Targeted disruption of Nphp1 causes male infertility due to defects in the later steps of sperm morphogenesis in mice

Si-Tse Jiang1, Yuan-Yow Chiou2, Ellian Wang4, Hsiu-Kuan Lin2, Sue-Ping Lee1, Hsin-Yi Lu1, Chi-Kuang Leo Wang5, Ming-Jer Tang3 and Hung Li1,6,*

1Institute of Molecular Biology, Academia Sinica, Taipei 115, Taiwan, 2Department of Pediatrics, Institute of Clinical Medicine and 3Department of Physiology, National Cheng Kung University Medical College, Tainan 704, Taiwan, 4Department of Life Sciences, National Taiwan Normal University, Taipei 106, Taiwan, 5National Laboratory Animal Center, National Applied Research Laboratories, Taipei 106, Taiwan and 6Institute of Biochemistry, National Yang-Ming University, Taipei 112, Taiwan

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Juvenile nephronophthisis type I is the most common genetic disorder causing end-stage renal failure in children and young adults. The defective gene responsible has been identified as NPHP1. Its gene product, nephrocystin-1, is a novel protein of uncertain function that is widely expressed in many tissues and not just confined to the kidney. To gain insight into the physiological function of nephrocystin, Nphp1-targeted mutant mice were generated by homologous recombination. Interestingly, homozygous Nphp1 mutant mice were viable without renal manifestations of nephronophthisis. They appeared normal, but males were infertile with oligoteratozoospermia. Histological analysis of the seminiferous tubules showed that spermatogenesis was blocked at the early stages of spermatid elongation, with degenerating spermatids sloughing off into the lumen. Electron microscopic analysis revealed detachment of early elongating spermatids from Sertoli cells, and a failure of sperm head and tail morphogenesis. However, a few mature spermatozoa were still deposited in the epididymis, though they were frequently dead, immotile, or malformed. These novel findings indicate that nephrocystin is critically required for the differentiation of early elongating spermatids into spermatozoa in mice. The possible roles of nephrocystin in the formation and maintenance of Sertoli–spermatid junctions are still under investigation.

INTRODUCTION

Juvenile nephronophthisis (nephronophthisis type I, NPH), an autosomal recessive cystic kidney disease, is the most frequent genetic cause of end-stage renal disease (ESRD) in children and young adults (1,2). It presents with renal symptoms such as polyuria, polydipsia, failure to thrive, pallor, unexplained fever and decreased urine concentrating ability at 4–6 years of age, culminating in ESRD at an average age of 13 years. NPH shows a characteristic pattern of renal histology, consisting of tubular basement membrane disruption, interstitial cell infiltration, tubular atrophy, and cyst formation at the corticomedullary border (3). This location of renal cyst formation is distinct from the more usual polycystic kidney disease, in which renal cysts are distributed uniformly throughout the entire kidney. Associations with various extra-renal manifestations including ocular motor apraxia, retinitis pigmentosa, coloboma of the optic nerve, cerebellar vermis aplasia, liver fibrosis and cone-shaped epiphyses are frequently observed (4–8). By linkage analysis a responsible gene NPHP1 has been identified (9). In ~80% of patients with mutation in NPHP1, large homozygous deletions involving this gene are found (10). This causal relationship should be further verified in animal models with targeted disruption of NPHP1 homologs.

NPHP1 encodes a 732-amino acid protein termed nephrocystin-1 (9). It contains an N-terminal coiled-coil...
structure, a Src homology 3 (SH3) domain flanked by two glutamatic acid-rich domains, and a nephrocystin homology domain (NHD) comprising the highly conserved C-terminal two-thirds protein (11). The coiled-coil domain is an amphipathic α-helical heptad repeat recognized for its ability to mediate protein–protein interaction. Such interactions have been described in hetero-dimerization of the two gene products of polycystic kidney disease (polycystin 1 and polycystin 2) (12). SH3 domains are modular protein-binding domains that are mostly found in adaptor proteins such as Crk in focal adhesion signaling complexes (13). The nephrocystin SH3 domain has been shown to interact with Crk-associated substrate p130Cas and proline-rich tyrosine kinase 2 (Pyk2), which are key mediators regulating cell–cell and cell-matrix signaling (14,15). The NHD is known to be involved in targeting of nephrocystin to epithelial cell–cell junctions, interacting with the actin-binding proteins known as filamins, and establishing cell polarity (15,16). Although little is known about the pathogenesis by which a defect in NPHP1 might lead to NPH, these research findings point to a possible role of nephrocystin-1 in adhesion-regulated signaling events that modulate the actin cytoskeleton. Recently, nephrocystin was shown to interact with and to colocalize with invesrin in the primary cilia of renal tubular epithelial cells (17). Mutations in INVS, encoding invesrin also cause the other type of nephronophthisis (infantile form NPH). This study demonstrates the link between nephrocystin-1 and the function of primary cilia. These functional hypotheses for nephrocystin-1 require further verification in different organisms.

Northern and in situ hybridization analyses of the tissue expression pattern of nephrocystin-1 in various animals have demonstrated widespread but relatively weak NPHP1 expression in human adults (11). However, the discrepancy between widespread tissue expression and the restriction of symptoms to the kidney is hard to reconcile. Although the extra-renal manifestations associated with NPH hint a possible role of nephrocystin-1 in these tissues, the molecular mechanism of the causal connection between NPHP1 disruption and NPH is still unclear. To resolve these issues, a mouse line with targeted disruption of Nphp1 should be generated. This would permit extensive studies into the physiological functions of nephrocystin-1 in various organs. In contrast to the ubiquitously low expression of NPHP1 in human, there was exceptionally high expression of Nphp1 in mouse testis, specifically in cell stages of the first meiotic division and thereafter (11). This prominent expression in mouse testis suggests an additional role in spermatogenesis. Here we report that nephrocystin is critically required for the differentiation of early elongating spermatids into spermatozoa in mice.

The functional unit of the mammalian testis that produces germ cells is the seminiferous tubule. This tubule is composed of two different populations of cells that have a complex structural and functional interrelationship during the process of sperm production (spermatogenesis). Sertoli cells form the structural framework of the epithelium and provide the physiological environment required for the development of sperm cells. Cells of the germ cell lineage lie between the Sertoli cells, and are attached to them by a specialized cell–cell actin-based adherens junction (AJ) type termed as the ectoplasmic specialization (EPS) (18,19). During spermatogenesis, diploid spermatogonia divide and differentiate into spermatocytes, which subsequently undergo two successive meiotic divisions to give rise to haploid round spermatids. The round spermatids proceed to elongate, condense nuclei, acquire flagella and acrosomes, and shed a prominent amount of cytoplasm to form spermatozoa. Additionally, the developing germ cells migrate progressively from the basal to the luminal edge of the adluminal compartment so that fully developed spermatids can be released into the lumen at spermiation. If the Sertoli–germ cell junctions are disrupted before spermiation, spermatogenic cells cannot orientate and migrate properly, which also leads to premature germ cell release from epithelium, resulting in infertility (20–22).

In this study, we deleted, by homologous recombination, the last exon of Nphp1 to disrupt its expression. Surprisingly, the mutant mouse homozygotes were viable with normal appearance, though there was a loss of intercellular attachment between elongating spermatic and Sertoli cells in homozygous Nphp1 mutant mice, leading to infertility with oligoteratozoospermia. This finding correlates well with the exceptionally high expression of nephrocystin-1 in testis, and describes a novel role for nephrocystin-1 in spermatogenesis. This mutant mouse also provides a promising tool for investigation into the physiological functions of nephrocystin-1.

RESULTS
Generation of Nphp1 mutant mice

We first targeted the Nphp1 gene in mice by constructing a vector-containing regions of the mouse Nphp1 gene that, upon homologous recombination, replaced exon 1 with a neomycin (neo) cassette. This event would remove the start codon of the Nphp1 coding sequence, resulting in a frameshift expected to create a null allele. The targeting vector was introduced into RW4 embryonic stem (ES) cells, and 1274 clones resistant to G418 and gancyclovir were picked and genotyped. Unfortunately, no correctly targeted clones were identified by Southern blotting. We then generated in a similar fashion a second targeting construct designed to replace exons 5–6, which encode SH3 domain of the protein. Once again, no targeted clones with homologous recombination were found by genotyping 1085 ES cell clones. Finally, an approach using a floxed Nphp1 for conditional inactivation of nephrocystin-1 by Cre/loxP-mediated gene targeting was successful, as described in Materials and Methods, and summarized in Figure 1A and B. In all, 262 correctly targeted independent ES cell clones were identified from a total of 685 genotyped clones. Two targeted clones (no. 472 and no. 483) with the neo cassette and all three loxP sites in the targeted allele (lox20) were injected into C57BL/6J blastocysts to produce chimeras. Chimeric mice were used to generate floxed Nphp1 heterozygous (Nphp1+lox20/) mice on an inbred genetic background by mating with C57BL/6J mice. The Nphp1 exon 20-deleted heterozygous (Nphp1+/del20) and homozygous (Nphp1−/−del20) mice were generated as described in Materials and Methods.

Characterization of Nphp1+/del20−/−del20 mice

Nphp1+del20 N10 progeny derived from clone #472 were intercrossed and the N10F1 progeny were genotyped at 3
weeks of age: 76 wild-type (Nphp1\(^{+/+}\)), 165 Nphp1\(^{+/+}\)/del\(^{20}\) and 85 Nphp1\(^{del20/del20}\) animals were found. Genotyping of the clone #483-N10F1 progeny showed 38 Nphp1\(^{+/+}\), 64 Nphp1\(^{+/+}\)/del\(^{20}\), and 31 Nphp1\(^{del20/del20}\) animals, similar to clone #472-N10F1 progeny. The ratio of Nphp1\(^{+/+}\)/Nphp1\(^{del20/del20}\) in either mouse line did not differ significantly from the 1:2:1 ratio expected for a non-lethal mutation. Most of the phenotypic analyses were performed with the #472-derived mouse line, but identical results were also obtained with the #483-derived line. Reverse transcription polymerase chain reaction (RT–PCR) analysis of total ribonucleic acid (RNA) from 60-day-old Nphp1\(^{+/+}\), Nphp1\(^{+/+}\)/del\(^{20}\) and Nphp1\(^{del20/del20}\) mouse testes was performed to confirm that the gene targeting resulted in

