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Defects in ciliary localization of Nek8 is associated with cystogenesis

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Abstract

Mutations in the human NIMA (Never in Mitosis gene A)-related kinase 8 (Nek8) are associated with a rare form of the juvenile renal cystic disease, nephronophthisis type 9, and mutations in murine Nek8 cause renal cysts in *jck* mice. Cystogenesis involves dysfunctional ciliary signaling, and we have previously reported that Nek8 localizes to the primary cilium in mouse kidney epithelial cells. We now report that in developing mouse kidney, Nek8 is detected in the cilia of a subset of ureteric-bud-derived tubules at embryonic day (E)15.5. An increasing proportion of ureteric-bud-derived tubules express ciliary Nek8 until E18.5. Postnatal day 1 and 7 Nek8 is observed with equal frequency in both ureteric-bud and non-ureteric-bud-derived tubules. To investigate the cell biological consequences of kinase-deficient and *jck* mutant forms of Nek8, we transiently expressed green fluorescent protein (GFP)-tagged constructs in vitro. Mutations in the kinase and C-terminal domains of Nek8 adversely affected ciliary targeting but did not affect ciliogenesis or ciliary length. Consistent with these in vitro observations, kidneys from homozygous *jck* mice revealed reduced ciliary expression of Nek8 compared with kidneys from

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heterozygous (unaffected) mice. These data indicate that the ciliary localization of Nek8 in a subset of ureteric-bud-derived kidney tubules is essential for maintaining the integrity of those tubules in the mammalian kidney.

Keywords

Polycystic kidney disease; Nephronophthisis; Cilia; Kinases

Introduction

The cystic renal diseases, including polycystic kidney disease (PKD) and nephronophthisis (NPHP) are caused by defects in ciliary signaling, but the precise pathways involved have not been elucidated [1]. Many proteins associated with cystic kidney disease localize to the cilium or the centrosome [2]. Additionally, defects in the assembly and maintenance of the cilium (ciliogenesis) cause cystic kidney disease [3]. Mutations in the NIMA (Never in Mitosis gene A)-related kinase 8 (Nek8) are associated with the formation of renal cysts in people (juvenile NPHP type 9) and in mice [in the juvenile cystic kidney (*jck*) model of PKD] [4, 5].

The *jck* kidney has many cellular characteristics of autosomal dominant PKD, including aberrant apical-basal polarity and increased proliferation, apoptosis, and cyclic adenosine monophosphate (AMP) levels [6]. Nek8 is a ciliary protein [7], and the cilia of *jck* kidney epithelia show mislocalization of polycystin-1 and polycystin-2 [6]. Finally, although the mechanism remains unknown, cystic kidney disease in *jck* mice is treatable by the cyclindependent kinase inhibitor roscovitine [8]. It is thus imperative to understand the role of Nek8 in the kidney.

Neks form a family of evolutionarily conserved cell cycle kinases that are defined by a conserved N-terminal Ser/Thr kinase domain and a variable C-terminal domain [9, 10]. The C-terminus of Nek8 contains a regulator of chromosome condensation 1 (RCC1) domain, a beta-propeller motif associated with protein–protein interactions. The causal mutation in *jck* mice is a missense mutation (G448V) within this RCC1 domain [5]. Similarly, three independent human mutations in the same domain are associated with a rare form of the juvenile cystic kidney disease: NPHP type 9 [4].

To understand the pathogenesis of kidneys inflicted with a mutant Nek8, we examined the expression of mouse Nek8 in the context of the organ itself. Mouse metanephric kidney development begins at embryonic day (E)10.5 where the ureteric bud forms in the Wolffian duct adjacent to the uninduced metanephric blastema. The ureteric bud extends and invades the metanephric mesenchyme, inducing it to differentiate. In turn, the induced metanephric mesenchyme causes the ureteric bud to bifurcate and continue growing, forming the branching collecting-duct tree. In this way, the ureteric bud and metanephric mesenchyme undergo reciprocal induction [11].

The induced mesenchyme condenses around the ureteric bud tip at E12.5 and undergoes a mesenchymal-to-epithelial transition to form renal vesicles or cysts in which apical cilia

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project into the lumen. Nephrogenesis continues as the cyst sprouts two buds: one furthest from the ureteric bud, forming the proximal tubule and glomerulus, resulting in a comma-shaped body, and one closest to the ureteric bud, which becomes the distal tubule, forming an S-shaped body. The distal tubule fuses with the adjacent ureteric bud connecting the nephron with the collecting duct. Through poorly defined mechanisms, the tubule elongates and undergoes patterning to form the complete functioning nephron [12, 13]. The temporal and spatial patterns of Nek8 expression within the kidney may provide clues to its function during development and therefore how mutations in Nek8 can cause cystic kidney diseases.

Herein we report that ciliary localization of Nek8 is restricted to tubules in the ureteric-bud lineage of the developing mouse kidney, where cysts form in *jck* mice [5]. We found subcellular mislocalization of transiently expressed kinase-deficient and *jck*-mutant forms of Nek8 and noted that these proteins do not have dominant effects on ciliogenesis or ciliary length in a mouse kidney epithelial cell line. These two lines of evidence led to the prediction that Nek8 is mislocalized in the *jck* kidney. Indeed, we observed substantially reduced ciliary localization of Nek8 in vivo in homozygous *jck* kidneys. Our data indicate that a defect in ciliary localization of Nek8 is associated with cystogenesis.

Methods

Mouse lines

CD1/129 mice were housed in the Animal Facility of The Hospital for Sick Children (Toronto, Canada), and C57BL/6J heterozygous and homozygous *jck* mice were housed in the NRB Animal Facility (Boston, MA, USA). Animal experiments were approved by the ethics committee at The Hospital for Sick Children and the Harvard Medical area standing committee on animals. Kidneys from different developmental stages from CD1/129 mice and C57BL/6J heterozygous and homozygous *jck* mice were dissected and embedded in paraffin using standard procedures. Sections were mounted on slides using a microtome.

Indirect immunofluorescence of kidney sections

Slide-mounted paraffin-wax-embedded 5-μm kidney sections were deparaffinized through incubations in xylene (Fisher Scientific) then sequentially with 100%, 95%, 70%, and 50% ethanol and washed in phosphate-buffered saline (PBS). Slides were microwaved in a pressure cooker in 10 mM sodium citrate (pH 6.0) and cooled. After three PBS washes, slides were incubated in blocking solution [PBS, 3% BSA (Sigma), 4.8% heat-inactivated goat serum (Invitrogen), 0.02% Tween-20 (Sigma)] for 1 h and incubated overnight at 4°C with one or more of the following primary antibodies and lectin in blocking solution: rabbit polyclonal anti-mouse Nek8 (2,000-fold dilution, [5]), mouse monoclonal anti-acetylated tubulin to label cilia (clone 6–11B-1, 500-fold dilution, Sigma), mouse monoclonal anti-calbindin-D-28K to label ureteric-bud-derived tubules (clone CB-955, 100-fold dilution, Sigma), mouse monoclonal anti-neural-cell adhesion molecule (NCAM) to label condensing mesenchyme (clone NCAM-0B11, 50-fold dilution, Sigma) and fluorescein isothiocyanate (FITC)-conjugated Dolichos biflorus agglutinin (DBA) to label ureteric-bud-derived tubules (DBA 20 ug/mL, Vector Labs). After three PBS washes, slides were incubated with one or more of the following secondary antibodies in blocking solution: Alexa 594-conjugated goat

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anti-mouse all immunoglobulin (Ig)G (1,000-fold dilution, Molecular Probes), Alexa 488-conjugated goat anti-rabbit (500-fold dilution, Molecular Probes), and Alexa 568-conjugated goat anti-rabbit (500-fold dilution, Molecular Probes). Slides were washed in PBS, incubated in 4′,6-diamidino-2-phenylindole (DAPI) (10 μg/mL), and mounted in Vectashield hardest mounting medium (Vector Labs). Fluorescence microscopy was performed using the Delta Vision system (Applied Precision) as described previously [14]. Heterozygous and homozygous *jck* kidney sections were stained with hematoxylin and eosin and imaged under brightfield illumination.

