Genetic and Informatic Analyses Implicate \textit{Kif12} as a Candidate Gene within the \textit{Mpkd2} Locus That Modulates Renal Cystic Disease Severity in the \textit{Cys1\textsuperscript{cpk}} Mouse

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\textbf{Abstract}

We have previously mapped the interval on Chromosome 4 for a major polycystic kidney disease modifier (\textit{Mpkd}) of the B6(Cg)-\textit{Cys1\textsuperscript{cpk}}/J mouse model of recessive polycystic kidney disease (PKD). Informatic analyses predicted that this interval contains at least three individual renal cystic disease severity-modulating loci (\textit{Mpkd1-3}). In the current study, we provide further validation of these predicted effects using a congenic mouse line carrying the entire CAST/EiJ (CAST)-derived \textit{Mpkd1-3} interval on the C57BL/6J background. We have also generated a derivative congenic line with a refined CAST-derived \textit{Mpkd1-2} interval and demonstrated its dominantly-acting disease-modulating effects (e.g., 4.2-fold increase in total cyst area; \textit{p}<0.001). The relative strength of these effects allowed the use of recombinants from these crosses to fine map the \textit{Mpkd2} effects to a <14 Mbp interval that contains 92 RefSeq sequences. One of them corresponds to the previously described positional \textit{Mpkd2} candidate gene, \textit{Kif12}. Among the positional \textit{Mpkd2} candidates, only expression of \textit{Kif12} correlates strongly with the expression pattern of \textit{Cys1} across multiple anatomical nephron structures and developmental time points. Also, we demonstrate that \textit{Kif12} encodes a primary cilium-associated protein. Together, these data provide genetic and informatic validation of the predicted renal cystic disease-modulating effects of \textit{Mpkd1-3} loci and implicate \textit{Kif12} as the candidate locus for \textit{Mpkd2}.
Mpkd Locus Modulates Renal Cystic Disease Progression

Introduction

The polycystic kidney diseases (PKD) are a major cause of end-stage renal disease [1]. Autosomal dominant PKD (ADPKD; MIM 173900) is caused by mutations in the PKD1 or PKD2 genes [2–5] and autosomal recessive PKD (ARPKD; MIM 263200) results from mutations in the PKHD1 gene [6, 7].

While ADPKD and ARPKD are considered to be classical Mendelian traits, the disease phenotypes in both forms of PKD are complex with respect to the severity of renal cystic disease and extrarenal manifestations. Such phenotypic variability is typical even among family members that share identical PKD mutations, suggesting modulating effects of other genetic (i.e., co-inherited modifier genes), epigenetic, or environmental factors (summarized by Mrug et al [8]). Among these modulators of PKD progression, co-inherited modifier gene effects are the most tractable for experimental investigation. Indeed, previous studies have identified several quantitative trait locus (QTL) intervals that harbor genetic modifiers of PKD progression.

To date, the most significant QTL that modulates the severity of renal cystic and biliary phenotypes has been mapped to mouse Chromosome (Chr) 4 [9–12]. In previous studies, we have performed intensive analyses of this interval and discriminated three individual QTL effects on Chr 4 [8]. These effects were designated as Mpkd1, Mpkd2 and Mpkd3 (MGI:3603220–3603222). Identification of specific candidate genes underlying the effects of the Mpkd1-3 loci has been complicated by the extensive span of the Chr 4 QTL complex (~50 cM corresponding to over 100 Mbp of genomic sequence with ~1000 RefSeq genes). Therefore, we prioritized the analyses of these positional candidates based on the reported expression in early postnatal kidneys and liver, differential renal expression in kidneys with slowly vs rapidly progressive cystic kidney disease, and comparative analyses of genomic sequence in selected candidates. These analyses implicated Kif12 as a strong positional candidate gene for the Mpkd2 effects [8]. All of these studies were performed in the well-characterized B6(Cg)-Cys1cpk/J (B6-Cys1cpk) mouse model that phenotypically mimics ARPKD [8, 13–16]. The Cys1-encoded protein cystin is a primary cilium-associated protein [13].

