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Mutations in NEK8 link multiple organ dysplasia with altered Hippo signalling and increased c-MYC expression

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Mutations affecting the integrity and function of cilia have been identified in various genes over the last decade accounting for a group of diseases called ciliopathies. Ciliopathies display a broad spectrum of phenotypes ranging from mild manifestations to lethal combinations of multiple severe symptoms and most of them share cystic kidneys as a common feature. Our starting point was a consanguineous pedigree with three affected fetuses showing an early embryonic phenotype with enlarged cystic kidneys, liver and pancreas and developmental heart disease. By genome-wide linkage analysis, we mapped the disease locus to chromosome 17q11 and identified a homozygous nonsense mutation in NEK8/NPHP9 that encodes a kinase involved in ciliary dynamics and cell cycle progression. Missense mutations in NEK8/NPHP9 have been identified in juvenile cystic kidney jck mice and in patients suffering from nephronophthisis (NPH), an autosomal-recessive cystic kidney disease. This work confirmed a complete loss of NEK8 expression in the affected fetuses due to nonsense-mediated decay. In cultured fibroblasts derived from these fetuses, the expression of prominent polygenic kidney disease genes (PKD1 and PKD2) was decreased, whereas the oncogene c-MYC was upregulated, providing potential explanations for the observed renal phenotype. We furthermore linked NEK8 with NPHP3, another NPH protein known to cause a very similar phenotype in case of null mutations. Both proteins interact and activate the Hippo effector TAZ. Taken together, our study demonstrates that NEK8 is essential for organ development and that the complete loss of NEK8 perturbs multiple signalling pathways resulting in a severe early embryonic phenotype.

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INTRODUCTION

During recent years, an emerging number of diseases have been related to the dysfunction of the primary cilium (1). In line with a new organizing principle, these disorders are now collectively termed ‘ciliopathies’. Primary cilia are sensory organelles with an axoneme that is composed of nine microtubule doublets derived from a modified centrosome, the basal body (2). Cilia are covered by plasma membrane and project like antennae from the cell surface. Primary cilia detect and orchestrate the cellular response to various extracellular stimuli through specific ciliary receptors that mediate chemo- and mechanosensation (3). Proper ciliary structure and function is crucial not only for early embryonic development, but also for tissue maintenance later in life. The broad range of phenotypes associated with ciliary defects is explained by their almost ubiquitous presence, the organ specificity of some phenotypes by the different properties and compositions of cilia in different tissues (4). Notably, renal cysts are a component of most ciliopathies.

Polycystic kidney disease (PKD) can be inherited dominantly or recessively and is characterized by enlarged cystic kidneys (5,6). Autosomal recessive PKD (ARPKD) is caused by mutations in PKHD1 encoding a type I single-pass transmembrane protein (fibrocystin; FC) (7). Most patients are severely affected and display a ‘Potter’ oligohydramnios phenotype with massively enlarged kidneys and hepatobiliary ductal plate malformation. The dominant counterpart autosomal dominant PKD (ADPKD) is typically characterized by adult onset of clinical symptoms (8); however, about 2% of patients present with early disease manifestations sometimes with significant perinatal morbidity and mortality indistinguishable from severe forms of ARPKD (9). In some of these severely affected patients, a dosage effect has been postulated as the most likely underlying pathomechanism. With a prevalence of 1 in 500–1000, ADPKD affects about 10–15 million individuals worldwide. About 80–85% carry a germinal mutation in PKD1, the others are thought to harbour a PKD2 mutation (8). Both ADPKD proteins polycystin-1 and polycystin-2 are glycosylated integral membrane proteins that interact via their C-terminal coiled-coil domains (10).

Many ADPKD patients are severely affected and display a ‘Potter’ oligohydramnios phenotype with massively enlarged kidneys and hepatobiliary ductal plate malformation. The dominant counterpart autosomal dominant PKD (ADPKD) is typically characterized by adult onset of clinical symptoms (8); however, about 2% of patients present with early disease manifestations sometimes with significant perinatal morbidity and mortality indistinguishable from severe forms of ARPKD (9). In some of these severely affected patients, a dosage effect has been postulated as the most likely underlying pathomechanism. With a prevalence of 1 in 500–1000, ADPKD affects about 10–15 million individuals worldwide. About 80–85% carry a germinal mutation in PKD1, the others are thought to harbour a PKD2 mutation (8). Both ADPKD proteins polycystin-1 and polycystin-2 are glycosylated integral membrane proteins that interact via their C-terminal coiled-coil domains (10).

RESULTS

In a third-degree consanguineous multiplex pedigree, three fetuses were affected with a multisystemic phenotype of the ciliopathy spectrum with enlarged organs resembling Ivemark’s syndrome. Two of the affected pregnancies were terminated because of predictable unfavourable disease outcome, one after intrauterine fetal death. The prenatal severe ultrasonographic features were confirmed by autopsy (for details, see Fig. 1 and Table 1). Chromosomal analyses showed a normal karyotype. To identify the causative disease locus in this family, we performed a whole-genome search for linkage by homozygosity mapping using the 250 K Affymetrix single nucleotide polymorphism (SNP) array that resulted in two peaks on chromosomes 9q34.3 (1.4 Mb interval) and 17q11.2-12 (6.3 Mb interval) for which the maximum logarithm of the odds (LOD) score of 3.6 was reached. We prioritized genes within the critical intervals for mutational screening on the basis of the chemo- and mechanosensation (3). Proper ciliary structure and function is crucial not only for early embryonic development, but also for tissue maintenance later in life. The broad range of phenotypes associated with ciliary defects is explained by their almost ubiquitous presence, the organ specificity of some phenotypes by the different properties and compositions of cilia in different tissues (4). Notably, renal cysts are a component of most ciliopathies.

In contrast to PKD, nephronophthisis (NPH) is a rather rare disease and kidney size in NPH patients is usually slightly reduced or normal (except for the infantile form caused by NPHP2/INVS mutations) (12). NPH comprises a clinically and genetically heterogeneous group of autosomal-recessive tubulo-interstitial cystic kidney disorders and is the leading genetic cause of end-stage renal disease in children and young adults (12). To date, almost 20 disease-causing NPHP genes have been identified that encode for the nephrocystin protein family (13–16). Mutations in these NPHP genes can cause pleiotropic phenotypes, and the cystic kidney disease in NPH is often associated with extrarenal manifestations of eye, brain and liver (12). For instance, mutations in NPHP3 lead to adolescent NPH, but may also mimic PKD with enlarged kidneys and prenatal manifestation of Potter’s sequence (C.B., own unpublished data). We and others have shown that the encoded nephrocystin-3 protein plays an important role in early embryonic development and left-right patterning (17,18). In line with this, NPHP3 null mutations can be identified in patients with renal-hepatic-pancreatic dysplasia and heterotaxy features as asplenia/polyplenia and cardiac defects (Ivemark’s syndrome) (17).

Here, we identify a complete loss of NEK8/NPHP9 to cause multiple organ dysplasia and developmental phenotypes. Single hypomorphic point mutations have previously been identified to cause human NPH (19) and juvenile cystic kidney disease in jck mice (20). We now describe a homozygous nonsense mutation in this gene leading to a complete loss of NEK8 expression, disturbances in several signalling pathways relevant in PKD and a developmental disease reminiscent to renal-hepatic-pancreatic dysplasia (Ivemark’s syndrome).
To analyse the expression of the mutated protein, we performed quantitative PCR in fibroblasts isolated from the fetuses and found a dramatic decrease in NEK8/NPHP9 mRNA-expression in the patients’ fibroblasts in comparison with age-related controls. Treatment with cycloheximide (CHX) led to an increased expression of NEK8/NPHP9 which is in line with the hypothesis that premature termination codons (PTCs) cause nonsense-mediated decay (NMD; Fig. 1C). Loss of NEK8/NPHP9 expression was further confirmed by western blot analyses using lysates from the fetal fibroblasts (Fig. 1D). Taken together, we were able to identify a previously undescribed homozygous nonsense mutation in NEK8/NPHP9 resulting in NMD in patients with a multisystemic phenotype resembling Ivemark’s syndrome.