**Figure 1.** Targeting of Nphp1 locus by homologous recombination and expression pattern of Nphp1 in testes. (A) Structure of wild-type, targeted and deleted Nphp1 alleles. Nphp1 is a 20-exon gene (the 3′-terminal exons are numbered and depicted by filled boxes). An inverted mcl promoter-driven neo-resistant gene (mcl-neo), floxed by two loxP sites (triangle flags), was inserted behind the last exon. The third loxP site was inserted into the intron 19. The floxed exon 20 and neo cassette were removed by Cre recombinase to generate the deleted allele, del\(^{20}\). A phosphoglycerate kinase (pgk) promoter-driven thymidine kinase gene (tk) was used for negative selection. Single arrows indicate the direction of transcription. Diagnostic restriction enzymes, probe for Southern blotting, primers for PCR analysis, and fragment size are indicated (B, BamHI; Sa, SacI; Sp, SpeI; X, XbaI). (B) Southern blot analysis of targeted mutant mice. DNA isolated from littermates derived from intercrossing of heterozygotes carrying the del\(^{20}\) allele was digested with SacI and hybridized with the probe outside the targeting vector. The 4.7 and 10.4 kb bands correspond to wild-type (+) and del\(^{20}\) alleles, respectively. (C) RT–PCR of Nphp1 transcripts in the wild-type (+/+), heterozygous (+/del\(^{20}\)), and homozygous (del\(^{20}/del^{20}\)) testes using primer sets F6-B11 and F16-B20 (see Materials and Methods for primer information). Amplification of β-actin shows the equivalence of the RNA loading and amplification. (D) Western blot analysis of nephrocystin-1 expression in testes. Three polyclonal antibodies against various regions of nephrocystin-1 (612–691 depicts the corresponding amino acids 612–691 of murine nephrocystin-1) all detect a ~90 kd band in wild-type (+/+), and heterozygous (+/del\(^{20}\)) but absent in homozygote (del\(^{20}/del^{20}\)). β-Actin was used as a control for equal loading of protein. (E) RT–PCR of Nphp1 transcripts in various ages of wild-type mouse testes from postnatal day 1 (D1) to day 365 (D365). All results are representative of at least three mice per genotype in two independent experiments.
Nphp1del mice are free from nephronophthisis

NPHP1 has long been known as the defect gene responsible for NPH in humans (9). Surprisingly, renal histology of Nphp1<sup>del20/del20</sup> mice did not reveal the pathological characteristics of nephronophthisis. The histological examination of the kidneys showed no obvious difference between Nphp1<sup>del20/del20</sup> mice and their control littermates at ages of 1, 2, 4, 8 and 18 months (data not shown). Epithelial basement membrane disruption and interstitial fibrosis were also not discernable by lamina immunostaining and Masson’s trichrome staining (data not shown).

Reproductive abnormalities of Nphp1<sup>del20/del20</sup> mice

Nphp1<sup>del20/del20</sup> mice were normal in appearance, size, growth, and development. Intercrossing Nphp1<sup>del20/del20</sup> mice never produced offspring. To determine which of them had reproductive abnormalities, we arranged several combinations of natural mating pairs. As shown in Table 1, it was the Nphp1<sup>+/del20</sup>del20 males that proved to be sterile; female Nphp1<sup>del20/del20</sup> mice did not show obvious reproduction defects. The body weights, seminal vesicle weights, and serum testosterone levels of Nphp1<sup>del20/del20</sup> males (Table 2) were comparable with those of Nphp1<sup>+/del20</sup> males or their control littermates, indicating normal development and androgen levels. But their testis weights, testicular sperm counts, and epididymal sperm counts were significantly lower than those of Nphp1<sup>+/del20</sup> mice or their control littermates (Table 2), suggesting a spermatogenesis defect. Testicular Nphp1 expression during postnatal testis development was analyzed at various ages by RT–PCR (Fig. 1E). Nphp1 transcripts were not detected until postnatal day 14, the fourth day of meiosis of the germ cells. In a previous study, the expression of Nphp1 in testes had been detected in germ cells after the first meiotic division but not in Leydig or Sertoli cells (11). Taken together, these findings hint at a direct role of Nphp1 in spermatogenesis.

Disruption of Nphp1 causes a maturation defect at the spermatid stage of spermatogenesis

Histological analyses of sectioned testes from Nphp1<sup>del20/del20</sup> mice of various ages did not reveal abnormalities in Leydig and Sertoli cells (Fig. 2A–F). Spermatogonia, spermatocytes, and round spermatids were normal in morphology and number, but the number of elongated spermatids and spermatozoa dramatically decreased in 1- or 2-month-old Nphp1<sup>del20/del20</sup> mice (Fig. 2A–D). Older Nphp1<sup>del20/del20</sup> males (8 months of age) had identical testicular phenotypes (Fig. 2E and F), suggesting that a delay in sexual maturation was not the cause of the defect. Mature spermatozoa with normal morphology were rarely observed in epididymides from adult Nphp1<sup>del20/del20</sup> males (Table 2), degenerated spermatids and severely malformed spermatozoa presented instead (Fig. 2G-J). Even the few mature spermatozoa found in the cauda epididymis were rarely viable and motile (<0.5%). These results indicate that the oligoteratozoospermia in Nphp1<sup>del20/del20</sup> mice is caused by a defect in maturation at the spermatid stage of spermatogenesis.

