A novel model of autosomal recessive polycystic kidney questions the role of the fibrocystin C-terminus in disease mechanism

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Autosomal recessive polycystic kidney disease (OMIM 263200) is a serious condition of the kidney and liver caused by mutations in a single gene, *PKHD1*. This gene encodes fibrocystin/polyductin (FPC, PD1), a large protein shown by *in vitro* studies to undergo Notch-like processing. Its cytoplasmic tail, reported to include a ciliary targeting sequence, a nuclear localization signal, and a polycystin-2 binding domain, is thought to traffic to the nucleus after cleavage. We now report a novel mouse line with a triple HA-epitope "knocked-in" to the C-terminus along with lox P sites flanking exon 67, which encodes most of the C-terminus (*Pkhd1*^{Flox67HA}). The triple HA-epitope has no functional effect as assayed by phenotype and allows *in vivo* tracking of Fibrocystin. We used the HA tag to identify previously predicted Fibrocystin cleavage products in tissue. In addition, we found that Polycystin-2 fails to co-precipitate with Fibrocystin in kidney samples. Immunofluorescence studies with anti-HA antibodies demonstrate that Fibrocystin is primarily present in a sub-apical location the in kidney, biliary duct, and pancreatic ducts, partially overlapping with the Golgi. In contrast to previous studies, the endogenous protein in the primary cilia was not detectable in mouse tissues. After Cre-mediated deletion, homozygous *Pkhd1*$^{\Delta 67}$ mice are completely normal. Thus, *Pkhd1*^{Flox67HA} is a valid model to track *Pkhd1*-derived products containing the C-terminus. Significantly, exon 67 containing the nuclear localization signal and the polycystin-2 binding domain is not essential for Fibrocystin function in our model.

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Autosomal recessive polycystic kidney disease (ARPKD) (OMIM 263200) is an often severe disorder that affects 1 in 20,000 live births.[1] It is a complex disease that presents with a wide range of clinical manifestations including enlarged echogenic kidneys primarily due to dilation of collecting ducts, cystic proliferation of biliary ducts, and congenital hepatic fibrosis.[1–3] Respiratory failure due to pulmonary hypoplasia is a leading cause of neonatal mortality, affecting 30% to 40%.[4] Although congenital hepatic fibrosis is an invariant finding in ARPKD, renal manifestations are highly variable, being most severe in neonatal disease but milder in patients who reach adulthood.[2,5–9]

Mutations of a single gene, *PKHD1*, are responsible for all typical forms of the disease.[10,11] The gene extends more than ~500 kb, and its longest open reading frame is encoded by a 67-exon transcript that produces a 4074aa protein, fibrocystin/polyductin (FPC). The gene is reported to undergo a complex pattern of tissue-specific splicing with several alternative exons, but the significance of this phenomenon is uncertain because mutations have thus far been exclusively found in the core 67 exons.[10,12,13] Mutations have been identified across the length of the gene, with 2 truncating mutations associated with a more severe phenotype than other mutational patterns.[2,9,13–15]

FPC is an ~500-kDa protein with a long extracellular N-terminal region, a single transmembrane span and an intracellular cytoplasmic domain (ICD).[10,11,16] It appears to be a ciliary protein, although it also has been localized to the basal body and the plasma membrane.[17–19] Although FPC's structure and its extracellular motifs suggest that it functions as a receptor, its ligand(s) remains unknown.

Previous *in vitro* studies of overexpressed recombinant, epitope-tagged human FPC showed that it undergoes Notch-like processing with multiple posttranslational proteolytic steps.[20,21] The polyductin extracellular domain (PECD) is first

cleaved by a likely proprotein convertase, and it then remains tethered to the stalk by disulfide bridges. The entire ectodomain undergoes regulated shedding, reportedly mediated by a metalloprotease, with γ-secretase–dependent release of an ICD that translocates to the nucleus where it may regulate transcriptional pathways.[20,21]

The ICD, encoded mostly by *Pkhd1* exon 67, is reported to have several functional motifs including a ciliary targeting sequence, a nuclear localization signal, and a polycystin-2 (PC2) binding domain.[21–26] PC2 is the protein encoded by the *PKD2* gene, which is linked to the less common form of human autosomal dominant polycystic kidney disease.[27,28] The interaction between FPC and PC2 is reported to regulate the latter's channel activity.[22] However, the functional relationship between PC2 and FPC has been questioned given the very different phenotypes associated with loss of either protein.[29] Previous studies, however, have reported a genetic interaction in mice between *Pkhd1* and either *Pkd1* or *Pkd2* suggesting that FPC and the polycystins may cooperatively modulate signaling pathways.[25,30,31]

Whether Notch-like processing of FPC occurs *in vivo* is still uncertain. Bakeberg *et al.*[32] have reported detection of a 450kDa product in exosome-like vesicles that decreases to ~390 kDa after deglycosylation, which they conclude is likely the shed ectodomain. However, biochemical analyses of samples from both wild-type mice and a mouse line with a double V5-tag knocked into exon 3 suggest that the uncleaved 500-kDa product is the predominant form present *in vivo*.[17,18,32] None of the studies report detection of the cleaved N-terminal fragment tethered to a C-terminal stalk *in vivo* or shorter C-terminal fragments.