All three fetuses presented with enlarged organs and cystic-dysplastic changes in kidney, liver and pancreas. The second
Table 1. Clinical and histological findings of patients described

<table>
<thead>
<tr>
<th>Patient</th>
<th>Ethnic origin</th>
<th>Termination of pregnancy</th>
<th>Gender</th>
<th>Pottery sequence with oligo-/anhydramnios</th>
<th>Kidneys</th>
<th>Liver</th>
<th>Pancreas</th>
<th>Spleen</th>
<th>Heart</th>
<th>Lungs</th>
<th>Skeletal findings</th>
<th>Others</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Dutch</td>
<td>21st gestational week</td>
<td>Female</td>
<td>+</td>
<td>Enlarged cystic-dysplastic kidneys</td>
<td></td>
<td></td>
<td></td>
<td>No abnormalities</td>
<td>Hypoplastic lungs with normal lobulation</td>
<td>Bowed femur diaphyses</td>
<td>Uterine agenesis</td>
</tr>
<tr>
<td>2</td>
<td>Dutch</td>
<td>18th gestational week after intrauterine fetal death</td>
<td>Female</td>
<td>-</td>
<td>Absent fetal lobulation, due to severe autolysis no histological examination</td>
<td>Due to severe autolysis no histological examination</td>
<td></td>
<td></td>
<td>Asplenia</td>
<td>Severe congenital heart defect with truncus arteriosus and unseptated atrium and ventricle</td>
<td>Lung lobulation defect</td>
<td>Shortened legs, information on bowing not available</td>
</tr>
<tr>
<td>3</td>
<td>Dutch</td>
<td>22nd gestational week</td>
<td>Male</td>
<td>+</td>
<td>Enlarged cystic-dysplastic kidneys</td>
<td></td>
<td></td>
<td></td>
<td>No abnormalities</td>
<td></td>
<td>No abnormalities</td>
<td>Bowed femur diaphyses with shortening of legs</td>
</tr>
</tbody>
</table>

affected fetus was severely autolytical that made fibroblast generation and histological examinations impossible. Other macroscopic manifestations found in at least one of the patients included severe heart defects, hypoplastic lungs, lobulation defects, asplenia, uterine agenesis and shortened legs due to bowed femur diaphyses as shown in the babygrams (Fig. 2A and Table 1). Detailed histological analysis was performed for kidney, liver and pancreas of two fetuses of the described family and a control fetus of similar age (Fig. 2B). Liver histology revealed a slight increase in immature mesenchyme around bigger portal fields. Pancreatic tissue presented with a rudimentary lobulation and severely reduced acinar units as well as the complete absence of islets of Langerhans. Cystic transformation of pancreatic ducts was recognizable in one of the two affected fetuses. Most strikingly, the kidney presented with severe cystic dysplasia, including predominance of immature mesenchyme and no zonal partition between cortical and medullary areas. Glomeruli were immature and decreased in number. Cartilaginous islands were not demarcated. Tubules were dilated and cysts lined with rather flattened epithelial cells. In summary, loss of NEK8/NPHP9 expression in this family resulted in the development of a cystic-dysplastic phenotype of multiple organs.

