Autosomal dominant polycystic kidney disease (ADPKD) is a common inherited nephropathy responsible for 4%–10% of end-stage renal disease cases. Mutations in the genes encoding polycystin-1 (PC1, \textit{PKD1}) or polycystin-2 (PC2, \textit{PKD2}) cause ADPKD, and \textit{PKD1} mutations are associated with more severe renal disease. PC1 has been shown to form a complex with PC2, and the severity of \textit{PKD1}-mediated disease is associated with the level of the mature PC1 glycoform. Here, we demonstrated that PC1 and PC2 first interact in the ER before PC1 cleavage at the GPS/GAIN site and determined that PC2 acts as an essential chaperone for PC1 maturation and surface localization. The chaperone function of PC2 was dependent on the presence of the distal coiled-coil domain and was disrupted by pathogenic missense mutations. In \textit{Pkd2} \textsuperscript{−/−} mice, complete loss of PC2 prevented PC1 maturation. In \textit{Pkd2} heterozygotes, the 50% PC2 reduction resulted in a nonequimolar reduction (20%–25%) of the mature PC1 glycoform. Interbreeding between various \textit{Pkd1} and \textit{Pkd2} models revealed that animals with reduced levels of functional PC1 and PC2 in the kidney exhibited severe, rapidly progressive disease, illustrating the importance of complexing of these proteins for function. Our results indicate that PC2 regulates PC1 maturation; therefore, mature PC1 levels are a determinant of disease severity in PKD2 as well as PKD1.
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ars typical of cis-Golgi–localized proteins. Consistent with previous findings from mouse tissue (20), PNGase F digestion showed that nearly all of human PC1 was cleaved at the GPS/GAIN site, and EndoH analysis indicated that approximately 50% of the PC1 N-terminal (NT) product was resistant (NTR; mature), and so likely surface localized, while the other approximately 50% was EndoH sensitive, suggesting ER localization (NTS) (Figure 1, A and D, and Supplemental Figure 1, A and B; supplemental material available online with this article; doi:10.1172/JCI76972DS1). All detectable PC2 was EndoH sensitive, consistent with an ER localization (13). