMCD cells under conditions utilized for shear stress assays

(A-C) IF of anti-acetylated tubulin (AT, blue) and anti-PC2 (PC2, red) antibodies on (A) WT, (B) PC2-knockdown (PC2KD) and (C) NEK8-knockdown (N8KD10) IMCD cilia (arrows mark the ends of cilia) confirms PC2 localization in (A) WT and (C) N8KD10 cilia and reduction of PC2 in (B) PC2KD cilia.