In the current study, we use congenic strain analyses to fine-map the predicted renal cystic disease-modulating effects of the Mpkd loci and provide further supportive evidence implicating Kif12 as the candidate Mpkd2 locus based upon genetic, informatic, and immunolocalization analyses.

Results

The congenic CAST/EiJ-derived interval containing the Mpkd1-3 loci modulates renal cystic disease severity

A congenic line homozygous for the CAST/EiJ (CAST)-derived proximal-medial segment of Chr 4 on the C57BL/6J (B6) genetic background (B6.CAST.4PM) was developed previously by mating (B6 × CAST)F1 females with B6 males; the male progeny with the desired microsatellite marker profile were backcrossed to B6 females; mice at the N6 generation or later were intercrossed. Homozygous lines were selected for propagation [17].

We used a series of microsatellite markers to confirm the CAST origin of the Chr 4 interval in the B6.CAST.4PM strain, to validate the B6 origin of the other Chromosomes, and to fine-map the break point between the proximal CAST and distal B6 intervals on Chr 4, defined by D4Mit11 and D4Mit204 (57.4–61.2 cM). We then intercrossed the Cys1cpk mutation into this mouse line using a (B6.CAST.4PM × B6-Cys1cpk/) backcross and established a new line, B6.CAST.4PM-Cys1cpk/+ . We used the same backcross to generate a control Cys1cpk/+ line in which the entire length of Chr 4 was derived from the B6 strain (B6.4PM-Cys1cpk/+). Since the
CAST-derived Chr 4 interval in B6.CAST.4PM-Cys1cpk/+ line spans the three Mpkd1, Mpkd2 and Mpkd3 loci (Fig 1a), this congenic line allowed us to more precisely evaluate the CAST-derived effects that were identified in our previous studies.

In our initial studies, we characterized the renal cystic disease severity of 10-d old Cys1cpk/cpk mice generated from the B6.CAST.4PM-Cys1cpk/+ cross and compared it to the renal phenotypes of Cys1cpk/cpk mice generated from the B6.4PM-Cys1cpk/+ cross. These analyses revealed strong disease-modulating effects of CAST-derived Mpkd1-3 loci, as evidenced by four indices of cystic disease severity (Fig 1b). Specifically, kidney length (KL) increased 1.4-fold; total kidney weight (KW), increased 3.3-fold (both p < 0.001 by t-test; n = 12 for B6 and n = 7 for CAST-derived Mpkd1-2 loci); KL adjusted for body length (crown to rump; BL), the KL/BL ratio, increased 1.6-fold; and KW adjusted for body weight (BW), the KW/BW ratio, increased by 4.2-fold (both p < 0.001). These results are consistent with strong renal cystic disease-modulating effects of the composite Mpkd1-3 locus. Since our experimental design compared the impact of homozygosity for CAST-derived vs B6-derived Mpkd1-3 loci, the observed differences reflect the sum of various (e.g., dominant, recessive, and additive) disease phenotype-modifying Mpkd1-3 effects.

The CAST-derived Mpkd1-2 locus has a dominantly-acting renal cystic disease accelerating effect

Our previous studies suggested strong interactions among the individual Mpkd1-3 loci [8]. As the first step towards determining whether these loci may also act independently, we generated a new Cys1cpk/+ congenic line, B6.CAST.4PM.P1, in which the CAST-derived Chr 4 interval included only the proximal segment of Ch 4 that contains Mpkd1 and Mpkd2, but not Mpkd3 (Fig 2a). The breakpoint between the proximal CAST and distal B6 intervals in this new line is defined by D4Mit80 and D4Mit175 (37.7 cM and 45.7 cM).