Because NEK8/NPHP9 has been demonstrated to regulate the expression and localization of both ADPKD proteins (21), we analysed PKD1 and PKD2 mRNA expression in the patients’ fibroblasts by quantitative PCR (qPCR). Interestingly, both PKD1 and PKD2 were significantly downregulated when compared with control fibroblasts (Fig. 3A). Because high levels of c-MYC can cause ADPKD in mice (22), we continued by analysing c-MYC levels. Strikingly, we also observed high c-MYC mRNA expression levels in the patients’ fibroblasts (Fig. 3B), which we could confirm by immunohistochemistry of the affected fetal kidneys. These stainings revealed increased c-MYC expression specifically in the epithelial cells lining the cystic tubules (Fig. 3C), suggesting that NEK8 may act as a negative canonical Wnt regulator.

NPHP3, another NPH-protein, has also been described to be a disease-causing gene for NPH and to cause renal-hepatic-pancreatic dysplasia in case of null mutations. Therefore, we investigated whether NPHP3 and NEK8/NPHP9 might share a common signalling function. A recent study demonstrated that NPHP proteins interact and form distinct protein complexes (23); however, an interaction between NPHP3 and NPHP9 has not yet been identified. Thus, we performed coimmunoprecipitation experiments which demonstrated that both proteins share a common protein complex (Fig. 4A). Inspired by our recent finding that NEK8/NPHP9 is able to activate the Hippo effector protein TAZ (24), we analysed the impact of both NEK8/NPHP9 and NPHP3 on TAZ signalling activity using luciferase based reporter assays. Interestingly, both proteins stimulated TAZ/TEAD transcriptional activity when co-expressed with TAZ. To investigate the impact of this finding in our patients’ cells, we analysed again the fetal fibroblasts focussing on genes involved in hippo signalling and regulated by the transcriptional co-activator TAZ. Quantitative PCR analysis revealed that Birc5/survivin and ITGB2, which represent well-established target genes of the Hippo pathway and have been implicated in ADPKD recently (25), were downregulated. These data are in line with a lack of NEK8 expression in the patients’ cells. CTGF, another known target gene of TAZ/YAP/TEAD (26), was upregulated; however, because CTGF expression is modulated by a variety of additional pathways [e.g. TGF-β signalling (27,28)], its upregulation might rather result from a more complex dysregulation of multiple signalling pathways in cells carrying the NEK8 mutation and is consistent with considerable enrichment of mesenchyme and fibrosis observed in the organs of our patients.
DISCUSSION

More than a decade ago, a recessive missense mutation in \textit{NEK8/NPHP9} was reported to cause juvenile cystic kidneys in a transgenic mouse model (\textit{jck}) (20). Only recently, \textit{NEK8/NPHP9} missense mutations have been described in patients with NPH and were shown to affect its ciliary and centrosomal localization (19). In contrast to these hypomorphic changes, our study clearly demonstrates that loss-of-function mutations in \textit{NEK8/NPHP9} lead to a more severe, multisystemic ciliopathy characterized by enlargement and dysplasia of several tissues. Thus, our findings support the hypothesis that tissue dysplasia and degeneration in NPH-related ciliopathies can be caused by different types of mutations within the same gene (15). Our sequencing data obtained from a large cohort of patients with different cystic kidney disease phenotypes and other ciliopathies indicate that mutations in \textit{NEK8/NPHP9} do not appear to be a frequent cause underlying these phenotypes.

\textit{NEK8/NPHP9} spans approximately 14 kb of genomic DNA and comprises 15 exons that encode the never in mitosis gene A-related serine/threonine protein kinase 8 (NEK8), a 692 amino acid protein. NEKs are cell cycle kinases and thought to coordinate the regulation of cilia and cell cycle progression. NEK8 localizes to centrosomes and the proximal segment of primary cilia in dividing and ciliated cells depending on the activity of its kinase domain and C-terminal non-catalytic RCC1 motif (29). The major transcript was found to be expressed in a wide variety of embryonic tissues, but not in corresponding adult organs, suggesting a crucial role in embryonic development. In humans, \textit{NEK8} missense mutations with preserved protein expression only affect tubular maintenance and cyst formation, but do not affect embryonic development (19). In contrast, complete absence of the protein has a dramatic effect in embryonic development affecting nearly all organs. Different phenotypic outcomes caused by either missense or complete loss-of-function mutations of \textit{NEK8} are also found in mice: Recently, \textit{Nek8} knockout mice have been described that display a much more severe phenotype when compared with \textit{jck} mutant mice carrying a missense mutation. Whereas \textit{jck} mice develop a cystic kidney disease during their first 2 months of life, \textit{Nek8} knockout mice die perinatally with left-right asymmetry defects, cardiac anomalies and glomerulocystic kidney disease (30).