Cell culture

Mouse inner medullary collecting duct (IMCD-3) cells were cultured in a humidified 37°C incubator with 5% CO_2 in a 1:1 mix of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 media with 10% fetal bovine serum (FBS) (all from Invitrogen).

Green fluorescent protein (GFP) constructs

The K33M (presumptive kinase deficient) and G448V (*jck*) mouse Nek8 (mNek8) cDNA constructs in pcDNA3.1 were polymerase chain reaction (PCR) amplified with *Sac*I- and *Sal*I-flanking restriction sites, digested, and subcloned into the *Sac*I/*Sal*I digested pEGFP-C2 vector (BD Biosciences Clontech) with an N-terminal GFP. The reading frame and mutations were verified by sequencing. The vectors were then purified by an Endotoxin-free Maxiprep kit (Qiagen). Transient transfections were performed using Lipofectamine 2000 (Invitrogen) according to manufacturer's protocol with the following modifications: cells grown on coverslips to 85–95% confluency (reached after 24–48 h) were incubated with DNA-Lipofectamine 2000 complexes in OptiMEM (Invitrogen) for 4–6 h, then washed with PBS and grown in DMEM/Ham's F12 media for 16–24 h. Mock transfected cells received no DNA. Transfection efficiency ranged from 18% to 55% for GFP alone and from 29% to 55% for the GFP-wt, GFP-K33M, and GFP-jck constructs.

Western blot

Transfected IMCD-3 cells were resuspended in 1x sodium dodecyl sulfate (SDS) sample buffer [10% glycerol, 50 mM Tris (pH 6.8), 2 mM ethylenediaminetetraacetate (EDTA), 2% SDS, 144 mM 2-mercaptoethanol, 0.01% bromophenol blue (all from Sigma)], and the suspension was sonicated to shear genomic DNA. The samples were separated by electrophoresis in an 8% SDS-polyacrylamide gel electrophoresis (PAGE) gel and transferred to Trans-Blot membrane (BioRad) using a wet transfer apparatus. The nitrocellulose membrane was blocked using 5% skim milk in Tris-buffered saline (TBS)-T for 1 h, followed by incubation in mouse anti-GFP antibody (clones 7.1 and 13.1, 0.4 μg/mL, Roche) overnight at 4°C. The membrane was then washed with TBS-T and incubated in horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody for 1 h at room temperature. GFP-conjugated proteins were visualized using an enhanced chemiluminescence kit (Amersham Biosciences) and X-OMAT film (Kodak).

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Indirect immunofluorescence of tissue culture cells

Transfected cells grown on coverslips were fixed by −20°C methanol for 10 min and then rehydrated by PBS. They were subsequently stained for immunofluorescence using monoclonal mouse anti-gamma tubulin (clone GTU-88, 1,000-fold dilution, Sigma) and monoclonal mouse IgG2b anti-acetylated tubulin (clone 6–11B-1, 7,500-fold dilution, Sigma) as primary antibodies and Alexa 594-conjugated goat anti-mouse all IgG (2,000-fold dilution, Molecular Probes) as a secondary antibody. Cell nuclei were stained with DAPI, 1 μg/mL. Coverslips were mounted using Mowiol (Calbiochem).

Quantification of cellular phenotypes of transiently transfected cells

In three independent experiments, immunostained untransfected and transfected cells were categorized as ciliated with a pair of centrioles, lacking cilia with a pair of centrioles, undergoing mitosis, multinucleate, or having an abnormal number of centrioles, including none at all. The ciliated mononucleate cells with low to medium levels of expression were then quantified for localization of GFP constructs to cilia, centrosomes, a perinuclear region, and/or cell periphery.

Quantification of Nek8 expression in developing kidney

Different embryonic stages of immunostained mouse kidney sections were scored for the presence or absence of Nek8 in the luminal cilia of tubules in the medullary and cortical regions judged by colocalization with acetylated tubulin or luminal orientation to cellular markers. At least three different sections from different kidneys were examined for each stage. In homozygous *jck* kidney sections, Nek8 localization was scored as positive if at least one cilium of a tubule contained Nek8.

Ciliary length measurements

We stained 5 μm postnatal day (P)7 kidney sections from heterozygous (+/*jck*) and homozygous (*jck*/*jck*) litter mates with monoclonal mouse IgG2b anti-acetylated tubulin (clone 6–11B-1, 500-fold dilution, Sigma) and rabbit polyclonal anti-mouse Nek8 (2,000-fold dilution, [5]) antibodies and processed as described above for indirect immunofluorescence. Noncystic tubules containing ciliary Nek8 were photographed, and up to five cilia (indicated by luminal acetylated tubulin) per tubule were measured using DeltaVision softWoRx v3.3.6 software to a total of 50 cilia per kidney region. The medulla and cortical regions were quantified separately, but the difference was not statistically significant (p=0.5 for +/*jck*) and were pooled for a total count of 100 cilia/kidney. Cystic tubules containing Nek8 and lacking Nek8 were separately quantified. Duplicate in vitro measurements were of 50 ciliated IMCD-3 cells, which were transfected with GFP fusion vectors and stained as indicated above. Values reported are average length ± standard deviation (SD).

Statistics

Statistical significance (p value) was assessed using a two-sample t test assuming unequal variances with two-tailed probability. P value and standard error of the mean (SEM) were calculated using Microsoft Excel 2003. P values below 0.05 were defined as significant.

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Trends were consistently reproduced through all three experiments but had *p* values above 0.05.

Results

Spatiotemporal Nek8 expression in developing kidney

To investigate the in vivo role of endogenous Nek8 in developing mouse kidney, we examined its localization pattern through indirect immunofluorescence of kidney sections at different embryonic stages. We found that Nek8 localized to the majority of luminal cilia within a given tubule or not at all in other ciliated tubules (Fig. 1). Also, Nek8 was rarely observed along the entire length of primary cilia but occurred in patches as previously reported in tissue culture cells [7].

Primary cilia are known to project into the lumen of kidney tubules and are expressed by mesenchymal, stromal, and epithelial cell types within the developing mouse kidney (AG; unpublished data). Analysis of temporal expression showed Nek8 was absent from luminal cilia in early stages of kidney development (E13.5–E14.5) and appeared at E15.5 in a subset of ciliated tubules in the medullary and cortical regions (Table 1). Nek8 also localized to more medullary tubules than cortical tubules throughout development (Table 1). In addition, a roughly increasing percentage of ciliated tubules of the medulla and cortex express Nek8 as development proceeds, to a maximum of 60–72% of medullary tubules and 40–56% of cortical tubules expressing Nek8 (Table 1). Because Nek8 is expressed from E15.5, we hypothesize that the effects of a mutation could manifest earlier than 2 weeks postnatal, as previously reported [5, 6].