To evaluate a subset of dominant renal cystic disease-promoting effects associated with CAST-derived Mpkd1-2 loci, we generated Cys1cpk/cpk mice heterozygous for the CAST-derived Mpkd1-2 loci and compared their renal cystic disease severity with Cys1cpk/cpk mice homozygous for B6-derived Mpkd1-2 loci. These two groups were generated in the same B6.CAST.4PM.P1 x B6-Cys1cpk/+ backcross. Phenotypic analysis at post-natal day 10 revealed strong disease-accelerating effects of CAST-derived Mpkd1-2 loci, as evidenced by our indices of cystic disease severity (Fig 2a). Specifically, KL increased by 1.3-fold; KW increased by 2.3-fold (both p < 0.001 by t-test; n = 14 for B6- and n = 9- for CAST-derived Mpkd1-2 loci); the KL/BL ratio increased by 1.2-fold; and KW adjusted for body weight (BW), the KW/BW ratio increased 1.9-fold (both p < 0.001). These results are consistent with a dominantly-acting, cystic disease-modulating effect of the CAST-derived Mpkd1-2 loci.

Similar to the weight- and length-derived phenotypes (i.e., KW and KL), renal cystic indices support the dominantly-acting cystic disease-modulating effects of the CAST-derived Mpkd1-2 locus. The total cyst area increased 4.2-fold, with the cyst area of the medulla increasing 3.9-fold and the cyst area of the cortex increasing 5.6-fold (all p < 0.001). However, the CAST-derived Mpkd1-2 locus had no effect on total cyst number or number of cysts in either the medulla or the cortex (Table 1). These data suggest that the Mpkd1-2 loci do not promote renal cystogenesis per se, but rather modulate renal cystic disease severity.

Fine mapping of the Mpkd2 effects

We adapted a deletion mapping approach [18, 19] to fine map the renal cystic disease-modulating effects of the Mpkd2 locus using 10-d old Cys1cpk/cpk Mpkd1-2 recombinants that were generated from the B6.CAST.4PM.P1 x B6-Cys1cpk/+ backcross (Fig 3). Using this approach,
we narrowed the Mpkd2 interval to 14 Mbp defined by D4Mit288 and D4Mit83 (Chr 4 position 56,769,379 and 70,962,376). This interval contains 175 gene entries in the NCBI Map Viewer (http://www.ncbi.nlm.nih.gov; NCBI Mus musculus Annotation Release 104), of which 116 are RefSeq sequences and 59 sequences correspond to predicted, but as yet uncharacterized genes. Among the RefSeq sequences, there are 92 protein-encoding genes, 3 microRNAs, and 21 pseudogenes.

We focused our subsequent evaluation of positional Mpkd2 candidates on the subset of the RefSeq sequences that are expressed in the kidney and liver, the two organs that predominantly express the recessive PKD phenotypes [8]. Based on NCBI Unigene data (http://www.ncbi.nlm.nih.gov), 33 of these RefSeq sequences are expressed in kidney and liver (Table 2), and of these, 19 contain a single nucleotide polymorphism (SNP) variant based on comparison of B6 vs CAST genome sequence data (Wellcome Trust Sanger Institute, Mouse Genome Project Data querying and visualization tool; http://www.sanger.ac.uk). Among these 19 sequences, 11 genes (Ikbkap, Svep1, A1314180, Fkbp15, Hdhd3, Kif12, Col27a1, Akna, Whrn, Tlr4 and Cdk5rap2) contain at least one amino acid change that in the context of disease would be predicted to be deleterious using software-based algorithms [20, 21]. In addition, among the 33 kidney and liver expressed RefSeq sequences mapped to the Mpkd2 interval, 6 genes (Ikbkap, Svep1, Fkbp15, Kif12, Col27a1 and Tlr4) contain an in frame insertion or deletion variant.

Fig 1. Congenic CAST-derived interval containing the Mpkd1-3 loci and its effects on renal cystic disease progression. a) The CAST-derived segment of Chr 4 corresponding to Mpkd1-3 interval is delimited by the distal marker, D4Mit11 (shaded area); the breakpoint between proximal CAST-derived and distal B6-derived segment of Chr 4 occurred between the markers, D4Mit11 (57.4 cM) and D4Mit204 (61.2 cM). b) The predicted cystic disease-modulating effects of the CAST-derived Mpkd1-3 loci were tested by comparing surrogates of renal cystic phenotypes (kidney length and weight) in Cys1 cpk/cpk mutants homozygous for the CAST-derived (CAST/Ei; n = 7) vs the B6-derived (B6; n = 12) segment of Chr 4. The genetic background for both groups was B6. Diamonds represent values for individual animals. The line indicates the mean value for each group.

