Individuals with mutations in XPNPEP3, which encodes a mitochondrial protein, develop a nephronophthisis-like nephropathy

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The autosomal recessive kidney disease nephronophthisis (NPHP) constitutes the most frequent genetic cause of terminal renal failure in the first 3 decades of life. Ten causative genes (NPHP1–NPHP9 and NPHP11), whose products localize to the primary cilium-centrosome complex, support the unifying concept that cystic kidney diseases are “ciliopathies”. Using genome-wide homozygosity mapping, we report here what we believe to be a new locus (NPHP-like 1 [NPHP1L1]) for an NPHP-like nephropathy. In 2 families with an NPHP-like phenotype, we detected homozygous frameshift and splice-site mutations, respectively, in the X-prolyl aminopeptidase 3 (XPNPEP3) gene. In contrast to all known NPHP proteins, XPNPEP3 localizes to mitochondria of renal cells. However, in vivo analyses also revealed a likely cilia-related function; suppression of zebrafish Xpnpep3 phenocopied the developmental phenotypes of ciliopathy morphants, and this effect was rescued by human XPNPEP3 that was devoid of a mitochondrial localization signal. Consistent with a role for XPNPEP3 in ciliary function, several ciliary cystogenic proteins were found to be XPNPEP3 substrates, for which resistance to N-terminal proline cleavage resulted in attenuated protein function in vivo in zebrafish. Our data highlight an emerging link between mitochondria and ciliary dysfunction, and suggest that further understanding the enzymatic activity and substrates of XPNPEP3 will illuminate novel cystogenic pathways.

Introduction

The autosomal recessive kidney disease nephronophthisis (NPHP) is the most frequent genetic cause of end-stage kidney disease in
Because mutations in the known NPHP genes are found in only 35% of NPHP families, and many questions remain regarding the molecular pathogenesis of NPHP, we sought to clone additional disease-causing genes using whole-genome homozygosity mapping. Here we report causative mutations in the gene encoding X-prolyl aminopeptidase 3 (XPNPEP3) as what we believe to be a new cause of an NPHP-like disorder. We demonstrate that XPNPEP3 localizes to mitochondria in renal cells in vitro and to kidney tubules in a cell type–specific pattern. Although, XPNPEP3 was undetectable in primary cilia or centrosomes, biochemical studies demonstrate that several cystogenic proteins are likely XPNPEP3 substrates, including NPHP6/CEP290, in which loss of function causes a range of ciliopathies, including NPHP (9, 23–27). Our data implicate for the what we believe to be first time a role for mitochondrial proteins in the development of ciliopathy-like phenotypes and offer a potential mechanism for the cystogenic effect of XPNPEP3 loss of function.

Results

Mutations in XPNPEP3 cause an NPHP-like kidney disease. We performed a whole-genome search for linkage in 116 consanguineous kindred with NPHP and NPHP-like phenotypes. Linkage analysis in a 5-generation consanguineous family from northern Finland (A131), with 3 members affected with an NPHP-like disorder, yielded a significant logarithm of odds (LOD) score of LODmax = 3.6, defining a new locus (NPHP-like 1, NPHPL1) on chromosome 22q13.2 (Figure 1A), within a 4.3-Mb interval flanked by markers SNP_A-1516630 and SNP_A-1649765 to a 4.3-Mb interval, which contains 101 positional candidate genes (per the UCSC sequence; http://genome.ucsc.edu/). Mutations were detected in XPNPEP3 (encircled red). (C) The XPNPEP3 gene extends over 70.8 kb and contains 10 exons (vertical hatches). (D) Exon structure of human full-length XPNPEP3 cDNA (3,056 bp). Positions of start codon (ATG) at nt +1 and of stop codon (TGA) are indicated. Exon sizes, ranging from 63 bp to 997 bp, are approximated. (E) Positions of the mitochondrial localization signal (Mito sign). The SMART program (http://smart.embl-heidelberg.de) predicts a putative N-terminal aminopeptidase P domain (AMP_N; [TGA] are indicated. Exon sizes, ranging from 63 bp to 997 bp, are approximated. (TGA) are indicated. Exon sizes, ranging from 63 bp to 997 bp, are approximated. (F) Two homozygous mutations of XPNPEP3 detected in families A131 and F543 with NPHPL1 (see Table 1).