Spermatogenesis in Nphp1<sup>del20/del20</sup> mice is impaired at earlier stages of spermatid elongation

To further characterize the stage of the maturation defect in Nphp1<sup>del20/del20</sup> male testes, we examined periodic acid-Schiff (PAS)-stained sections from Nphp1<sup>del20/del20</sup> mice and their control littermates at 6 months of age (Fig. 3). Sixteen separate stages of mouse spermiogenesis have been defined (23). Spermatids of Nphp1<sup>del20/del20</sup> testes did not show obvious abnormalities in morphology and number as compared with their control littermates before stage X (Fig. 3A–F), but from stage XI to XII the number of elongating spermatids was significantly lower in Nphp1<sup>del20/del20</sup> than control littermates, with many spermatids degenerating and sloughing off into the lumen (Fig. 3G and H). Thereafter, the number of Nphp1<sup>del20/del20</sup> elongated spermatids was gradually reduced as stages proceeded (inner layer of the seminiferous epithelium in Fig. 3A–D). However, the surviving elongated spermatids commonly did not complete the normal maturation process: the sperm had larger, rounder and amorphous heads, and usually no or only rudimentary tails. These degenerating spermatids and immature/malformed spermatozoa that sloughed off into the lumen were subsequently passively propelled into the epididymis (Fig. 2H and J). These results delineate

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### Table 1. Reproductive phenotypes of Nphp1 mutant mice

<table>
<thead>
<tr>
<th>Male</th>
<th>Female</th>
<th>Total pups (litters)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Genotypes of pups</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+</td>
<td>+/+</td>
<td>73 (11)</td>
<td>+/+ del20</td>
</tr>
<tr>
<td>del20/ del20 (4)</td>
<td>87 (12)</td>
<td>–</td>
<td>del20/ del20 (4)</td>
</tr>
<tr>
<td>+/+ del20 (4)</td>
<td>57 (9)</td>
<td>30</td>
<td>27</td>
</tr>
<tr>
<td>+/+ del20 (4)</td>
<td>79 (11)</td>
<td>22</td>
<td>38</td>
</tr>
<tr>
<td>del20/ del20 (4)</td>
<td>63 (10)</td>
<td>–</td>
<td>36</td>
</tr>
<tr>
<td>del20/ del20 (4)</td>
<td>+/+ del20 (4)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

<sup>b</sup>N: number of mice used for intercross mating.

<sup>b</sup>Total pup number in each paring group was summated during an 3-months mating period.
the crucial role of Nphp1 in morphogenesis of elongating spermatids at earlier stages.

Disorganized structure of spermatid–Sertoli cell junctions in Nphp1del20/del20 mouse testes

To explore possible changes at the ultrastructural level in Nphp1del20/del20 mouse testes, we examined testicular sections by transmission electron microscopy (TEM). Round spermatids before stage VII were as well developed as those of control littermates; both had normal acrosomal formation and all other morphological signs of polarization (data not shown). The morphological abnormalities in Nphp1del20/del20 testes were first noticed in the elongating spermatids of stages VIII–X. Many of them were found to have prematurely detached from Sertoli

### Table 2. Body weights, organ weights, numbers of sperm, and sperm viability in Nphp1 mutant mice

<table>
<thead>
<tr>
<th>Nphp1 genotype</th>
<th>Body weight (g)</th>
<th>Seminal vesicle weight (mg)</th>
<th>Testosterone (ng/dl)</th>
<th>Testis weight (mg)</th>
<th>Cauda epididymis weight (mg)</th>
<th>Sperm head per testis (10^5)</th>
<th>Sperm per cauda epididymis (10^5)</th>
<th>% Viable sperm</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+</td>
<td>30.2 ± 2.2</td>
<td>302 ± 31</td>
<td>115 ± 43</td>
<td>94 ± 4</td>
<td>39 ± 2</td>
<td>255 ± 19</td>
<td>169 ± 17</td>
<td>32.0 ± 3.7</td>
</tr>
<tr>
<td>+/del20</td>
<td>29.1 ± 2.9</td>
<td>293 ± 30</td>
<td>93 ± 37</td>
<td>98 ± 5</td>
<td>38 ± 2</td>
<td>242 ± 14</td>
<td>160 ± 21</td>
<td>30.8 ± 4.5</td>
</tr>
<tr>
<td>del20/del20</td>
<td>29.2 ± 2.4</td>
<td>295 ± 34</td>
<td>98 ± 30</td>
<td>80 ± 8</td>
<td>37 ± 2</td>
<td>42 ± 7</td>
<td>7 ± 1</td>
<td>0.3 ± 0.5</td>
</tr>
</tbody>
</table>

*Mice (n = 8) were used at 24 weeks of age.

Serum testosterone levels were qualified using a commercially available RIA kit (Diagnostic Products, Los Angeles, CA, USA).

Sperm of cauda epididymis were incubated for 90 min, then diluted to ~1:10 and stained with SYBR-14 and propidium iodide as described in Materials and Methods. All values are means ± standard errors.
cells, resulting in impairment of further steps of differentiation (Fig. 4, right panels). The detached spermatids generally degenerated (Fig. 4H) and then sloughed off into the lumen (Fig. 3H). This premature detachment from Sertoli cells was also observed in elongated spermatids of later stages (Fig. 5B). The flagella of normal spermatozoa in control epididymides showed a continuous and well-organized membrane which surrounds the typical 9+2 structure of the axonema (Fig. 5C). In contrast, the flagellar membrane of the rare surviving spermatozoa in Nphp1del20/del20 epididymides was usually ruptured, and the electron-dense microtubules of flagellar axonema became dispersed and/or disorganized (Fig. 5D).