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Among the Mpkd2-associated genes, Kif12 expression most closely correlates with the structural and developmental expression pattern of Cys1.

To evaluate the relationships between the renal expression patterns of Cys1 and the genes that map to the Mpkd2 interval, we compared the transcriptional profiles across 18 different nephron-derived anatomical structures and several development time points using the Genitourinary Molecular Anatomy Project (GUDMAP) Database [http://www.gudmap.org; [22]].

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Congenic CAST-derived interval containing the Mpkd1-2 loci and its effects on renal cystic disease progression. a) The CAST-derived segment of Chr 4 corresponding to Mpkd1-2 interval is delimited by the marker, D4Mit80 (shaded area). The breakpoint between the proximal CAST/E-derived and distal B6-derived segment of Chr 4 occurred between markers D4Mit80 (37.7 cM) and D4Mit175 (45.7 cM). b) The predicted cystic disease-modulating effects of the CAST-derived Mpkd1-2 loci was tested by comparing the renal cystic phenotypes in Cys1cpk/cpk mutants that were heterozygotes for the CAST-derived (CAST/Ei; n = 9) Mpkd1-2 interval to Cys1cpk/cpk mutants from the same cross that were homozygous B6 for the Mpkd1-2 interval (n = 14). The genetic background of both groups was B6. Diamonds represent values for individual animals. The line indicates the mean value for each group.

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### Table 1. Renal cystic indices of mice with one CAST/Ei-derived and one C57BL/6J-derived Mpkd1-2 locus (CAST/Ei.B6) vs. two C57BL/6J-derived Mpkd1-2 loci (B6.B6)

| Mpkd1-2 locus | Total Cyst Number | Total Tissue Area (TA) | Total Cyst Area (CA) | % CA/TA | Medulla Cysts Number | Medulla Area (MA) | Medulla Cyst Area (MCA) | % MCA/MA | Cortex Cysts Number | Cortex Area (CoA) | Cortex Cyst Area (CoCA) | % CoCA/CoA |
|---------------|-------------------|------------------------|---------------------|---------|----------------------|-------------------|------------------------|---------|-------------------|-----------------|------------------------|-----------|
| B6.B6 average | 554               | 13.4                   | 1.9                 | 14.2    | 371                  | 6.9               | 1.6                    | 23.6    | 184               | 6.6             | 0.3                    | 4.3        |
| B6.B6 SEM     | 14                | 0.2                    | 0.0                 | 0.2     | 9                    | 0.1               | 0.0                    | 0.3     | 6                 | 0.1             | 0.0                    | 0.1        |
| CAST/Ei.B6 average | 532 | 19.7                   | 7.9                 | 37.1    | 335                  | 11.8              | 6.4                    | 50.2    | 197               | 7.8             | 1.6                    | 17.9       |
| CAST/Ei.B6 SEM | 11                | 0.7                    | 0.5                 | 1.4     | 8                    | 0.5               | 0.4                    | 1.5     | 6                 | 0.2             | 0.1                    | 1.2        |
| *p*-value     | 0.750             | **<0.001**              | **<0.001**          | **<0.001** | 0.424              | **0.001**         | **<0.001**              | **<0.001** | 0.659             | 0.057         | **<0.001**              | **<0.001** |

All reported areas are in mm²; SEM = standard error of the mean; *p*-value = significance of differences between the B6.B6 and CAST/Ei.B6 averages.