Mechanistically, several hypotheses have been put forth to explain the mechanism of cystogenesis in the absence of functional cilia. A mechanosensory-based hypothesis suggested that abnormal flow sensing is causally associated with cyst formation (16), whereas more recent data have indicated that defects in the axis of cell division and polarization of renal epithelial cells might drive the cystic phenotype (17). Interestingly, both models implicate defective ciliary extension movements during gastrulation (22, 23).
Table 1

<table>
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<th>Family</th>
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<th>A131 (II-2)</th>
<th>A131 (II-3)</th>
<th>A131 (II-4)</th>
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All mutations were absent from more than 150 healthy control subjects (see text). Amino acid residue conservation is indicated for lowest species with continuous conservation.

Chromosomal analysis. We detected no patients with 2 recessive mutations in any of 823 families with an NPHP-like phenotype and in 100 index cases with isolated RCC1 deficiency. Ten novel heterozygous mutations of unknown pathogenicity were identified in 10 patients. We did not find any kindred with 2 recessive mutations in any of the 643 families with NPHP-like phenotype (Table 1).

We thus identified mutations in XPNPEP3 as a novel cause to our knowledge of an NPHP-like nephropathy (NPHP-L1). XPNPEP3 is contained in the M24B subfamily of X-prolyl peptidases that comprises aminopeptidase P and the dipeptidase prolidase. XPNPEP3 has a predicted C-terminal catalytic domain and has greatest overall sequence identity (32%) to E. coli aminopeptidase P. This high level of conservation, which includes the catalytic domain, suggests that XPNPEP3 is a functional aminopeptidase P and is 1 out of 3 X-prolyl aminopeptidases known to exist in mammals (31) (Figure 1E). The E. coli ortholog and the human XPNEP3 contain 2 manganese ions that are required for peptidase activity, sites that are also conserved in XPNEP3 (31–33). Studies performed with the E. coli ortholog (34) indicate that XPNEP3 recognizes N-terminal peptides that have proline in their second position as substrate and cleaves the peptide bond between the first and second amino acid residues, releasing the N-terminal amino acid. In addition, Mito-Prot II analysis of XPNPEP3 detected an N-terminal mitochondrial localization signal with a cleavage site after amino acid 53, resulting in a 51-kDa protein of likely mitochondrial function (Figure 1E).

Clinical and biochemical phenotype of individuals with NPHP-L1. Clinical data of kindred A131 and F543 are presented in Table 1. Three affected individuals of kindred A131 had moderate renal insufficiency (glomerular filtration rate, 30%–40% of normal) at between 20 and 29 years of age (Table 1). Two individuals...
of the northern Finnish family A131 had undergone renal biopsy, which yielded characteristic features of NPHP (Figure 2). Renal ultrasound revealed features characteristic of NPHP (Table 1), while extrarenal manifestations included essential tremor in all 3 affected individuals. Two affected individuals (A131 II-3 and A131 II-4) also had high frequency sensorineural hearing loss; A131 II-3 had arachnoid cysts on brain imaging, while A131 II-1 and A131 II-3 also had gout.

In the Turkish kindred (F543), the 2 affected individuals exhibited end-stage kidney disease by 8 and 9 years, respectively (Table 1). Renal ultrasound and renal histology demonstrated NPHP (data not shown). In addition, both affected individuals manifested a mitochonrdiopathy, with an isolated complex I deficiency and decreased NADH-CoQ oxidoreductase activity. This complex I deficiency was not found in fibroblast cultures from family A131, with the splice-site mutation (Supplemental Tables 2 and 3), but in family F543, with a homozygous frame-shift mutation, it was detected in a muscle biopsy of F543-I (Supplemental Tables 4 and 5) and in a fibroblast culture of F543-II (Supplemental Tables 6 and 7). Additionally, both affected individuals had seizures and hypertrophic dilated cardiomyopathy. F543 II-1 also demonstrated chronic pancreatitis with pancreatic cysts, while F543 II-2 had a hepatopathy.