Genetically modified mice are invaluable tools for elucidating the function of a gene and the protein it encodes in a physiologic setting. To better track the C-terminal ICD fragment of FPC and to determine its physiologic role *in vivo*, we targeted exon 67 and the 3′ UTR of the *Pkhd1* gene. We introduced Lox P sites into intron 66 and adjacent to the 3′ UTR. In addition, we knocked a triple hemagglutinin (HA) epitope into the C-terminus of FPC. Using this model, we find that the C-terminus of FPC can be detected *in vivo*. We also unexpectedly discovered that mice lacking exon 67, which encodes the nuclear localization signal and PC2 binding domain, are completely normal.

RESULTS

Generation of a conditional *Pkhd1*^{Flox67HA} knock-in mouse

To investigate the functional role of the C-terminal tail of FPC, we used a homologous recombination gene–targeting strategy to flox exon 67 and to introduce a triple HA epitope-tag into the mouse *Pkhd1* gene locus (*Pkhd1*^{Flox67HA}) (Figure 1a–e). To make the targeting vector, 3 HA tags were cloned in the frame at the end of *Pkhd1* exon 67, just before the stop codon (Figure 1a and b). The HA tags allow *in vivo* tracking of full-length FPC (FPC-HA) and any cleavage fragments containing the C-terminus of the protein. A neomycin cassette, flanked by 2 FLP recombinase target (FRT) sites, was added after the 3′ UTR and LoxP sites were inserted into intron 66 and adjacent to the neomycin cassette (Figure 1b and d). This design allows Cre-mediated deletion of the C-terminal 137 amino acids of FPC (Figure 1a). This segment harbors the nuclear localization signal and the PC2 binding domain and is a site for pathogenic mutations in humans (Figure 1a and c)[7,9,33] and RWTH Aachen University *PKHD1* mutation database, http://www.humgen.rwth-aachen.de/index.php).

Once we obtained germline transmission of the targeted allele, we deleted *Pkhd1* exon 67 by breeding *Pkhd1*^{Flox67HA} mice with a Meox2-Cre transgenic line (*Meox2*$^{tm1(cre)Sor}$/J) to produce *Pkhd1*$^{\Delta 67}$ offspring.[34] Cre-recombinase–mediated deletion also results in the loss of the HA tag. We devised a single polymerase chain reaction (PCR) genotyping strategy that allowed us to distinguish 3 products corresponding to the untagged, wild-type *Pkhd1* (188 bp, *Pkd1*^{wt}), HA-tagged *Pkhd1* (320 bp, *Pkhd1*^{Flox67HA}), and exon 67–deleted *Pkhd1* (268 bp, *Pkhd1*$^{\Delta 67}$) (Figure 1e).

Pkhd1^{Flox67HA} mice can be used to study native FPC

Pkhd1^{Flox67HA} homozygous mice were viable and fertile with normal behavior throughout postnatal and adult life (Supplementary Table S1). Renal, hepatic, and pancreatic histology from adult *Pkhd1*^{Flox67HA} mice was indistinguishable from that of *Pkd1*^{wt} control littermates (Figure 2a–d), suggesting that epitope tagging of FPC at its C-terminus does not have overt functional consequences.

We took advantage of the C-terminal HA tag to survey FPC expression. We prepared total lysates from murine tissues and probed Western blots with anti-HA antibody. In neonatal and adult kidney as well as in adult pancreas, we detected a band of ~500 kDa, compatible with the predicted size of full-length tagged FPC (FPC-HA) as well as a few fainter, less consistently detected high molecular weight bands ranging in size from 180 kDa to just less than 500 kDa in P0 kidney (Figure 2e–g). Anti-HA did not detect tagged FPC in negative controls lacking HA, including wild-type (*Pkhd1*^{wt}) and *Pkhd1*$^{\Delta 67}$ mice in which Cre recombinase deleted the HA tag. A new rat monoclonal antibody generated against the C-terminal region of FPC (mFPC-ct) recognized a ~500-kDa protein, expressed at comparable levels, in *Pkhd1*^{wt} and *Pkhd1*^{Flox67HA} kidneys (Figure 2e and f). The mFPC-ct antibody did not detect FPC in either *Pkhd1*$^{\Delta 67}$ or *Pkhd1*^{LSL} kidneys, indicating that this antibody is specific (Figure 2f). *Pkhd1*^{LSL} is a previously described null allele that produces no *Pkhd1* mRNA.[32]

We used immunoprecipitation with anti-HA affinity agarose beads followed by immunoblotting with anti-HA to enhance detection of FPC-HA. Using this enrichment strategy, we not only identified FPC-HA in the pancreas and kidney but also in the liver (Figure 2h and Supplementary Figure S1A and B). Low hepatic FPC levels are likely due to restricted expression of FPC in cholangiocytes, which make up a small fraction of the cell types found in the liver. FPC-HA could not be detected in adult heart or lung, indicating that FPC is either absent or present in very low abundance (Supplementary Figure S1A). In some gels, FPC