To identify the subset of tubules expressing Nek8, E18.5 kidney sections were stained with a variety of kidney-cell-type markers. Nek8 was not observed in the cilia of cells during the early stages of mesenchyme condensation, demarked as NCAM-positive cells (0% costaining, n=91), although NCAM-positive cells still contain cilia (Fig. S1). In fact, the majority of Nek8-containing tubules positively stain for calbindin (83%, n=90) and DBA (82%, n=28), indicating that tubules of ureteric-bud origin, which include the developing and maturing collecting ducts contain Nek8 in their cilia (Fig. 2a,b). Conversely, Nek8 was detected in the cilia of only 36% of DBA (n=42) and 52% of calbindin-positive (n=136) tubules. Thus, Nek8 is specifically in the primary cilia of tubules of ureteric-bud origin, but not all ureteric-bud tubules or collecting ducts have detectable Nek8 in their primary cilia.

We next examined the spatiotemporal expression pattern of Nek8 specifically in ureteric-bud-derived tubules, shown by anti-calbindin antibody staining. Early in development (E15.5–E18.5), the majority of tubules with ciliary Nek8 are of ureteric-bud origin (Fig. 2c). However, after birth (P1–P7), Nek8 is expressed in a higher percentage of non-ureteric-bud tubules (Fig. 2c). Similarly, tubules of ureteric-bud origin have ciliary Nek8 starting on E15.5, with the highest percentage of tubules with ciliary Nek8 at E16.5. As development proceeds, a smaller proportion of ureteric-bud-derived tubules have ciliary Nek8 (Fig. 2c). Therefore, during mouse kidney development, Nek8 first localizes to the cilium at E15.5 in tubules of ureteric-bud origin. After birth, non-ureteric-bud-derived tubules also begin to

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express ciliary Nek8. At all time points examined, there was a population of tubules without ciliary Nek8.

Transiently expressed mutant forms of Nek8 are mislocalized but have no effect on overall ciliary assembly

Mutations in the RCC1 domain of Nek8 cause renal cysts in both mouse models and human NPHP patients. To assay the effect of the K33M (kinase deficient) and *jck* mutations, a mouse kidney epithelial cell line (IMCD-3 cells) was transiently transfected with GFP-tagged full-length mouse Nek8 cDNA, encoding wild type or mutant forms of the protein, as monitored by Western blot (Fig. 3a). Transiently expressed GFP alone was cytoplasmic, but GFP-tagged wild-type Nek8 localized to cilia (58% of cells, p=0.01 vs. GFP alone), centrosomes (78%, p=0.05), a perinuclear region (92%, p=0.002), which was statistically significant, and possibly also to the cell periphery (22%, p=0.3) (Figs. 3 and 4a). However, when either the presumptive kinase dead K33M or the *jck* G448V mutations were introduced into GFP-Nek8, a trend was observed, showing decreased localization to the cilia (19%, p=0.09 and 25%, p=0.03 respectively, vs. GFP-wild type Nek8). Kinase-deficient GFP-Nek8 also showed reduced localization to the centrosomes (6%, p=0.0002) and possibly cell periphery (1.5%, p=0.096) compared with GFP-wild type Nek8, whereas the *jck* mutation did not (40%, p=0.11 and 23%, p=0.97, respectively) (Fig. 3 and 4a). These data suggest that the K33M and *jck* mutations in Nek8 affect the subcellular localization of this protein and the K33M mutation is more severe.

Members of the NIMA-related kinase (Nek) family function in both cell cycle and ciliary regulation while localizing to cilia and/or centrosomes [15]. To investigate the cellular effects of transient expression of mutant forms of Nek8, we quantified the percentage of transfected cells that had a pair of centrioles with a cilium, had a pair of centrioles without a cilium, were mitotic, were multinucleate, or had an abnormal number of centrioles (Fig. 4b). Transfection of GFP-wild-type Nek8 and GFP-mutant Nek8 had no effect on ciliogenesis, as the percentage of transfected ciliated cells was similar to GFP alone (p> 0.69). There was no obvious effect on mitosis (p>0.15) or number of centrioles (p>0.44, Fig 4b). In addition, transfection of any form of GFP-Nek8 caused multinucleate cells, which is possibly due to a defect in cytokinesis, as the total number of cells in mitosis and spindle formation in transfected cells appeared normal. Liu et al. [5] previously reported that overexpression of kinase-deficient and *jck* Nek8 caused multinucleate cells, but we now demonstrate that overexpression of wild-type Nek8 caused the same phenotype; therefore, this phenotype is not likely to be related to the etiology of renal cysts. Note that because the quantification was mutually exclusive, the decrease of cells in the category “2 centrioles, no cilia” in GFP-Nek8 wt, K33M, and *jck* transfectants was due to an increase in multinucleate cells, which contained a range of ciliated and non-ciliated cells. Expression of the GFP-tagged constructs was many fold higher than endogenous Nek8 expression (data not shown). Nevertheless, our data indicate that overexpression of wild-type, kinase-deficient, or *jck* Nek8 do not have dominant effects on ciliogenesis.

We next wanted to determine whether any form of Nek8 affected control of ciliary length. Overall, there were no trends that were consistent between duplicate experiments (Fig. S2).

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But, if the duplicate numbers are averaged, a statistically significant although small decrease (0.6 μm to 1.1 μm) in ciliary length was observed in cells overexpressing any form of GFP-Nek8 (wild type, K33M, and *jck*) compared with mock transfection or overexpression of GFP alone ($p<0.05$, Fig. S2). There was no difference in ciliary length between wild-type and mutant forms of overexpressed Nek8 (Fig. S2).

Reduced ciliary Nek8 localization in early *jck* kidneys

Having established that Nek8 localizes to cilia of the developing kidney, we examined whether this was affected in diseased *jck* mice. Sectioned kidneys of P7 mice reveal the earliest reported example of cysts within the *jck* mouse. In hematoxylin-and-eosin-stained sections, cysts are primarily localized to the corticomedullary region (Fig. S3), a phenotype characteristic of human nephronophthisis, in which Nek8 has recently been identified as a causal mutation [4]. Heterozygous *jck* litter mates were unaffected (Fig. S3).