A signal peptide traffics XPNPEP3 to mitochondria. To study the expression and subcellular localization of XPNPEP3, we characterized a rabbit polyclonal anti-human XPNPEP3 antibody (HPA000527, Sigma-Aldrich), hereafter called α-XPNPEP3. Upon immunoblotting, α-XPNPEP3 specifically recognized transiently transfected V5-tagged human XPNPEP3 (XPNPEP3-V5) as well as endogenous XPNPEP3 in lysate from murine inner medullary collecting duct (IMCD3) cells (Supplemental Figure 3B). It also specifically detected overexpressed XPNPEP3-V5 in mitochondria, where it colocalized with the anti-V5 antibody (Supplemental Figure 3, A and C–E) as determined by immunofluorescent staining. Upon immunoblotting of cell lines from human, monkey, or dog, the α-XPNPEP3 cross-reacted with single bands at 51 kDa, representing the expected size for full-length XPNPEP3, following cleavage of the mitochondrial signal peptide (data not shown). Immunoblotting of multiple tissues from adult mouse detected single bands in cell lysates of kidney, heart, liver, skeletal muscle, brain, and testis (Supplemental Figure 4).

Because of the predicted targeting of XPNPEP3 to mitochondria, we fractionated whole kidney homogenates from mice into mitochondrial and cytosolic fractions. Immunoblotting with α-XPNPEP3 yielded a single band in the mitochondrial fraction at approximately 51 kDa, consistent with the product predicted to result from cleavage of the mitochondrial signal peptide following mitochondrial import (Figure 3A). The cytosolic fraction showed a doublet at approximately 57 kDa, compatible with unprocessed XPNPEP3. Immunofluorescent microscopy in IMCD3 cells stably expressing human full-length XPNPEP3-GFP, which contains the mitochondrial leader sequence, were localized to mitochondria (Figure 3B). In contrast, in IMCD3 cells that stably express a CDNA, which lacks the mitochondrial leader sequence (AN-XPNPEP3-GFP), XPNPEP3 was found diffusely in the cytoplasm (Figure 3C). Expression of XPNPEP3 in mitochondria was also confirmed by transmission electron microscopy in ultrathin rat kidney sections, using immunogold-labeled α-XPNPEP3 (Figure 3D).

No evidence for XPNPEP3 in primary cilia, basal bodies, and centrosomes in vitro. NPHP is considered a ciliopathy, because 10 different causative genes (NPHP1–NPHP9 NPHP11) share the feature of subcellular function of their respective gene products at primary cilia, basal bodies, or centrosomes (1). We therefore examined XPNPEP3 for organellar targeting to the cilia/basal body/centrosome complex. In IMCD3 cells that stably express the human full-length XPNPEP3-GFP, which contains the mitochondrial leader sequence, XPNPEP3-GFP was not detected in primary cilia, basal bodies, or centrosomes (Figure 4). We conclude that loss of XPNPEP3 leads to an NPHP-like phenotype (NPHPL1), without any apparent ciliary localization, departing from the paradigm of all other genes mutated in NPHP.

XPNPEP3 exhibits cell type–specific expression in kidney. As the most prominent phenotype in individuals with suppressed XPNPEP3 function was manifested in the kidney, we performed immunofluorescent microscopy of rat kidney sections to study cell type–specific expression. We found XPNPEP3 to be expressed specifically in distal convoluted tubule and cortical collecting duct cells (Supplemental Figure 5). Of note, XPNPEP3 expression was detected not in principal cells but in intercalated cells, which are known to lack primary cilia (Supplemental Figure 5).

xpnpep3 suppression phenocopies ciliary and basal body zebrafish morphants. Depletion of several cystogenic proteins, including NPHP2, CEP290/NPHP6, and numerous BBS proteins, have been shown to perturb convergence and extension movements in zebrafish (18, 22, 25, 35), a noncanonical Wnt phenotype, which in the mammalian nephron manifests as failure of orientation of the axis of cell division and has been causally linked to tubular dilatation (20). We therefore wondered whether suppression of xpnpep3 might phenocopy this defect or whether loss of this gene/protein might affect hitherto unknown pathways. We therefore designed a translation-blocking morpholino (MO) against the sole Danio rerio ortholog (by reciprocal BLAST), a transcript that is expressed as early as 12 hours.