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Among the top 100 of the ~40,000 GUDMAP transcript entries that most closely correlate with the Cys1 expression pattern (S1 Fig), Kif12 ranked fourth in rank correlation ($r = 0.819$) and was the only gene that mapped to the Mpkd2 interval. Based on a comparison that used Cys1 as the query to which all other transcripts in the GUDMAP Database were compared, the Cys1 expression pattern was also highly correlated with the expression of several key cystogenic genes (Fig 4), including Pkhd1 (correlation rank 69), the orthologue of principal human ARPKD gene, and Hnf1b (correlation rank 75), which encodes the transcription factor, hepatocyte nuclear factor-1beta (HNF1B) that directly regulates the expression of several cystogenic genes. The Mpkd2 locus modulates renal cystic disease progression via a deletion mapping approach to fine map the dominant cystic disease-modulating effects of the CAST-derived Mpkd2 locus using genetic recombinants for the Mpkd1-2 interval that were generated from the B6.CAST.4PM.P1-Cys1$^{f^{286}x}} \times B6-Cys1^{f^{286}x}$ cross. Specifically, we refined the boundaries of the Mpkd2 interval with CAST-containing Mpkd1-2 recombinants whose phenotypes (i.e., kidney length) were less than $\sim 2$ SD of the phenotype distribution in the CAST Mpkd1-2 interval homozygotes (black-filled diamonds). The Mpkd1-2 interval is designated by light shading on upper panel. The refined Mpkd2 interval (dark shaded area) is delimited by D4Mit288 (28.6 cM, 56.8 Mbp) and D4Mit83 (35.6 cM, 71.0 Mbp). It contains 92 RefSeq sequences and Kif12 maps to the center of this interval (dashed line). Consistent with the predicted cystic disease-modulating effects of the Mpkd2 locus, recombinants carrying the refined Mpkd2 interval of CAST origin had more severe phenotypes (e.g., increased kidney length by 20%; $p = 0.004$) when compared to recombinants that did not carry the CAST-derived Mpkd2 interval.

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genes including *Pkhd1* [summarized by Igarashi et al. [23]], *Cys1* [24] and *Kif12* [25]. Reciprocal analyses using *Kif12* as the query revealed that *Kif12* expression is correlated most strongly with that of the *Cys1* gene. *Kif12* expression was also highly correlated with the expression of *Pkhd1* (correlation rank 26), and *Hnf1b* (correlation rank 15).

The *Mpkd2* locus-associated gene *Kif12* encodes a primary cilium-associated protein

We have also demonstrated that the *Kif12*-encoded protein, member 12 (kinesin 12), co-localizes with the primary cilia markers somatostatin receptor 3 [26] and α-tubulin in a principal...
cell line derived from the mouse internal medullary collecting duct (mIMCD; Fig 5). This observation provides the first evidence that an Mpkd2 locus-associated gene encodes a primary cilium-associated protein. Since the protein product of Cys1 localizes to primary cilia together with most genes involved in renal cystic disease, localization of the Kif12 protein product to primary cilia provides further support for this gene as an Mpkd2 locus candidate.

Discussion

The strong renal cystic disease-modulating effects observed in Cys1pk/cpk mice carrying the CAST-derived Mpkd1-3 interval or its Mpkd1-2 derivative, provide further validation of our initial QTL mapping studies that identified a major effect QTL on Chr 4 [8]. Importantly, the current study demonstrates that the Mpkd1-3 complex exerts its effects in a fashion that is independent of previously identified non-Chr 4 QTL [8]. In addition, while our previous studies predicted interactions among genes in the Chr 4 QTL complex [8], the current study shows that an isolated Mpkd1-2 interval, without the Mpkd3 locus, is sufficient to exert a strong,
dominantly-acting effect on renal cystic disease severity. This \textit{Mpkd1-2} QTL complex also modulated the cystic area in the renal cortex and medulla, but did not influence cyst number. Therefore, we propose that the \textit{Mpkd1-2} locus most likely modulates progression of existing renal cystic disease, rather than initiating new cyst formation, which may be regulated by other factors known to impact cyst number, such as the renoprotective enzyme, heme oxygenase-1 \cite{16}.

We then went on to determine whether the \textit{Mpkd2} interval alone contains a gene (or multiple genes) with strong cystic disease-modulating effects. We developed a complementary set of strategies to assess whether the \textit{Mpkd2} effect could result from a coding sequence variant within a subset of positional gene candidates that are expressed in kidney and liver, the two organs that predominantly express the recessive PKD phenotypes. This approach identified 11 promising positional \textit{Mpkd2} candidates.