Indirect immunofluorescence of kidney sections showed that wild type and heterozygous *jck* ciliated tubules expressed Nek8 in the majority of cilia within a given tubule (Fig. 5a,b). In homozygous *jck*-ciliated tubules, Nek8 localized to fewer cilia within a given tubule in both cystic and, interestingly, non-cystic tubules (Fig. 5c,d). Moreover, fewer kidney tubules expressed Nek8 in cilia. Only 29% of medullary and 23% of cortical non-cystic ciliated tubules of the *jck* homozygote expressed Nek8, whereas 69% of medullary and 49% of cortical ciliated tubules had ciliary Nek8 in heterozygous litter mates (Table 2). Cystic tubules in the *jck* homozygote also had decreased ciliary Nek8 localization similar to non-cystic tubules (26%, Table 2). Furthermore, the decreased percentage of tubules expressing Nek8 in *jck* homozygotes provides a conservative estimation of the overall effect of the mutation, because any ciliated tubule with at least one patch of Nek8 colocalizing with the cilia was scored as a tubule that expressed Nek8. Thus, in *jck*-homozygous mice, Nek8 is expressed in fewer tubules, and these tubules have fewer cilia that colocalize with Nek8. This result correlates with our cell culture data in which GFP-*jck* shows reduced ciliary localization (Figs. 3 and 4) and with previously published data reporting the lack of ciliary Nek8 localization in *jck* mouse kidney primary culture cells [6].

We also observed differences in ciliary localization of *jck* Nek8 in P7 collecting ducts, where homozygous *jck* tubules showed decreased ciliary Nek8 (22%, $p=0.04$) compared with heterozygous litter mates (Fig. 6a). However, fewer tubules of all origins expressed ciliary Nek8, and thus the percentage of Nek8-positive tubules that were of ureteric-bud origin was not statistically significant ($p=0.07$) between homozygous and heterozygous *jck* kidneys (Fig. 6b). We note that at P7, 60% of cysts ($n=175$) were of ureteric-bud origin (data not shown). Importantly, some of these cysts expressed ciliary Nek8 (Fig. 5 and Table 2), indicating that it is not a complete absence of ciliary Nek8 that triggers cystogenesis. Indeed, many tubules in a healthy individual express no ciliary Nek8. Nevertheless, we noted that even tubules and cysts that are scored positive for ciliary Nek8 in the *jck*/*jck* kidney have fewer cilia, and fewer of these cilia express Nek8.

Smith et al. [6] reported anecdotal observations of longer cilia in the cortical collecting ducts of homozygous *jck* kidneys [6]. We further investigated this by indirect immunofluorescence on heterozygous (+/*jck*) and homozygous (*jck*/*jck*) kidney sections. In medullary and

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cortical +/*jck* tubules with ciliary Nek8, the average length for cilia was 4.77±2.17 μm (Fig. 6c). Interestingly, this was not statistically different than the ciliary length (4.86± 2.00 μm) of Nek8-positive medullary and cortical non-cystic tubules of *jck*/*jck* mice (p=0.8, Fig. 6c). However, *jck*/*jck* cysts with ciliary Nek8 have slightly longer cilia (5.82± 2.67 μm) than +/*jck* tubules (p=0.02) and cilia of noncystic *jck*/*jck* tubules (p=0.03, Fig. 6c). In *jck*/*jck* cysts, cilia that lack Nek8 are the same length as cilia that express Nek8 (p=0.8; Fig. 6c). These data indicate that ciliary Nek8 does not control ciliary length.

Discussion

The effect of the *jck* mutation on Nek8 in the developing kidney

The *jck* missense mutation was responsible for decreased ciliary localization of Nek8 both in vitro and in vivo. In *jck* homozygous mice, reduced ciliary localization was observed in both cystic and non-cystic tissue. This result correlates with loss of apical Nek8 localization in *jck* kidney tubules reported in Liu et al. [5] and Nek8 absence in cilia of primary kidney cell culture from *jck* mice reported by Smith et al. [6]. Our data indicate that a small portion of cilia within the *jck* kidney still colocalized with Nek8, and suggest that the *jck* mutation is hypomorphic, reducing the ability of Nek8 to localize to cilia in both cystic and non-cystic tubules.

Similar loss of ciliary Nek8 localization was observed in overexpression studies with the K33M-kinase-deficient mutation. Additionally, kinase-deficient Nek8 failed to localize to the centrosomes, unlike wild-type and *jck* forms of the protein. These data indicate that in addition to the RCC1 domain, kinase activity is required for ciliary targeting via the centrosome. Dependence of the kinase domain for correct subcellular localization has been demonstrated with another Nek family member, Nek1, which carries the causal mutation of the *kat* (kidney, anemia, testes) mouse model of cystic kidney disease (Mark White and Lynne Quarmby, unpublished data) [16]. We conclude that ciliary Nek8 plays an essential role in normal signaling between cilia and the machinery of cell differentiation/proliferation and that inadequate localization of Nek8 to cilia leads to the formation of cysts.

Contribution of Nek8 to ciliogenesis

The role of ciliary signaling in normal tubular architecture in the kidney remains enigmatic, but we do have a few hints. In *Tcf2* mice, a defect in oriented cellular division precedes cyst formation in kidney tubules, thus implicating the planar cell polarity pathway in cystogenesis [17]. Other defects in PKD include increased proliferation and apoptosis, as well as defects in mechanosensation of luminal flow in kidney tubules [18]. Failure to build a cilium can also cause polycystic kidneys, as exemplified by the *orpk*/Tg737 mouse, which is defective in intraflagellar transport, a process necessary for assembly, maintenance, and disassembly of the cilium [19]. Yet, the presence of cilia in *jck* mice, in cells overexpressing Nek8, and in cells with RNAi knockdown of Nek8 [7] indicates that Nek8 plays no role in the assembly or maintenance of cilia. We observed a minimal, but still statistically significant, increase in ciliary length in cysts. However, cystic cilia were longer than tubular cilia, regardless of whether those cilia expressed Nek8. We conclude that ciliary lengthening is a consequence rather than a cause of cystogenesis.

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Ciliary localization of Nek8 in developing kidneys

The earliest developmental stage at which we observed Nek8 in the cilia of embryonic mouse kidneys was E15.5. At this stage, Nek8 appears in a subset of tubules of ureteric-bud origin and continues to be expressed until at least P7. However, cells associated with tubules in this lineage are ciliated earlier than E15.5, indicating temporal as well as spatial specificity of Nek8 localization to cilia. The spatial localization of endogenous Nek8 is consistent with the collecting-duct origin of *jck* cysts at P14 [5] and 26 days after birth [6]. This suggests a cell autonomous function of Nek8, as defects in ciliary localization due to the *jck* mutation correlates with cyst formation of that tubule.

Onset of Nek8 expression coincides with a series of events occurring at E15.5, including epithelial-to-mesenchymal transition, nephrogenesis, and onset of kidney function and urine flow [11]. It is tempting to speculate that Nek8 may play a role in one or more of these processes, perhaps in conjunction with other cystoproteins that are expressed in the developing kidney. For example, polycystin-1 is expressed at the basal surface of ureteric-bud epithelium in the developing kidney medulla in humans and mice [20, 21]. Polycystin-2 is expressed from mouse E14.5 in the ureteric-bud, at P0 in distal tubules, and in the adult in the distal convoluted tubule and collecting ducts [22, 23]. Both polycystin-1 and polycystin-2 seem to be absent from the nephrogenic mesenchyme, S-bodies, and glomeruli, similar to our findings with Nek8 [20, 22]. Fibrocystin-1, defective in autosomal recessive PKD, is found in mouse ureteric-bud branches from E15 to E19 and in medullary and cortical collecting ducts and the ascending loop of Henle in adult human kidney [24]. Inversin, which is defective in NPHP type 2, localizes to cortical collecting ducts and strongly to the medulla to the ascending loop of Henle and distal tubules of the adult kidney [25]. Specific spatiotemporal expression in the developing kidney has not been demonstrated for NPHP1 (nephrocystin), NPHP3, NPHP4 (nephroretinin), NPHP5 (IQCB1), or NPHP6 (CEP290).