However, the numbers of apoptotic cells in seminiferous epithelia of Nphp1del20/del20 testes were similar to control littermates (by TUNEL analysis and TEM examination, data not shown). Even the spermatids prematurely sloughing off were TUNEL-negative, demonstrating that apoptosis was not involved in the underlying mechanism of these defects. In the control seminiferous tubules, the acrosome-facing surface of elongating and

Figure 4. Electron microscope sections of wild-type and Nphp1del20/del20 seminiferous tubules. Low magnification views of elongating spermatids at stage IX in wild-type (A) and Nphp1del20/del20 (B) testes. High magnification views of normal apical ES in wild-type stage IX (C), stage X (E), and late-elongated (G) spermatids. High magnification views of defective apical ES formation in stage IX mutant elongating spermatids (D, F). Degeneration of mutant elongating spermatid is shown in (H). A, acrosome; aEPS, apical ectoplasmic specialization; N, nucleolus; S, Sertoli cell.

Figure 5. Electron microscopic sections of wild-type and Nphp1del20/del20 seminiferous tubules and epididymides. Low magnification views of stage V–VI wild-type (A) and Nphp1del20/del20 (B) tubules. Electron micrographs of the cross-section of normal and defective sperm axonemes in wild-type (C) and Nphp1del20/del20 (D) epididymides, respectively.

Adhesive protein components on spermatid side of apical ES are normal in Nphp1del20/del20 testes

Assembly of an F-actin scaffold at the apical EPS between spermatids and Sertoli cells requires the interaction of cell
adhesion molecules on both sides. To date, five adherent proteins: the cadherins, CAR, JAM-C, laminin $\alpha_{3}\beta_{3}$,$\alpha_{3}\beta_{3}$, and nectin-3, are found on the spermatid side of the apical ES (18). Nectin-3 and laminin $\alpha_{3}\beta_{3}$,$\alpha_{3}\beta_{3}$ associate with nectin-2 and $\alpha_{6}\beta_{1}$-integrin, respectively, on the Sertoli cell side. Cadherins, CAR, and JAM-C are also present on the Sertoli cell side, and homotypically interact between the two cell types across the apical EPS. Because the $Nphp1$ expression in the testis is exclusively in germ cells, the defect of the apical EPS in $Nphp1^{del20/del20}$ testes must be on the spermatid side.

As shown in Figure 6, neither the expression level nor the localization of these proteins on the spermatid side changed in $Nphp1^{del20/del20}$ testes. Cadherins and JAM-C were not able to be demonstrated in the testis due to a lack of suitable antibodies for immunohistochemistry, but the western blot data clarify that they were not be affected by the $Nphp1$ disruption and could not contribute to the apical EPS destruction.

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Figure 6. Normal expression level and localization of adhesion molecules at apical ES, and higher filamin B content in $Nphp1^{del20/del20}$ testes. (A) Protein content of the nephrocystin-1-interacting molecules and adhesion molecules at apical ES in wild-type, $Nphp1^{+/del20}$, and $Nphp1^{del20/del20}$ testes. $\alpha$-Tubulin was used as a control for equal loading of protein. Localization of nectin-3 (B, E), CAR (C, F), and LAMC3 (D, G) in elongation spermatids of wild-type (B–D) and $Nphp1^{del20/del20}$ (E–G) testes.

F-actin disorganization contributes to the failure of apical EPS construction caused by $Nphp1$ disruption

Nephrocystin-1 has been shown to interact with p130(CAS), Pyk2, and filamins and all proteins localized to cell–cell contacts of polarized epithelial cells as described in Introduction. Although these proteins are proved to be expressed in murine testes, their roles in spermatogenesis are still unexplored (24–27). We compared their expression levels in testes of $Nphp1^{del20/del20}$ mice with those of control littermates, and found a significant increase in filamin B (Fig. 6A). Its content was 2.3-fold higher than that of control. Filamins are actin-binding proteins which are ubiquitous in eukaryotes. Mice have three filamin: filamin A and B are ubiquitously expressed, while filamin C is predominantly expressed in muscle. Filamins have a bipolar mode of action: they cross-link F-actin into orthogonal networks below a threshold filamin concentration and bundle them above that threshold in vitro (28). Since assembly of an F-actin scaffold is essential for the spermatid–Sertoli cell junction, the increased filamin B level induced by $Nphp1$ disruption could affect the architecture and mechanics of F-actin networks leading to apical ES abnormality. Indeed, targeted disruption of $Nphp1$ in mice caused abnormal F-actin distribution at the apical ES. F-actin covered the head of elongating spermatids in normal testes, but failed to

Figure 7. Abnormal F-actin distribution in elongating spermatids of $Nphp1^{del20/del20}$ testes. Confocal images of cross-sections from wild-type (A, B), and $Nphp1^{del20/del20}$ (C, D) seminiferous tubules. Nuclei are labeled with DAPI (blue). Rhodamine-phalloidin staining F-actin (red) is polarized at wild-type Sertoli–spermatid plaques (arrows) but is disorganized in $Nphp1^{del20/del20}$ (arrow heads). The orientation of the seminiferous tubules are indicated as L, lumen site and BM, basement membrane site.
explained by one of the following reasons.