Although mutations in \textit{NEK8/NPHP9} affect the protein localization at cilia and centrosomes, they did not affect ciliogenesis. In contrast, it was recently demonstrated that the activity and expression of \textit{NEK8/NPHP9} is linked to the ciliogenesis process itself (31). Our data demonstrating a connection between \textit{NEK8/NPHP9} and NPHP3 are consistent with the finding that both proteins co-localize in the proximal segment of primary cilia, where they are anchored by Inversin/NPHP2 (29). Our signalling data imply that \textit{NEK8/NPHP9} and NPHP3 have a common function in stimulating the activity of the Hippo downstream effector TAZ. Originally discovered in \textit{Drosophila}, the Hippo pathway has gained increasing interest over the last couple of years as an important regulator of proliferation and organ growth with most of its components possessing either tumour suppressor or oncogenic properties. In accordance, many processes defective in
coliopathies, such as epithelial and planar cell polarity, are also known to play crucial roles in tumourigenesis. Recently, we have identified NPHP4 and NEK8/NPHP9 to be inhibitors of the Hippo pathway (24,32) and we now add NPHP3 to this functional complex. In addition, this work demonstrates a downregulation of TAZ target genes in patients' cells that is an important confirmation of the data derived from the reporter assays.

In addition, we found low expression levels of the classical ADPKD genes, PKD1 and PKD2, in the patients’ cells, as well as high levels of c-MYC. These high c-MYC levels were also detectable in the analysed patients’ kidneys by immunohistochemical analysis. Given previous reports on high c-MYC levels in multiple models of PKD as well as in patients with ADPKD (33) and the fact that c-MYC transgenic mice develop an ADPKD-like phenotype (22,34), the observed high c-MYC levels could help to explain the development of a cystic kidney phenotype in the fetuses described in this paper. As c-MYC is a classical target gene of the canonical Wnt pathway, we hypothesize that NEK8/NPHP9 might inhibit canonical Wnt signaling. NPHP3 has been demonstrated to negatively regulate canonical Wnt signalling (17), and crosstalk between Wnt and Hippo signalling has previously been reported (35–38). Therefore, our data suggest that both NPHP3 and NEK8/NPHP9 are involved in regulating the Hippo-Wnt signalling network.

With the exception of c-MYC, we could not confirm the fibroblast expression data in tissues from the affected fetuses due to technical limitations in fetal material. This weakens our findings regarding differential gene expression to some extent because fibroblasts are not the causative cell type leading to the observed phenotype. Yet, the recent identification of NEK8 as an activator of TAZ-dependent transcription in epithelial cells (24) strongly suggests that NEK8 deficiency might result in decreased epithelial TAZ target gene expression. Taken together, the phenotype caused by loss of NEK8 in the patients of our study is associated with transcriptional changes in TAZ target gene expression and with expression changes of PKD1, PKD2 and c-MYC, each of which are known to promote cystogenesis and proliferation.

**MATERIALS AND METHODS**

**Genetics**

DNA samples were available from parents, and all affected fetuses were obtained after informed consent had been given. Chromosomal analyses showed a normal karyotype.
We performed homozygosity mapping using a 250 K Affymetrix SNP array that resulted in two peaks, each reaching the maximum LOD score of 3.6. Exon primers used for PCR and direct sequencing are given in Supplementary Material, Table S1.