We propose that Nek8 is an essential component of a ciliary signaling pathway that promotes correct tubule morphogenesis. Identification of the targeting mechanism of Nek8, through identification of proteins that interact with the RCC1 domain as well as substrates for the kinase activity of Nek8, will reveal important elements of the elusive signaling pathway that is defective in cystogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Guay-Woodford LM (2006) Renal cystic diseases: diverse phenotypes converge on the cilium/centrosome complex. Pediatr Nephrol 21:1369–1376 [PubMed: 16823577]
2. Hildebrandt F, Otto E (2005) Cilia and centrosomes: a unifying pathogenic concept for cystic kidney disease? Nat Rev Genet 6:928–940 [PubMed: 16341073]
3. Pazour GJ (2004) Intraflagellar transport and cilia-dependent renal disease: the ciliary hypothesis of polycystic kidney disease. J Am Soc Nephrol 15:2528–2536 [PubMed: 15466257]
4. Otto EA, Trapp ML, Schultheiss UT, Helou J, Quarmby LM, Hildebrant F (2007) Mutations in NIMA-related kinase NEK8 affects ciliary and centrosomal localization and may cause nephronophthisis in humans (in press)
5. Liu S, Lu W, Obara T, Kuida S, Lehoczky J, Dewar K, Drummond IA, Beier DR (2002) A defect in a novel Nek-family kinase causes cystic kidney disease in the mouse and in the zebrafish. Development 129:5839–5846 [PubMed: 12421721]
6. Smith LA, Bukanov NO, Husson H, Russo RJ, Barry TC, Taylor AL, Beier DR, Ibraghimov-Beskrovnaya O (2006) Development of polycystic kidney disease in juvenile cystic kidney mice: insights into pathogenesis, ciliary abnormalities, and common features with human disease. J Am Soc Nephrol 17:2821–2831 [PubMed: 16928806]
7. Mahjoub MR, Trapp ML, Quarmby LM (2005) NIMA-related kinases defective in murine models of polycystic kidney diseases localize to primary cilia and centrosomes. J Am Soc Nephrol 16:3485–3489 [PubMed: 16267153]
8. Bukanov NO, Smith LA, Klinger KW, Ledbetter SR, Ibraghimov-Beskrovnaya O (2007) Long-lasting arrest of murine polycystic kidney disease with CDK inhibitor roscovitine. Nature 444:949–952
9. Parker JD, Bradley BA, Mooers AO, Quarmby LM (2007) Phylogenetic analysis of the neks reveals early diversification of ciliary-cell cycle kinases. PLoS ONE 2:e1076 [PubMed: 17957258]
10. O'Connell MJ, Krien MJ, Hunter T (2003) Never say never. The NIMA-related protein kinases in mitotic control. Trends Cell Biol 13:221–228 [PubMed: 12742165]
11. Davies JA, Bard JB (1998) The development of the kidney. Curr Top Dev Biol 39:245–301 [PubMed: 9476003]
12. Schedl A, Hastie ND (2000) Cross-talk in kidney development. Curr Opin Genet Dev 10:543–549 [PubMed: 10980433]
13. Dressler GR (2006) The cellular basis of kidney development. Annu Rev Cell Dev Biol 22:509–529 [PubMed: 16822174]
14. Mahjoub MR, Rasi MQ, Quarmby LM (2004) A NIMA-related kinase, Fa2p, localizes to a novel site in the proximal cilia of Chlamydomonas and mouse kidney cells. Mol Biol Cell 15: 5172–5286 [PubMed: 15371535]
15. Quarmby LM, Mahjoub MR (2005) Caught Nek-ing: cilia and centrioles. J Cell Sci 118:5161–5169 [PubMed: 16280549]
16. Upadhya P, Birkenmeier EH, Birkenmeier CS, Barker JE (2000) Mutations in a NIMA-related kinase gene, Nek1, cause pleiotropic effects including a progressive polycystic kidney disease in mice. Proc Natl Acad Sci USA 97:217–221 [PubMed: 10618398]
17. Fischer E, Legue E, Doyen A, Nato F, Nicolas JF, Torres V, Yaniv M, Pontoglio M (2006) Defective planar cell polarity in polycystic kidney disease. Nat Genet 38:21–23 [PubMed: 16341222]
18. Bissler JJ, Dixon BP (2005) A mechanistic approach to inherited polycystic kidney disease. Pediatr Nephrol 20:558–566 [PubMed: 15719257]
19. Pazour GJ, Dickert BL, Vucica Y, Seeley ES, Rosenbaum JL, Witman GB, Cole DG (2000) Chlamydomonas IFT88 and its mouse homologue, polycystic kidney disease gene tg737, are required for assembly of cilia and flagella. J Cell Biol 151: 709–718 [PubMed: 11062270]
20. Polgar K, Burrow CR, Hyink DP, Fernandez H, Thornton K, Li X, Gusella GL, Wilson PD (2005) Disruption of polycystin-1 function interferes with branching morphogenesis of the ureteric bud in developing mouse kidney. Dev Biol 286:16–30 [PubMed: 16122726]

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21. Guillaume R, D'agati V, Daoust M, Trudel M (1999) Murine Pkd1 is a developmentally regulated gene from morula to adulthood: role in tissue condensation and patterning. Dev Dyn 214:337–348 [PubMed: 10213389]

22. Foggensteiner L, Bevan AP, Thomas R, Coleman N, Boulter C, Bradley J, Ibraghimov-Beskrovnaya O, Klinger K, Sandford R (2000) Cellular and subcellular distribution of polycystin-2, the protein product of the PKD2 gene. J Am Soc Nephrol 11: 814–827 [PubMed: 10770959]

23. Chauvet V, Qian F, Boute N, Cai Y, Phakdeekitacharoen B, Onuchic LF, Attie-Bitach T, Guicharnaud L, Devuyst O, Germino GG, Gubler MC (2002) Expression of PKD1 and PKD2 transcripts and proteins in human embryo and during normal kidney development. Am J Pathol 160:973–983 [PubMed: 11891195]

24. Menezes LF, Cai Y, Nagasawa Y, Silva AM, Watkins ML, da Silva AM, Somolo S, Guay-Woodford LM, Germino GG, Onuchic LF (2004) Polyductin, the PKHD1 gene product, comprises isoforms expressed in plasma membrane, primary cilium, and cytoplasm. Kidney Int 66:1345–1355 [PubMed: 15458427]

25. Nürnberger J, Kavapurackal R, Zhang SJ, Saez AO, Heusch G, Philipp T, Pietruck F, Kribben A (2006) Differential tissue distribution of the Invs gene product inversin. Cell Tissue Res 323:147–155 [PubMed: 16007506]