While multiple coding variant changes are present within the 11 genes that map to the this interval (Table 2), only the expression of the previously described candidate, \textit{Kif12} \cite{8}, is strongly correlated with the expression of \textit{Cys1} across the multiple anatomical nephron.
structures and developmental time points catalogued in the GUDMAP Database. While a complete systematic evaluation of each Mpkd2 positional candidate on protein level is confounded by limited immunoreagents, we have demonstrated that kinesin 12, the protein encoded by Kif12, is a primary cilia-associated protein. Together, these data implicate Kif12 as a candidate genetic modifier within the Mpkd2 interval.

Our interpretation is further supported by the recently described localization of kinesin 12 to polycystin 1 (PC1) positive urinary exosome like vesicles [ELVs; [27]] together with Cys1, Pkhd1 and Pkd2 encoded proteins. In addition, the expression of Kif12, Pkhd1 and Cys1, are directly regulated by HNF1β [25]. Since Kif12 expression also strongly correlates with the expression of HNF1 β, as well as Pkhd1 and Cys1 in the GUDMAP datasets, we propose that these four genes may define a functional complex that together with Pkd1 [28, 29] modulates the progression of recessive PKD (Fig 6).

Additional supporting evidence implicating Kif12 as the Mpkd2 modifier gene candidate is provided by recent studies that demonstrate renal cystic disease-attenuating effects of Kif3a deletion in Pkd1 and Pkd2 mouse models [30]. Similarities between the N-terminal kinesin motor domain and the C-terminal cargo-binding domain between kinesin 12 and kinesin 3a [31] as well as kinesin 17 [32] suggest that kinesin 12 may complement the function of these motor ciliary proteins. We speculate that the C57BL/6J-associated five amino acid deletion within the kinesin 12 motor domain may impair function of this protein and have cystic disease-inhibiting effects similar to the Kif3a deletion. Kinesin 12 may also complement the function of other kinesins that direct biogenesis and function of primary cilia, e.g. through regulation of microtubular dynamics [kinesin 7 [33]] or nucleation of cilia at centrioles [kinesin 24 [34]]. In addition, we note that kinesin 12 is expressed by macrophages [http://www.ncbi.nlm.nih.gov/geo; e.g., GDS3554 and GDS3555 [35], and GDS2430 [36]] and the C57BL/6J-associated deletion may alter their function. Macrophages induce proliferation of cystic epithelial cells in the Cys1cpk and Pkd1 models [37, 38] and their markers are abundantly expressed in kidneys from ARPKD patients [15, 39, 40] as well as Cys1cpk/cpk mice carrying the CAST-derived Kif12 allele [8, 15, 39].

However, it must be noted that unlike the well-studied anterograde IFT kinesins (e.g., kinesin 3a) that are conserved in most eukaryotic cells, kinesin 12 likely emerged later in evolution [41], its orthologues appear only in mammals and birds together with orthologues of Pkhd1. Cys1 so far has only been detected in mammals (based on NCBI Homologene; http://www.ncbi.nlm.nih.gov). Therefore, while the data presented in this report and supporting evidence from other recent studies are intriguing, they provide only circumstantial evidence for Kif12 as the Mpkd2 candidate gene. Further rigorous analyses, such targeted gene conversion experiments, are required to validate Kif12 as the Mpkd2 gene.

While the impact of modifier genes in modulating the ARPKD phenotype is suggested by intrafamilial variability in disease expression [42–44], this proposition also has yet to be systematically evaluated. Such studies are complicated by compound heterozygosity for the majority of low frequency PKHD1 mutations. However, a recent study in South African Afrikaners demonstrated a founder effect in which most ARPKD patients are homozygous for a single PKHD1 mutation and yet the clinical phenotypes in this cohort are variable [45]. Such populations provide powerful experimental resources for future studies to test the impact of KIF12 and other specific candidate modifier genes on disease progression in human ARPKD. In addition, KIF12 variants may modulate the severity of a broader spectrum of hepato-renal fibrocystic disorders or even trigger a ciliopathy such as KIF7 mutation-induced Joubert syndrome [33].