We subsequently performed NEK8 mutation analysis in a large cohort of 288 patients with different (poly)cyctic kidney disease phenotypes and other ciliopathies using a targeted next-generation sequencing approach. All exons and adjacent intronic boundaries of 129 and recently 258 genes (including NEK8) known or hypothesized to cause ciliopathies were targeted by a custom SeqCap EZ choice sequence capture library (NimbleGen, Madison, WI, USA) and subsequently sequenced on a Roche 454 GS FLX or an Illumina MiSeq platform (2 × 150 PE) according to the manufacturer’s protocol. Patients were analysed with an average coverage of 60-fold (GS FLX) or 120-fold (MiSeq), respectively. Bioinformatic analysis was performed using the Roche GS Reference MapperTM software (v2.6), SeqPilot SeqNext module™ (v3.5.2, JSI medical systems, Kippenheim, Germany) and an in-house bioinformatic pipeline established at Bioscientia. No further NEK8 mutation thought to be of pathogenic relevance for the disease phenotype was present among the above patient cohort.

Histology

Kidneys, pancreas and liver tissues were prepared as formalin-fixed, paraffin-embedded tissue blocks. From each block, 1–2 μm sections were cut and stained with haematoxylin and eosin, Pas (Kidneys) and Elastic van Gieson for liver.

Cell culture

Human embryonic fibroblast cells were cultured in RPMI-1640 medium supplemented with 10% foetal bovine serum (FBS). HEK293T cells were maintained in DMEM supplemented with 10% FBS and transfected as described previously (39).

Luciferase assays

HEK293T cells were transfected in a 96-well format using Lipofectamine LTX (Invitrogen) as transfection reagent. The firefly luciferase reporter plasmid (pGBD-Hyg-Luc) was transfected together with an activator plasmid (pGal4-TEAD), as well as Renilla luciferase pGL4.74 (Promega) for normalization and the indicated experimental plasmids (TAZ, NPHP3, NPHP9; balanced as required using empty pcDNA6 vector) (32). Renilla luciferase and firefly luciferase activities were measured using a dual-luciferase reporter assay system (Promega) in a luminometer (Mithras LB940, Berthold) 24 h after transfection. Transfections and measurements were done in triplicate for each single experiment, and each experiment was repeated at least three times. Error bars shown in the figures represent standard error of the mean (SEM). Equal expression of the transfected proteins was confirmed by western blot analysis.
Coimmunoprecipitation and immunoblotting

HEK293T cells were transiently transfected using the calcium phosphate method (39). The following day, cells were harvested with ice cold PBS. A small aliquot of this cell suspension was immediately removed and lysed directly in SDS-PAGE sample buffer as a whole cell lysate. The remaining harvested cells were lysed in a 1% Triton X-100 buffer [1% Triton X-100, 20 mM Tris–HCl, pH 7.5, 50 mM NaCl, 50 mM NaF, 15 mM Na2P2O7, 2 mM Na3VO4 and protease inhibitors (protease inhibitor mix complete, Roche) for 15 min on ice. After centrifugation at 15000g (30 min, 4°C), the supernatants were incubated for 2 h at 4°C with anti-FLAG(M2)-antibody covalently coupled to agarose beads (Sigma) or with 1 μg of the appropriate first antibody and 20 μl of protein-G-sepharose beads (GE). Before the addition of antibodies, a small aliquot of each supernatant was preserved and diluted with 2 × SDS-PAGE sample buffer for subsequent western blot analysis (lysate). After incubation, the beads were washed extensively with lysis buffer and diluted with 2 × SDS-PAGE sample buffer. Bound proteins were resolved by SDS-PAGE, blotted on to polyvinylidene difluoride-membranes and visualized with enhanced chemiluminescence after incubation with primary antibodies as indicated in the figure legends (32). To analyse Nek8 expression in fibroblasts derived from the fetuses, cells were lysed in 1% Triton X-100 buffer as described above followed by centrifugation at 15000g (15 min, 4°C). The supernatant was diluted with 2 × SDS-PAGE sample buffer, incubated at 95°C for 5 min and analysed by immunoblotting. Equal total protein amount was confirmed by staining anti-actin.