Figure 2
Renal histology of patient A131 II-4. Morphologic changes are characteristic for NPHP and include (a) thickening, splitting, and attenuation of tubular basement membranes in tubules with disorganized epithelium (thick arrows); (b) atrophic tubules that contain protein casts (thin arrows); (c) dilated collecting ducts (cd), which are lined by exceptionally tall epithelium; and (d) a mild degree of interstitial fibrosis. Most glomeruli appeared normal, but there were a few scattered obsolescent glomeruli (not shown). Scale bar: 30 μm.
after fertilization, as determined by RT-PCR (data not shown). At the mid-somite stage, embryos injected with xpnpep3 MO (n = 50–100 embryos/injection; scored blind) displayed quantifiable gastrulation defects reminiscent of ciliary gene morphants (22, 25, 35); these included a shortened body axis, small anterior structures, broadening and kinking of the notochord, and elongated somites (Supplemental Figure 6). These phenotypes were unlikely to be caused by nonspecific action of the MO: not only did we observe an increase in affected embryos correlated with dose, but rescue with capped full-length human XPNPEP3 mRNA resulted in significant amelioration of the phenotype (χ² = 34.45, P < 0.0001). These phenotypes are likely independent of the XPNPEP3 mitochondrial activity: coinjection of xpnpep3 MO with a human rescue construct devoid of the mitochondrial localization signal (ΔN-XPNPEP3) rescued the phenotype in a manner indistinguishable from that of the WT human construct (χ² = 3.47, P = 0.17). Therefore, targeting of XPNPEP3 to the mitochondrion is not required to rescue the convergence and extension phenotypes, suggesting that this protein might also have mitochondrial-independent activity.

Cleavage of known cystic disease proteins by XPNPEP3. Our data led us to hypothesize that, in addition to its mitochondrial functions (which potentially explain the RCCI defects observed in family F543), XPNPEP3 might affect the function of ciliary proteins biochemically, most likely through its N-terminal proline cleavage activity. To identify such candidate substrates, we parsed the ciliary proteome (28) for proteins with a proline in the second position (after N-terminal methionine cleavage). To reduce incidence of false-positives, we used a stringent version of the proteome that was restricted to reciprocal orthologs (minimal E value of 10⁻³⁰) identified in at least 2 independent ciliary studies, thereby parsing 426 likely ciliary proteins for substrate candidacy. We identified 51 proteins with sequences that fulfilled our search criteria (Supplemental Table 8). Interestingly, 3 candidate substrates (centrosomal protein 290 kDa/NPHP6 [CEP290/NPHP6], Alstrom syndrome 1 [ALMS1], and leucine rich repeat containing 50 [LRRC50]) are known to cause cystic renal disease (9, 36, 37).

To verify our computational predictions, we next sought to test whether XPNPEP3 can cleave these 3 candidate substrates. Expression of recombinant human XPNPEP3 in bacteria was not pos-
more than 99% homogeneity as detected by Coomassie stain processed by ecAPP (data not shown). Importantly, the cleavage of XPNPEP3, ecAPP, which we were able to express and purify to sufficient purity, by ecAPP (Figure 5C). By contrast, peptides containing residues other than proline in the second position could not be processed by ecAPP (data not shown). Importantly, the cleavage activity of the enzyme is not promiscuous, since we found that dynein, another ciliary protein that also contains a proline in the second residue but is not involved in cystic kidney diseases, is poorly digested by ecAPP (Figure 5C). These data suggested that XPNPEP3 cleavage of other cystogenic proteins might be relevant to their biological function(s).