NPHP1 expression of a partially defective nephrocystin-1, but this Nphp1 nuclease, leading to decreased protein expression. Thus, our without its poly(A) tail should be rapidly destroyed by exo-

are essential for correct cell–cell junctional targeting of residues and a poly(A) signal. The final 77 C-terminal residues deletion of the last exon which encodes the C-terminal 90

remain obscure. We generated a -targeted mutant mouse seems to exhibit a diminishing Nphp1del testes (Fig. 7). These data delineate one possible mechanism of nephrocystin-1 role in the organization of the apical EPS. This novel mechanism should be further investigated to clarify the functional role of nephrocystin-1 in spermatogenesis.

DISCUSSION

Gene targeting by homologous recombination in mouse embryonic stem (ES) cells is a valuable strategy to generate knockouts, knockins or conditional alleles of target genes. The targeting efficiency in ES cells is highly dependent on length and sequence of the homologous arms flanking the positive selector on a targeting vector. In this study, we found the DNA fragments flanking either exon 1 or exon 5–6 of Nphp1 are inefficient arms for homologous recombination in ES cells: the targeting frequencies were $<10^{-3}$ in both cases, and we were unable to generate null or hypomorphic allele with severe interruption in Nphp1 expression or function. However, deletion of the last exon of Nphp1 was more successful, with a much higher targeting frequency in ES cells, suggesting the DNA fragments around exon 20 are very useful for homologous recombination. As the homolo-
gous arms of all three target constructs are similar in length, and none of them have tandem repeated sequences longer than 100 nucleotides, the dramatic difference in targeting frequencies must be attributed to specific sequence differ-
ences. It is still very difficult to predict the targeting efficiency of the homologous arms based on their sequence, and investi-
gators are usually not sure if their targeting constructs will be viable. Our Nphp1-targeted mutant is therefore probably not the best but is currently the only accessible model for loss-of-function study in mice.

NPHP1 was first described as the most common defective gene responsible for NPH, though the pathogenesis and the normal functional role of the gene product nephrocystin-1 remain obscure. We generated a Nphp1-targeted mutant by deletion of the last exon which encodes the C-terminal 90 residues and a poly(A) signal. The final 77 C-terminal residues are essential for correct cell–cell junctional targeting of nephrocystin-1 (16). In addition, the truncated mRNA without its poly(A) tail should be rapidly destroyed by exonucleases, leading to decreased protein expression. Thus, our Nphp1-targeted mutant mouse seems to exhibit a diminishing expression of a partially defective nephrocystin-1, but this defect does not mimic the large homozygous deletion of NPHP1 in the majority of NPH patients (29), and does not recapitulate human NPH. This unexpected result could be explained by one of the following reasons.

The first is that defects in the NPHP1 gene alone could be insufficient to cause NPH, although this disease is by defi-
nition a monogenic disorder. Some other unknown modifier genes and/or environmental factors are perhaps also required to induce this disease: NPH is a genetically heterogeneous dis-
order. To date, five genes (NPHP1 through 5) have been impli-
cated in infantile, juvenile, or adolescent forms of NPH which differ in the onset of ESRD (30). The gene products of NPHP2, NPHP3 and NPHP4 are known to associate with nephrocystin-1, suggesting assembly into a large, multiprotein complex (17,31,32). In this regard, functional redundancy among these NPHP genes is possible. In fact, NPHP1 and NPHP4 proteins in Caenorhabditis elegans play redundant roles in facilitating ciliary sensory signal transduction (33). In another autosomal recessive disorder, Bardet–Biedl syndrome (BBS), which shares clinical features with NPH, mutations in more than one gene have been detected, suggesting the presence of ‘oligogenicity’ (34). Recently, oli-
gogenicity has also been demonstrated in NPH patients with homozygous NPHP1 deletions, and it has been suggested that a potential third mutation in another NPHP gene exerts an epistatic effect on the phenotype (35). In addition, one case report described four rare NPH patients diagnosed in adulthood, with homozygous deletion of the NPHP1 gene (36). The uncommon late occurrence of ESRD observed in these adult patients also suggests that the onset and pro-
gression of NPH may be altered by unidentified modifier genes and environmental factors.

A second possibility is cross-species variation of nephrocystin-1 function between human and mouse. Physio-

NPHP1 homozygous deletions, and it has been suggested that a potential third mutation in another NPHP gene exerts an epistatic effect on the phenotype (35). In addition, one case report described four rare NPH patients diagnosed in adulthood, with homozygous deletion of the NPHP1 gene (36). The uncommon late occurrence of ESRD observed in these adult patients also suggests that the onset and progression of NPH may be altered by unidentified modifier genes and environmental factors.