qPCR

Human fetal fibroblasts cultured with or without serum starvation were harvested in Qiazol (Qiagen), and RNA was isolated using the phenol–chloroform method. After DNase treatment (Ambion), reverse transcription was performed using ABI’s HighCapacity cDNA Kit and qPCR analysis was performed as previously described (32). ABI Taqman assays were used to evaluate CTGF (Hs00164957_m1) and ITGB2 (Hs00170014_m1) levels. ActB (4326315E) served as endogenous control. The expression levels of NPHP9, PKD1, PKD2, Birc5 and c-MYC were evaluated with SYBR Green qPCR (NPHP9 fp: 5′-CTTCTTCAGCTGCCTGTGACTT-3′; NPHP9 rp: 5′-GGCCCTTTCGGCAGGCACAGGT-3′; c-MYC fp: 5′-TTTCCGTTAGTGAAAAACCA-3′; c-MYC rp: 5′-CCACGAGTCGTAGTGGAGTT-3′; Birc5 fp: 5′-CCACTGA GAACAGGCCAGACTTGA-3′; Birc5 rp: 5′-AGAAAGGGA AAGCGAACCAG-3′; PKD1 fp: 5′-tcttggagttcttggagaa-3′; PKD1 rp: 5′-cctttgccatgtgctgttgact-3′; PKD2 fp: 5′-GGAAAGGAGCAGCTACCCAGG-3′; PKD2 rp: 5′-CACATTGTTGAGCTCATCTAG-3′), HPR1 served as endogenous control (HPR1 fp: 5′-ttgagctgtgagctgtgac-3′; HPR1 rp: 5′-gccttctttcagcagcag-3′). All qPCR experiments were performed on an ABI 7900HT System. RNA was isolated from fibroblasts of fetus 1, fetus 3 and a control fetus and analysed in three biological replicates. Data were combined according to the genotype. Error bars shown in the figures represent SEM.

Plasmids and reagents

The GAL4-TEAD reporter system (pGBD-Hyg-Luc; pGal4-TEAD) was purchased from Biomyx (San Diego, CA, USA). Eps, NPHP3, NPHP9 and TAZ constructs were described before (17,24,32). CHX was purchased from Sigma. Cells were treated with 100 μg/ml CHX for 8 h prior to harvest. Antibodies were from Sigma (mouse anti-FLAG/ M2 #F3165: western blot 1:10,000, mouse anti-actin #A2288: western blot 1:1,000), Serotec (mouse anti-V5 #MCA1360: western blot 1:5,000), Millipore (rabbit anti-V5 AB3792: western blot 1:2,000) and Genetex (rabbit anti-Nek8/Nphp9 #GTX112027: western blot 1:500).

Immunohistochemistry

Slides of fixed and paraffin-embedded mouse kidneys were de-paraffinized using Xylool and descending concentrations of ethanol (40). Antigen retrieval was carried out by warming kidney slides in citrate buffer (10 mM, pH 6) for 10 min using a microwave. After blocking with 3% H2O2 and Avidin and Biotin (Vector Laboratories, Inc.) for 15 min each, slides were sequentially incubated with the antibodies indicated [mice c-myc (9E10; Roche); biotinylated antimouse IgG (H + L), Vector Laboratories, Inc.]. Kidney slides were labelled with ABC kit (Vector Laboratories, Inc.), and development was carried out using diaminobenzidine solution (Thermo Scientific). For counterstaining, we used haematoxylin for 10 s. Afterwards, the kidney slides were dehydrated, mounted with Histomount (National Diagnostics) and analysed using brightfield microscopy. Pictures were taken with an inverted microscope (Axiovert 200, obj.: Plan Apochromat 20×/0.8 NA; Zeiss) equipped with a camera (AxioCam ICC1; Zeiss) using Axiovision 4.8 software for acquisition and subsequent image processing.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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