Requirement of XPNPEP3 N-terminal cleavage for early gastrulation in zebrafish. To probe the importance of the likely XPNPEP3 cleavage sites in vivo, we asked whether cleavage resistance was deleterious to protein function. Focusing on LRRC50 (each of CEP290/ NPHP6 and ALMS1 encode mRNA more than 7 kb, rendering them difficult to transcribe in vitro at sufficient purity), we investigated whether a cleavage-resistant human capped mRNA can rescue the gastrulation phenotypes caused by MO-driven suppression of endogenous lrrc50. Injection of 4.5 ng of a translation-blocking MO induced early gastrulation phenotypes that include all the hallmarks of cilia-related convergent extension defects and which could be rescued by coinjection of 150 pg of WT human capped LRRC50 mRNA (Figure 6). However, coinjection of MO with mRNA encoding an N-terminal Pro-Val point mutation, followed by blind scoring of embryos, showed complete failure of rescue (WT vs. Pro-Val, \( \chi^2 = 39.20, P < 0.0001; n = 80–110\) embryos/injection). This experiment could not exclude the possibility that the observed loss of function could be due to the presence of the Val at this position. However, repetition of this assay with Asp or Arg yielded similar data (Figure 6). Still, the possibility remained that the observed failure to rescue might still be driven by the loss of the proline for reasons other than cleavage. To assess this possibility, we searched the zebrafish genome for other N-terminal aminopeptidases and identified an alanyl aminopeptidase (ZFIN ID, ZDB-Gene-030131-1253), which predicts that Ala at the position of the N-terminal proline should render lrrc50 subject to cleavage. Consistent with the notion that it is the cleavage that functionalizes lrrc50, injection of MO with an Ala-encoding human mRNA rescued the phenotypes in a manner indistinguishable from WT (Figure 6; \( \chi^2 = 0.26, P < 0.87 \)).

Discussion

Using a whole-genome homozygosity mapping approach, we hereby identify recessive mutations in XPNPEP3 as a new cause of an NPHP-like disease. The phenotypic severity and organ involvement vary widely between the 2 different families with XPNPEP3 mutations, whereas the phenotypic spectrum is concordant for affected individuals within the same family (Table 1). Specifically, 3 affected individuals of family A131 exhibited only a mild form of an NPHP-like kidney disease (Figure 2), with residual kidney function beyond the age of 20 years, at which time virtually all patients with “classic” NPHP would have reached ESRD (1). Extrarenal manifestations were limited to mild neurologic involvement with sensorineural hearing loss and essential tremor (Table 1). In contrast, the 2 affected siblings of F543 exhibited early-onset renal failure, leading to renal replacement therapy at 8 and 9 years of age, with extrarenal manifestations of mental retardation, seizures, and cardiomyopathy. The observed phenotypic variability might be due to different degrees of loss of function for the 2 different homozygous XPNPEP3 alleles. Whereas in family A131, residual correct splicing of the 80% conserved splice consensus may produce some WT splice product and thereby residual function (Supplemental Figure 2), the frameshift mutation of F543 will result in complete truncation of the C-terminal third of XPNPEP3 and likely renders the mutant message substrate for nonsense-mediated decay. This notion is also supported by the finding that there was a measurable reduction in RCCI function in muscle biopsy samples of F543-I and fibroblasts of F543-II but not those of family A131.
Alternatively, modifiers in either family might potentiate or protect the effect of the respective primary mutations, as has been suggested for other ciliopathies (23, 38).

NPHP is considered a ciliopathy because 10 different causative genes (NPHP1–NPHP9 and NPHP11) share the feature of subcellular expression of their respective gene products at primary cilia, basal bodies, or centrosomes (1). The absence of XPNPEP3 in cilia or basal body from in vitro cell culture could be due to the limitation of antibody affinity, or epitope internalization within cilium, or requirement of further posttranslational procession of XPNPEP3 before localizing to that organelle. It is also possible that trafficking of XPNPEP3 to the cilium or basal body requires some specific physiological stimulus, as shown for Smo (39).

Nevertheless, our data suggest a mitochondria-independent, yet cilia-related function, for XPNPEP3. Suppression of xpnpep3 in zebrafish phenocopies the developmental phenotypes induced by the suppression of numerous ciliary and basal body–encoding genes (22, 23, 25). Importantly, these phenotypes can be rescued by the xpnpep3 isoform devoid of mitochondrial targeting sequence, which in mammalian cells leads to cytoplasmic diffusion of the protein. We propose that the ciliary phenotypes unmasked by loss of XPNPEP3 might arise from the loss of XPNPEP3-dependent processing of ciliary proteins. This idea is reinforced by the observed requirement to cleave the N-terminal proline of 3 cystogenic pro- teins, most likely executed by XPNPEP3, and by the demonstration that, at least for LRRC50, N-terminal proline cleavage is necessary for correct gastrulation movements. Given the observed localization of XPNPEP3 in mammalian renal cells, we speculate that such processing might occur outside the cilium, especially since there is mounting evidence for the requirement of ciliary proteins in preciliary functions that include vesicular transport to the apical membrane/transition zone (41) and for the assembly of some intraflagellar transport particles in the cytoplasm (42). Although, in the present study CEP290/NPHP6, ALMS1, and LRRC50 (loss of each of which can induce renal cysts), have been identified as likely targets of XPNPEP3, we do not know whether it is the dys- function of these molecules that drives NPHP in the 2 families with XPNPEP3 mutations. It will be important to assay the maturation of these 3 proteins in patient cells and to examine the possible anatomical and signaling defects of primary renal cilia in the absence of XPNPEP3.