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The last one is that the renal phenotype of NPH may be non-

The NPHP1 gene is ubiquitously expressed at a relatively low level in the majority of human organs (11). In NPH, there is little correlation of this tissue expression pattern with the clinical phenotype, which exhibits pathological changes only in the kidney (9). Although the extra-renal mani-
festations of in eyes, brain, and liver may be present in NPH, the total occurrence is $<10\%$ of all affected families (30). Whether patients with extra-renal disorders carry an additional mutation in an unidentified modifier gene is an open question.
These findings indicate that NPHP1 seem to be essential only for kidney development and maintenance, but it still could play some role in other organs. In this study, we have demonstrated the crucial role of Nphp1 in mouse spermatogenesis. Whether this finding can be duplicated in humans or not is still unknown, since exceptionally high expression of Nphp1 in testis is only observed in mouse. On the other hand, NPH patients manifest with ESRD at the median age of 13 years, and ESRD-induced significant deterioration of both spermatogenesis and steroidogenesis has been recognized for a long time (38). The dysfunction of spermatogenesis induced by renal failure is not corrected by maintenance hemodialysis or renal transplantation when the patient was subject to ESRD before or during adolescence, the crucial period for spermatogenesis (39,40). In this regard, a spermatogenesis defect is expected in human NPH as a secondary effect of NPHP1 deletion, if the patients live to adulthood. However, a direct disruption of spermatogenesis in human testis by NPHP1 deletion is still a possibility, and is worth investigating further.

Nephrocystin-1 has been reported to localize to the cell–cell junctions in polarized epithelial cells (14–16). Moreover, it interacts with the adapter protein p130(CAS) and the actin-binding proteins tensin and the filamins. These findings suggest a functional role for nephrocystin-1 as a docking protein involved in regulating the actin cytoskeleton at sites of epithelial cell–cell adhesion. The spermatogenesis defects in our Nphp1-targeted mutant began to emerge during spermatic elongation, in which F-actin distribution and apical EPS construction were abnormal. The apical EPS is a specialized actin-based AJ in seminiferous epithelium. The F-actin scaffold at the apical EPS has critical roles during spermatogenesis, and requires several adhesion protein complexes identified in spermatids and Sertoli cells. These include CAR, JAM-C, cadherins, nectins, and α6β1-integrin–lamin α3β3γ3 complex that anchor onto the F-actin scaffold via corresponding adaptors (18). Disruption or depletion of these adhesion proteins could cause apical EPS destruction, leading to premature germ cell release from the testicular epithelium (20–22), similar with what we found in Nphp1del20/del20 testes. Nephrocystin-1, with supposed function at epithelial junctions, could also exert a similar action on the apical EPS, regulating the actin cytoskeleton at these sites. The abnormal actin bundling at the apical EPS in Nphp1del20/del20 testes could be evidence of this, even though both expression level and localization of most adhesion proteins at the apical EPS were normal. This argument could be resolved by locatering nephrocystin-1 at the apical EPS, but that was not possible due to absence of suitable antibodies for immunohistochemistry. Transgenic mouse expressing epitope-tagged-nephrocystin-1 is an alternative to resolve this predicament.

The subcellular localization of nephrocystin-1 is not confined to the AJ. Nephrocystin-1 also localizes to primary cilia, predominantly at the ciliary base in several types of epithelial cells (41). Primary cilia are non-motile cilia, and their association with nephronphthisis and other cystic kidney disorders has been widely reviewed (30,42). Our Nphp1-targeted mutants failed to recapitulate NPH in the mouse kidney, indicating that their renal primary cilia function normally, but they were infertile owing to malformation of sperm heads and flagella. Although primary cilia and sperm flagella have different internal structures, sperm flagella have nine peripheral doublets of microtubules with a centrally located pair (9 + 2 pattern) whereas primary cilia lack the centrally located pair (9 + 0), there are overall similarities. They both extend from a specialized centriole, the basal body. The centriolar triplet microtubular structure converts within the transition zone into the axonemal doublet microtubular structure. Nephrocystin-1 exactly localizes to the transition zone of primary cilia, where protein transfer to and from the ciliary compartment occurs (41). Whether nephrocystin-1 also localizes to the transition zone of sperm flagella is still unclear and needs further validation. Components of the intraflagellar transport machinery and ciliary cargo assemble at or near the transition zone. Thus, nephrocystin-1 might be a component of a functional protein complex at the transition zone that is crucial for organization of both primary cilia and sperm flagella. In fact, proteins that are mutated in the related disease BBS are components of the basal body transitional zone. Targeted disruption Bbs2 or Bbs4 in mice led to defects in both primary cilia and sperm flagella (43,44). However, the sperm head morphogenesis still proceeded normally in these Bbs mutants. In contrast, targeted inactivation of nectin-2 in mice caused abnormal actin bundling at apical ES, resulting to malformations of sperm heads only (21). These data suggest that nephrocystin-1 might have multiple functions during sperm morphogenesis, depending on its localization and association with distinct protein complexes.

Overall, we have established the first Nphp1-targeted mutant mouse even though it is not a null mutant and does not recapitulate the renal phenotype in human NPH. In this study, we focused on the spermatogenesis defects in this mutant to decipher the functional roles of Nphp1 during sperm morphogenesis at a molecular level. The other extra-renal manifestations in this mutant are still under investigation and hence will not be described here. To further examine the correlation between Nphp1 and NPH in mouse, we have began to generate a new Nphp1-targeted mutant in mouse ES cells through an alternative targeting strategy using intact bacterial artificial chromosomes as targeting constructs. This new Nphp1-targeted mutant should also prove useful in untangling the complicated role of nephrocystin-1 in human and murine physiology and disease.