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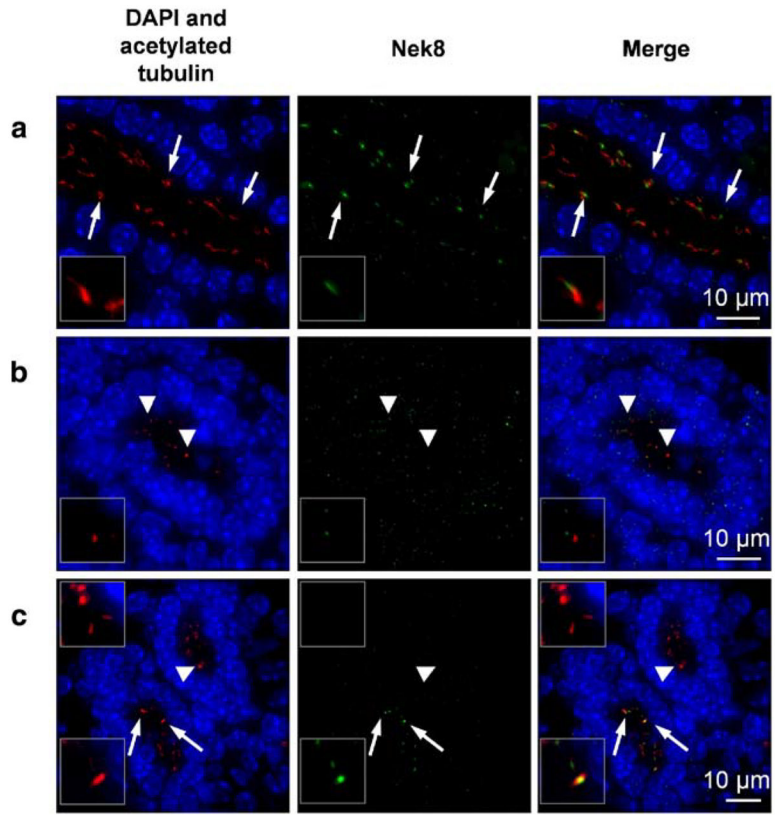

Fig. 1.

Variable spatial expression of ciliary NIMA (Never in Mitosis gene A)-related kinase 8 (Nek8) in developing mouse kidney. Paraffin-embedded sections of CD1/129 mouse embryonic kidney were stained with 4′,6-diamidino-2-phenylin-dole (DAPI) for nuclei (*blue*), anti-acetylated tubulin for cilia (*red*), and anti-Nek8 for endogenous protein (*green*). **a** A medullary tubule from embryonic day (E)18.5 with Nek8 colocalizing with the majority of cilia (arrows). **b** A cortical tubule from E16.5 with Nek8 absent in cilia (arrowheads). **c** Tubules from E16.5. Nek8 colocalizes with the majority of cilia in a medullary tubule (arrows and bottom inset) but exhibits no colocalization with cilia in an adjacent tubule (*arrowhead and top inset*). Insets are x3 magnification

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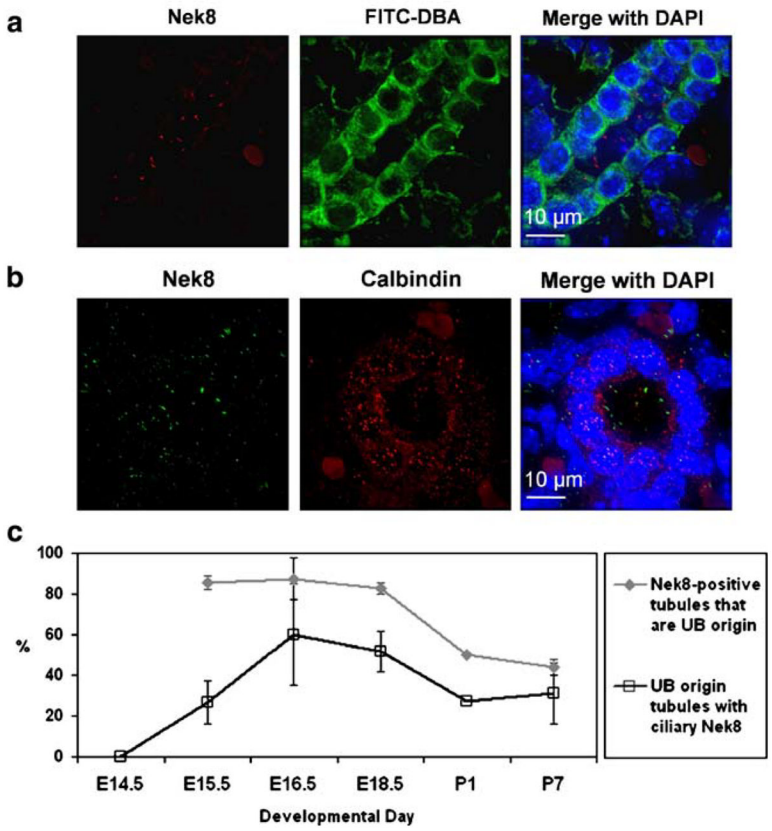

Fig. 2.

Tubules of ureteric-bud origin express NIMA (Never in Mitosis gene A)-related kinase 8 (Nek8). Paraffin-embedded sections of CD1/129 mouse kidneys stained with anti-Nek8 antibody, markers denoting kidney cell types, and 4′,6-diamidino-2-phenylindole (DAPI) (*blue*). **a** An example of an embryonic day (E)18.5 tubule in the medulla labeled with fluorescein isothiocyanate (FITC)-co6njugated Dolichos biflorus agglutinin (DBA) (*green*) expressing luminal Nek8 (*red*). **b** An example of an E18.5 tubule in the medulla labeled with anti-calbindin (*red*) expressing luminal Nek8 (*green*). **c** Kidney sections at different developmental stages were stained with anti-Nek8 and anti-calbindin antibodies. Shown are percentages of Nek8-positive tubules of ureteric-bud (*UB*) origin (*grey diamonds*) and tubules of ureteric-bud origin that have ciliary Nek8 (*black squares*). *Error bars* = standard error of mean

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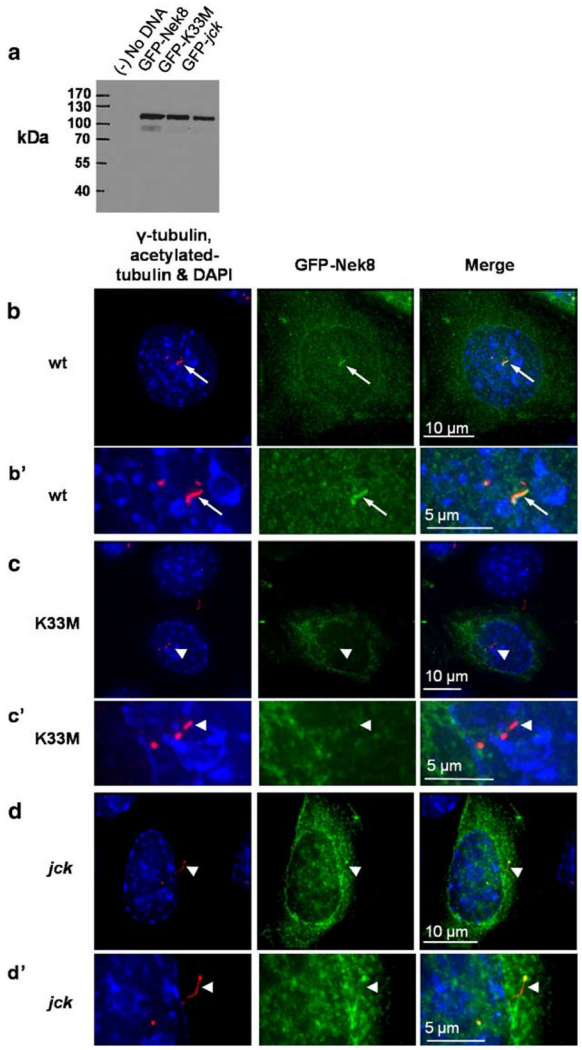

Fig. 3.