Methods

Study subjects. We obtained blood samples, clinical data, and pedigree information after obtaining informed consent from patients with NPHP and/or their parents. Approval for these studies was obtained from the University of Michigan Institutional Review Board. The diagnosis of NPHP, as defined by the Online Mendelian Inheritance in Man database, was based

![Figure 5](http://www.jci.org)
on the clinical course and renal ultrasound or renal biopsy that were compatible with the diagnosis of NPHP, as judged by a pediatric nephrologist. Patients were selected for additional mutation analysis using the criteria that they had an isolated RCCI deficiency and a residual RCCI activity beneath the lowest control value from 5% to 90%, with a median of 51%.

Renal involvement was recorded for 11 out of 100 of those patients. Ten patients had renal tubular acidosis and one had renal insufficiency.

**SNP genotyping and sequencing.** Whole-genome search for linkage was performed in 116 consanguineous families with NPHP using an Affymetrix SNP array (50 K). Data were evaluated with nonparametric LOD

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**Figure 6**

N-terminal cleavage of LRRC50 is required to rescue lrrc50 morphant phenotypes in zebrafish. (A) Quantitative representation of the effect of lrrc50 MO on gastrulation development and rescue efficiency of different human lrrc50 RNA isoforms. The developmental phenotype of embryos was scored as Class I–II as described previously (22). Normal, indistinguishable from WT; Class I, mildly affected with a shortened body axis, small anterior structures, mild somite defects; Class II, severely affected with a short body axis, poorly defined head and eyes, broadening and kinking of the notochord, broad, thin somites, and tail extension defects. (B) Representative examples of embryos showing the gastrulation defect caused by lrrc50 MO, which could be rescued by WT and proline-to-alanine human LRRC50 but not other mutants. PA, proline to alanine; PV, proline to valine; PD, proline to aspartic acid; PR, proline to arginine.
scores across the entire genome to identify regions of homozygosity by descent as reported previously (43). Genomic segments of homozygos- ity were confirmed by high-resolution haplotype analysis within those regions using microsatellite markers. Additional SNPs were typed by direct sequencing. The GeneHunter program was used to calculate multipoint LOD scores, assuming recessive inheritance with complete penetrance, a disease allele frequency of 0.001, and allele frequencies for northern European descent (Affymetrix).

**Mutational analysis of candidate genes.** Exon PCR of candidate genes was performed using genomic DNA from affected individuals. Exon-flank- ing primers were designed using the University of California, Santa Cruz sequence (http://genome.ucsc.edu/) and Primer3 software (http://ihg2. helmholtz-muenchen.de/igh/ExonPrimer.html). PCR products were purified (Marligen Biosciences) prior to direct sequencing (Genetic Analyzer 3700, Applied Biosystems). Sequence data were analyzed using the software Mutation Surveyor (SoftGenetics) and Sequencer (Gene Codes). One hundred ninety healthy control individuals were screened as negative controls for each mutation identified.