In summary, we have performed functional validation of the renal cystic disease-modulating effects associated with the Mpkd1-3 interval. We have demonstrated that the CAST-derived Mpkd1-2 interval promotes cystic disease progression independently of other loci and in a
dominantly-acting fashion. We have used Mpkd1-2 recombinants to fine-map the Mpkd2 locus to a 14 Mbp interval with 92 RefSeq sequences and developed convergent lines of experimental evidence that implicate Kif12 as the principal candidate for the Mpkd2 effects. This work sets the stage for direct hypothesis testing of Kif12 as a genetic modifier using targeted gene conversion experiments in cpk mice, as well as for initial directed studies of KIF12 as a genetic modifier in specific human ARPKD populations, such as the Afrikaner cohort.

Materials and Methods

Mice

The C57BL/6J-Cys1<sup>pk/+</sup> mice were obtained from the Jackson Laboratory (Bar Harbor, ME). The Cys1 mutation arose spontaneously on the B6 background. The congenic line carrying the CAST-derived proximal to medial segment of chromosome 4 (B6.CAST.4PM) on a B6 background [17]. This CAST fragment corresponds to the Mpkd1-3 interval identified by our previous QTL mapping [8]. We have used non-recombinant F2 mice generated from a (B6.CAST.4PM x B6-Cys1cpk/+)F1 intercross to establish homozygous B6.CAST.4PM-Cys1cpk/+ and B6.4PM-Cys1<sup>pk/+</sup> mouse lines. Cystic disease severity in 10-d old Cys1<sup>pk/cpk</sup> mice from the B6.CAST.4PM-Cys1cpk/+ cross was compared to those generated in the B6.4PM-Cys1<sup>pk/+</sup> cross. Scoring of phenotypes and genotyping with Cys1<sup>cpk</sup> allele-specific assay has been previously described [8, 13].

Additional crosses were established to generate the congenic line B6.CAST.4PM.P1, with the proximal Chr 4 QTL interval that contained the Mpkd1-2 loci. To evaluate the cystic disease-modulating effects of the CAST-derived Mpkd1-2 interval, we backcrossed Cys1<sup>pk/+</sup> F1 mice from the B6.CAST.4PM x B6-Cys1<sup>pk/+</sup> cross to B6-Cys1<sup>pk/+</sup> mice. The resulting Cys1<sup>pk/cpk</sup> mice were screened for recombinants containing CAST-derived segments spanning the proximal or distal portion of the Mpkd1-2 interval.

Fig 6. Proposed integration of Kif12 into recessive PKD genetic pathway. Hnf1β regulates the transcription of several cystogenic genes including Pkhd1, the mouse orthologue of the principal ARPKD gene; Cys1, the gene mutated in the cpk model of ARPKD, and Kif12, a candidate modifier of the Cys1<sup>pk/cpk</sup> phenotype. Therefore, we propose that the four proteins encoded by Hnf1b, Cys1, Kif12, and Pkd1 may define a functional complex that modulates the progression of recessive PKD (black font). This hypothesis is supported by the very high correlations among structural and developmental expression patterns of these four genes (Fig 4). In addition, components of the recessive PKD-associated pathway may interact at different levels with functional components of ADPKD-associated pathways (white font). For example, HNF1β also regulates the expression of Pkd2, an ADPKD gene. Furthermore, genetic interaction studies suggest that abnormal Pkd1 expression sensitizes renal tubular cells to Pkhd1 defects [28, 29]. The latter interaction may physically occur within the primary apical cilium (shaded oval), where these proteins all co-localize. The proposed Kif12 modulating effect on Cys1 function is designated by dashed line, solid lines correspond to previously described relationships.

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The integrity of CAST-derived Chr 4 intervals and the remaining B6 genome in these mouse lines was confirmed using a microsatellite marker-based genome scan [8]. Microsatellite makers were also used to fine-map the boundaries of CAST-B6 breakpoints on Chr 4.

All protocols were approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee. The University of Alabama at Birmingham is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International.