**MATERIALS AND METHODS**

**Generation of Nphp1 mutant mice and breeding scheme**

A targeting plasmid was constructed using genomic DNA fragments derived from 129X1/SvJ mouse strain. A loxP site, a neo cassette flanked by two loxP sites, and a thymidine kinase cassette were introduced into the Nphp1 locus (Fig. 1A) by ES cell electroporation, selection, and screening that was performed using standard gene targeting techniques. Briefly, genomic DNA was isolated from neo and gancyclovir double-resistant ES cell clones, screened for a specific targeting event by PCR (PCR1 in Fig. 1A) using a forward primer in the neo sequence (5’-TCG CCA ATG ACA AGA CGC TG-3’) and a reverse primer outside the targeting vector and downstream of last exon (5’-CAC CAG GAC TGT TTC CAG GAT G-3’).
To demonstrate the presence of the loxP in intron 19, one set of primers located at 5′ upstream from the loxP site (5′-TAG AAC AAC AGT GGG TGA GGT GCG-3′) and neo (5′-TGC TCC TGC CGA GAA AGT ATCC-3′) were used. All the PCR products amplified from positive clones were confirmed by sequencing. The correct targeting of PCR positive clones was confirmed by Southern blot analysis. For this analysis, 10 μg of DNA from selected clones was digested with SacI, and DNA was electrophoresed and transferred onto nylon membranes using capillary transfer. Blots were hybridized with P32-labeled probe external to the targeting vector (Fig. 1A). Blots were also hybridized with neo-specific probes to verify the absence of additional random integration of the targeting vector. Positive clones were injected into C57BL/6J blastocysts to generate chimeras. The chimeras were bred with C57BL/6J inbred strain mice. The floxed exon 20 and neo cassette were excised by mating with the E2a-Cre transgenic mice in C57BL/6J strain. Mice positive for both the Cre transgene and the deleted allele were backcrossed with wild-type C57BL/6J mice to segregate the Cre transgene, and the excision events were identified by PCR (PCR2 in Fig. 1A) and Southern blot analysis. The Nphp1 exon 20-deleted heterozygous (Nphp1+/del120) mice were backcrossed with C57BL/6J mice for a further nine generations (N10 progeny), when homozygotes (N10F1 progeny) were crossed with C57BL/6J mice for a further nine generations (N10 progeny). Heterozygotes and homozygote status were determined by Southern blot of tail genomic DNA (Fig. 1B).

**Assessment of sperm viability**

The proportions of living and dead sperm were assessed by living-cell nucleic acid stain, SYBR-14, in combination with the conventional dead-cell nucleic acid stain, propidium iodide (46). The SYBR-14 stained the nucleus of living sperm green, while dead or membrane-damaged sperm were stained red by propidium iodide. At least 100 sperm were counted for each sample.

**Antibodies**

A rabbit antiserum to GST fusion protein corresponding to amino acids 612–691 of mouse nephrocystin was purchased from Sigma (St Louis, MO, USA). Purified goat antibodies raised against a peptide mapping within an internal region and near the C-terminus of mouse nephrocystin-1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyclonal antibodies against nectin-3, CAR, LAMC3, filamin A, and filamin B was purchased from Santa Cruz Biotechnology. Polyclonal antibody against JAM-C was purchased from Zymed Laboratories (South San Francisco, CA, USA). Mouse monoclonal antibodies against E-cadherin, N-cadherin, p130Cas and Pyk2 were purchased from BD Biosciences (Franklin Lakes, NY, USA). Phosphi-Pyk2 (Tyr402) and phospho-p130Cas (Tyr165) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA).

**Western blot analysis**

For western blot analysis, total protein was measured by Bio-Rad protein assay, for which 35 μg/lane tissue extracts were separated on SDS-polyacrylamide gels at 80 V at room temperature. Protein was transferred onto polyvinylidene difluoride (PVDF) membrane at 35 V for 16 h at 4°C in Tris-glycine transfer buffer. The membrane was blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween-20 and incubated with primary antibodies. The immunoreactive protein complexes were visualized by enhanced chemiluminescence. The blots were scanned and quantitated with software, Image Gauge, version 3.45, that was installed on a FUJIFILM LAS-1000 plus picrotography 3000 (Fuji Inc.).

**Histology and immunohistochemistry studies**

For light microscopic analysis, specimens were fixed in Bouin’s solution, embedded in paraffin, sectioned at 4 μm, and stained with either hematoxylin and eosin (H&E) or PAS stain. Tissues for electron microscopy were fixed at 4°C with 4% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, postfixed with 1% OsO4 in cacodylate buffer, dehydrated in an ethanol series, equilibrated in propylene oxide and then embedded in epoxy resin. Thin sections were stained with uranyl acetate and lead citrate and examined in a Zeiss EM902A electron microscope. For immunostaining, a standard immunoperoxidase protocol (Vectastain ABC kit, Vector Laboratories) was used. After blocking with goat serum, sections were incubated with primary antibodies for...
1 h at room temperature, rinsed in phosphate-buffered saline, incubated with biotinylated goat anti-rabbit or anti-mouse secondary antibodies, rinsed, then incubated with streptavidin-conjugated peroxidase, rinsed, then incubated with 3-amino-9-ethyl-carbazole or diaminobenzidine as a chromogen, counterstained with hematoxylin, and examined by light microscopy.

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**Conflict of Interest statement.** None declared.

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