High-resolution melting analysis was used for mutation scanning technology to analyze the coding region of XPNPEP3 in 100 patients with isolated RCCI deficiency. XPNPEP3 exons were PCR amplified from 5 ng genomic DNA, with a final denaturation step at 94°C for 1 minute (0.25 units Thermo-Start Taq DNA polymerase [Abgene], 1x LCGreen Plus [BIOKE], 0.25 μM of each primer; Supplemental Tables 1–7). High-resolution melting analysis was performed on a LightScanner instrument (Idaho Technology). In the presence of the saturating double-stranded DNA-bind- ing dye, ampiclons were denatured, starting at 77°C, while fluorescence intensities were recorded continuously. Melting curves were analyzed by LightScanner software (Idaho Technology), with normalized, tempera- ture-shifted curves displayed as difference plots (−dF/dT). Detected sam- ples with altered melting curves, compared with the average of multiple WT results, were directly sequenced with the BigDye Cycle sequencing kit (Applied Biosystems) (44).

**Antibodies and protein expression studies.** The rabbit polyclonal α-XPNPEP3 was from Sigma-Aldrich (HPA000527). Secondary antibodies to rabbit, mouse, and goat IgG were conjugated with either Alexa Fluor 488 or 594 (Molecular Probes). Antibodies for β-actin and actinylated α-tubulin and γ-tubulin were from Sigma-Aldrich; V5 was from Invitrogen; and pericen- trin was from Novus. The aquaporin-2 antibody was a mouse monoclonal antibody from Santa Cruz Biotechnology Inc. (sc-9882). The cytochrome C oxidase antibody was a mouse monoclonal antibody from Santa Cruz Biotechnology Inc. (sc-658348).

**Cloning.** Full-length XPNPEP3 and ΔN-XPNPEP3 (amino acid 79–507) were PCR amplified from pENTR-XPNPEP3 (NM_022098.2, clone IOH5999; Invitrogen), with the termination codon removed and inserted into the pDONR221 vector, using BP Clonase II (Invitrogen), and subse- quently transferred into pEF5/FRT/VS-DEST (Invitrogen) and p-G-AP5 (C-terminal GFP-Lap tag) (45) or pCS2+ vectors, using LR Clonase II (Invitrogen). Human LRRCS0 was cloned into the pCS2+ vector; vectors encoding mutated LRRCS0, with the third proline residue replaced by alanine, valine, aspartic acid, or arginine, were generated by site-directed mutagenesis. The fidelity of all constructs was confirmed by sequence analysis. MitoProt II was used for prediction of mitochondrial localization sig- nal (MitoProt, http://ihg2.helmholtz-muenchen.de/igh/mitoprot.html).

**Immunoblotting.** Immunoblotting was performed as described previ- ously (9). For mitochondrial fractionation studies, whole kidneys from 6-month-old male J2956 mice were isolated, homogenized, and fraction- ated with differential centrifugation into mitochondrial and cytosolic fractions. Then, 50 μg total protein from each fraction was loaded and separated on a denaturing 4%–12% SDS-PAGE gradient gel and trans- ferred to PVDF membrane. Total protein was visualized using Ponceau S staining. Membranes were then immunoblotted with α-XPNPEP3 over- night at 4°C at a titer of 1:500.

**Immunoelectron microscopy.** Kidney cortex and medulla from 4-month- old Sprague-Dawley rats were used for immunocytochemical analysis. Tissues were fixed lightly for 5 minutes at 4°C with 3% formaldehyde and 20 mM ethylacetimide in HEPES-buffered saline (30 mM HEPES, 151 mM NaCl, 4.7 mM KCl, 1.2 mM MgCl2, 7.8 mM glucose, pH 7.3) at 4°C. These samples were washed, further fixed in 3% formaldehyde containing 0.25% glutaraldehyde in HEPES-buffered saline at 4°C for 45 minutes, then dehydrated in ethanol and embedded in LR White Resin (Polysciences Inc.). Thin sections were blocked with PBS containing 1% w/v BSA and 0.01% w/v Tween 20 (PBT). Grids were then incubated with α-XPNPEP3 (HAP000527, Sigma-Aldrich) at 1:10 and 1:25 dilution in PBT for 12 hours at 4°C. Negative controls included normal rabbit serum and PBT replaced as the primary antibody. After washing, grids were incubated for 1 hour in 10-nm gold-conjugated goat anti-rabbit IgG (Amersham Life Sciences) diluted 1:30 in PBT, rinsed with PBS, and fixed with glutaraldehyde to sta- bilize the gold particles. Samples were stained with uranyl acetate and lead citrate, then examined in a Zeiss CEM902 electron microscope.