Histomorphometry
Paraffin-embedded kidneys were sectioned through the long axis and the hilus and stained with hematoxylin and eosin. The histomorphometry was performed without knowledge of experimental classifications using Bioquant Osteo 2013 Version 13.2.60 image analysis software. The data was collected by examining: (i) Total tissue area; (ii) Total cyst area (the area and number of cysts in the entire kidney was measured using void and outline filters in the software, the cystic space criteria were set to >4X diameters of the normal proximal tubule spaces; (iii) Medullary area (we estimated the boundary by using the glomeruli as cortical controls), (iv) Medullary cyst area (by selecting the pixels in the cystic area within the medulla boundary, we defined the medullary cysts using the same void and outline criteria as above; these analyses also provided the medulla cyst count); (v) Boundary cysts: to optimize the accuracy of cyst counting, we created an array that allowed us to subtract the cysts from the medulla if over 50% of the area was within the cortex. All other reported data were calculated as derivatives of the above data. All data were independently validated by an additional reader using a different microscope and image analysis software according to a previously described protocol [16].

Cell culture and Immunostaining
The IMCD-K2 cell line derived from SV40 transformed internal medullary collecting duct cells (mIMCD-K2; [46]) is a well-established renal principal cell line [39]. Cells were plated at confluence and grown in DMEM/F12 medium containing Earle balanced salt solution supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin (Thermo Fisher Scientific Inc; Waltham, MA), in 5% CO₂/95% air at 37°C. Immunostaining was performed after 3–4 d in culture.

The mIMCD-K2 cell line was stained using standard laboratory protocols. Briefly, the cells were quickly fixed in -20°C methanol and rehydrated in PBS. We used polyclonal anti-mouse α-tubulin antibody (Invitrogen Corporation, Carlsbad CA) and polyclonal anti-human kinesin 12 (N-18) antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibodies were obtained from Invitrogen. Immunostaining was performed after blocking tissue sections for 30 min with PBS containing 1% bovine serum albumin (Sigma). Primary antibody diluted in blocking buffer was incubated with the tissues for 12-hours at 4°C, followed by four rinses with PBS. Nuclei were stained with Hoechst No. 33528 (Sigma) diluted 1:1,000 in PBS, rinsed 3 times in PBS, then mounted in Prolong Gold antifade mounting media (Molecular Probes). A similar approach was applied to immunostaining of a mIMCD cell line stably expressing a GFP-tagged somatostatin receptor 3; Sstr3-GFP [26]. Stained samples were analyzed using a fluorescent Leica HC microscope (Leica, Allendale, NJ) and MetaMorph software (Molecular Devices, Sunnyvale, CA).
GUDMAP analyses
This study utilized data and analytical tools available from the NIDDK GUDMAP developing kidney gene expression atlas database (http://www.gudmap.org; April 2011) including the genome wide gene-expression datasets for developing kidney [22].

Statistical analyses
Statistical evaluations were performed with SPSS 11.5 statistical software package (SPSS Inc.). Renal cystic phenotypes for both kidneys were combined to give an average phenotypic score for each mouse. Significance of the differences between the two groups was determined by an unpaired, two-tailed t-test.

Supporting Information
S1 Fig. Global genome-wide correlation of gene expression patterns to the one of Cys1. Among ~40,000 transcripts profiled in 18 different renal anatomical structures at different developmental time-points that were deposited into the GUDMAP Database, the Cys1 expression was highly correlated with the Mpkd2 gene candidate, Kif12. (PNG)

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Author Contributions
Conceived and designed the experiments: MM BJA XC LMG-W. Performed the experiments: MM JZ CY. Analyzed the data: MM JZ CY BJA XC TRS GPS LMG-W. Wrote the paper: MM TRS GPS BKY LMG-W. Interpreted the results of experiments: MM BJA GPS BKY LMG-W. Prepared the figures: MM JZ CY. Drafted the manuscript: MM. Edited and revised manuscript: BJA BKY LMGW. Approved final version of manuscript: MM JZ CY BJA XC TRS GPS BKY LMG-W.

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