Overexpression of mutant forms of NIMA (Never in Mitosis gene A)-related kinase 8 (Nek8) shows differential localization. Transient overexpression of N-terminal green fluorescent protein (GFP)-tagged mouse Nek8 cDNA in mouse inner medullary collecting duct (IMCD-3) cells. **a** Western blot of transfected cell extracts using anti-GFP antibody shows expected band size of 102kDa for the GFP-Nek8 fusion proteins. **b–d** Cells were fixed and stained for anti-acetylated tubulin for cilia and anti-gamma tubulin for centrosomes (both red) and 4′,6-diamidino-2-phenylindole (DAPI) to indicate the nucleus (*blue*). **b** Wild-type GFP-Nek8 localizes to the cytoplasm, centrosomes and cilia (*arrow*). **c** GFP-kinase-deficient (K33M) Nek8 localizes to the cytoplasm but not centrosomes and cilia (*arrowhead*) in this example. **d** GFP-*jck* Nek8 localizes to the cytoplasm and centrosomes but not cilia (*arrowhead*) in this example. Single cells are shown as representatives. **b'**, **c'**, and **d'** are higher magnification images of the cells shown in **b**, **c**, and **d**

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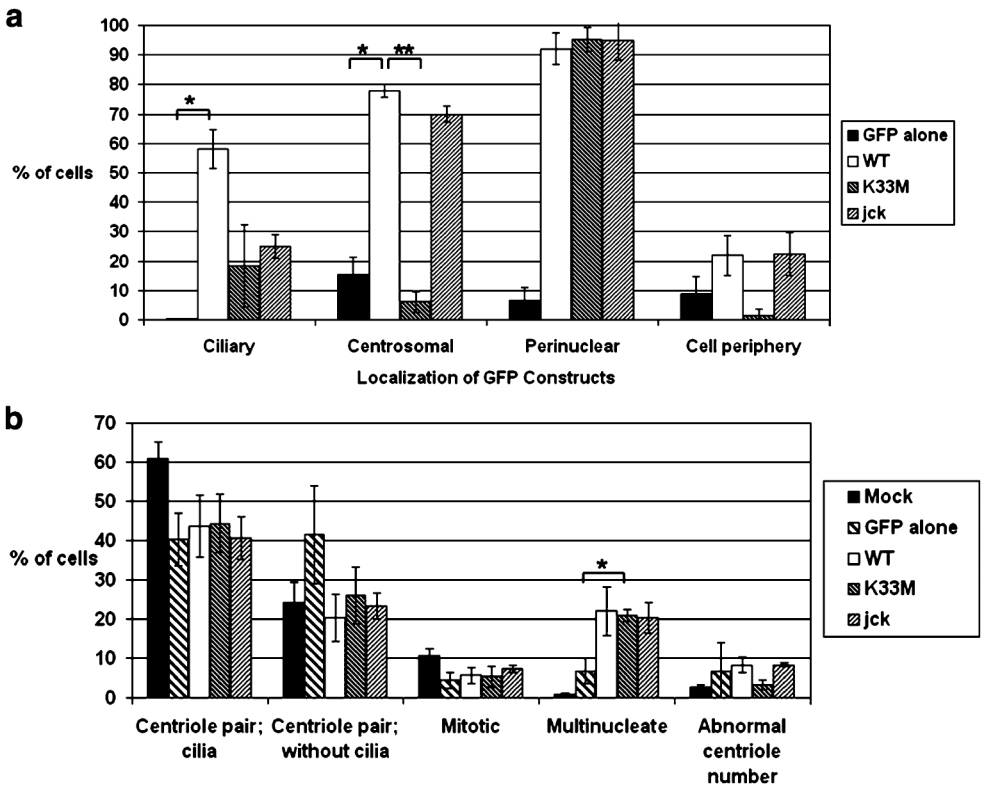

Fig. 4.

In vitro expression of green fluorescent protein (GFP)-NIMA (Never in Mitosis gene A)-related kinase 8 (Nek8) mutations. **a** Quantification of differential subcellular localization of GFP-tagged Nek8 constructs. Transiently transfected ciliated, mononucleate inner medullary collecting duct (IMCD)-3 cells with low to medium levels of expression were quantified for localization of various forms of GFP-Nek8 to cilia, centrosomes, a perinuclear region, and/or cell periphery. The total number of cells counted from three independent experiments was n=45, 50, 65, and 40, respectively, for GFP alone, wt, K33M, and *jck*. *Error bars* = standard error of mean (SEM). **b** Overexpression of Nek8 has no effect on overall ciliogenesis. Transfected and untransfected IMCD-3 cells expressing various forms of GFP-Nek8 were categorized as ciliated with a pair of centrioles, lacking cilia with a pair of centrioles, undergoing mitosis, multinucleate, or having an abnormal number of centrioles, including none at all. The total number of cells counted from three independent experiments was n=950, 250, 300, 300, and 300, respectively for untransfected, GFP alone, wt, K33M, and *jck*. Error bars = SEM. * p< 0.01, ** p<0.001

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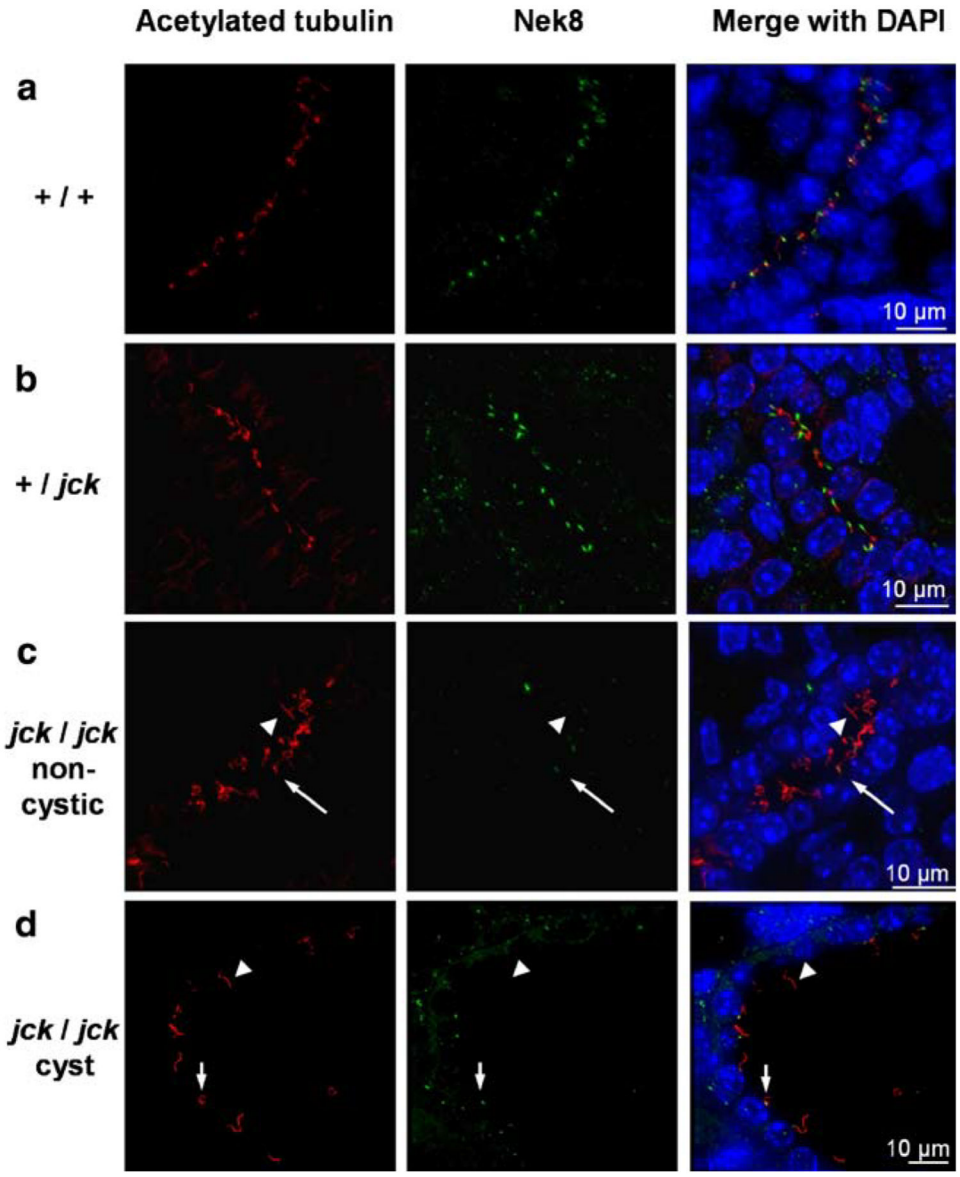

Fig. 5.

Differential NIMA (Never in Mitosis gene A)-related kinase 8 (Nek8) ciliary localization in diseased *jck* kidney. Paraffin-embedded sections of postnatal day 7 mouse kidneys from **a** wild type (+/+), **b** heterozygous (+/*jck*), and **c, d** homozygous *jck* (*jck*/*jck*) mice were stained with 4′,6-diamidino-2-phenylindole (DAPI) (*blue*), anti-acetylated tubulin for cilia (*red*), and anti-Nek8 for endogenous protein (*green*). The heterozygous and homozygous mice were litter mates. Shown are ciliated tubules within the medulla. *Arrows* indicate Nek8 localization, and *arrowheads* indicate lack of Nek8 in cilia

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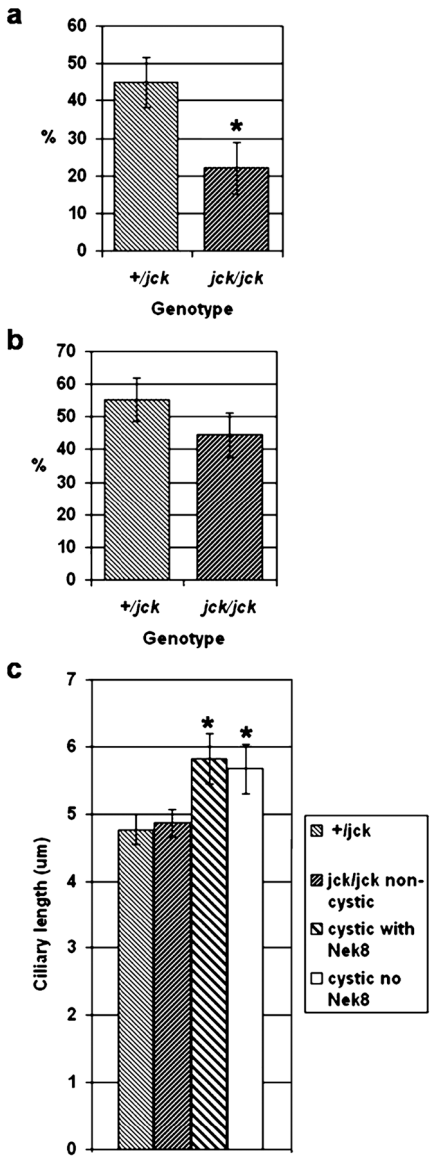

Fig. 6.

NIMA (Never in Mitosis gene A)-related kinase 8 (Nek8) expression in ureteric-bud-derived tubules and ciliary lengthening in *jck* kidneys. **a, b** Paraffin-embedded sections of postnatal day (P)7 mouse kidney from heterozygous (+/*jck*) and homozygous (*jck*/*jck*) *jck* mouse litter mates were costained with anti-calbindin D-28K and anti-Nek8 antibodies. **a** *Percentages* indicate calbindin D-28K-positive tubules that express Nek8 in luminal cilia. **b** *Percentages* indicate Nek8-positive tubules that are of ureteric-bud origin. **c** P7 heterozygous (+/*jck*) and homozygous (*jck*/*jck*) *jck* mouse litter mates were costained with anti-acetylated tubulin and anti-Nek8 antibodies. The two bars on the *left* show the average length of Nek8-expressing cilia of non-cystic +/*jck* and *jck*/*jck* tubules. The *two* bars on the *right* are the average lengths of cilia of *jck*/*jck* cysts, where the cilia either express or do not express Nek8. *$p<0.05$

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Table 1

Temporal expression of NIMA (Never in Mitosis gene A)-related kinase 8 (Nek8) in developing kidney

Stage	Medullary tubules	Cortical tubules
E14.5	0% (*n*=11)	0% (*n*=16)
E15.5	42% (*n*=26)	13% (*n*=23)
E16.5	44% (*n*=55)	38% (*n*=53)
E18.5	60% (*n*=78)	29% (*n*=91)
P1	72% (*n*=110)	41% (*n*=100)
P7	66% (*n*=83)	56% (*n*=87)

Paraffin-embedded CD1/129 mouse kidney sections were stained with anti-acetylated tubulin to denote luminal cilia and anti-Nek8 to denote endogenous expression. Percentages indicate the number of ciliated tubules expressing Nek8 in the medullary and cortical regions of wild-type developing mouse kidney; *n* = number of ciliated tubules counted

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Table 2

Decreased NIMA (Never in Mitosis gene A)-related kinase 8 (Nek8) expression in *jck* kidneys

Phenotype	Medullary tubules	Cortical tubules
+/jck non-cystic	69% (*n*=148)	49% (*n*=116)
jck/jck non-cystic	29% (*n*=49)	23% (*n*=52)
jck/jck cystic	26% (*n*=42)	26% (*n*=42)

Paraffin-embedded sections of postnatal day 7 mouse kidneys from heterozygous (+/*jck*) and homozygous *jck* (*jck*/*jck*) mouse litter mates were stained with anti-acetylated tubulin and anti-Nek8 antibodies. Percentages indicate the number of cystic and non-cystic tubules in the medulla and cortex of the kidney that express Nek8 in luminal cilia. Any tubule with at least one cilium colocalizing with Nek8 was scored as positive; *n* = number of ciliated tubules counted