**Tissue culture.** XPNPEP3-V5- and XPNPEP3-LAP-expressing cell lines were generated by cotransflecting IMCD3 Flp-In cells (Invitrogen) with pOG44 and pDEST EF/FRT XPNPEP3-V5 or p-G-LAP-XPNPEP3, accord- ing to the manufacturer’s instructions. Stable cells were generated by selection with hygromycin (800 μg/ml).

Measurements of mitochondrial respiratory complex function were performed in muscle biopsy samples from family F543 as described previously (46, 47). In individual A131 II-4, mitochondria were isolated from a muscle biopsy sample using the method of Rasmussen et al. (48), and the respira- tory chain complex activities were measured according to the method of Rustin et al. (49). An Amino DC-DW2 Dual-Wavelength Spectrophotometer was used to record the enzyme kinetics.

**Immunofluorescence studies in IMCD3 cells.** Cells were grown on 24-well glass bottom plates, stained with Mitotracker Red CMTMRos (250 nM) (Invitrogen) for 30 minutes at 37°C in 5% CO2, and fixed with 4% paraformaldehyde. For indirect immunofluorescence studies, fixed cells were permeabilized with 0.1% Triton 1% BSA/PBS, pH 7.4, and stained with primary antibodies for 1 hour at 25°C, followed by secondary antibody immunolabeling. Nuclei were counterstained with Hoechst (Invitrogen). Epifluorescence microscopy images were acquired using a Zeiss AxioObserver.Z1, with a 63× 1.4 numerical aperture oil objective, and a Hamamatsu ORCA-ER CCD camera, with image stacks (0.5-μm steps) deconvolved and pertinent images of the cell z-projected, using Axiovision 3.1 software (Zeiss).

**Kidney histology and immunofluorescence.** Human kidney biopsies were fixed in Dusbois-Brasil solution and embedded in paraffin; 2- to 6-μm sections were stained with hematoxylin-eosin, PAS, PAS methenamine silver, Masson’s trichrome, and Congo red for routine diagnostics. Immunofluorescence of rat kidney sections was performed as previously described (50).

**Protein purification and mass spectrometry.** The E. coli ortholog of XPNPEP3, ecAPP, was cloned into the pET28a vector (Novagen) to express a fusion protein with a His tag at the N terminus. The resulting construct was transformed into E. coli BL21 (DE3) codon plus RIL (Strat- agene). After induction by IPTG, ecAPP was purified with Ni-NTA tech- nology (Qiagen). The 9-amino acid peptides identical to the N-terminals of NPHP6, ALMS1, LRRC50, and dynein without methionine were syn- thesized by Genscript. In a typical enzymatic reaction, 100 μM peptide substrate was incubated with 100 nM purified ecAPP, which was preac- tivated by 20 times excess MnCl2 in 20 mM Tris-HCl, pH 7.4, (0.75 mM MnCl2). The reaction was stopped by boiling samples at 100°C for
5 minutes, and product was detected by ESI-LC-MS (Agilent Technologies). Candidate cleavage substrates were identified from the Ciliary proteome database (http://cilia.proteome.org).

Rescue of gastrulation phenotypes in zebrafish. M0s (Gene Tools) targeting the Danio rerio ortholog of XPNPEP3 or LRRCC5 were resuspended to appropriate concentrations, using sterile deionized water, and injected into WT embryos at the 2-cell stage. To rescue mid-somite morphant phenotypes, we generated capped mRNA for MO coinjections, using linearized pCS2+ XPNPEP3 (full length or ΔN-XPNPEP3) or pCS2+ LRRCC5 (WT or mutants) as a template to transcribe mRNA with the SP6 mMessage mMachine kit (Ambion). xpnpep3 rescue studies were carried out with 4 ng translation blocker MO and 50 pg mRNA. For brc50 rescue studies, 4.5 ng of brc50 translation blocker MO was cojected with 150 pg of RNA. Gastrulation phenotypes were scored at the 10 somite stage.

Statistics. To evaluate zebrafish knockdown phenotypes, Pearson χ² test was used; P values of less than 0.05 were considered significant (Figure 6).

Putative ciliary targets of XPNPEP3 were ranked using expectant values (Supplemental Table 8).

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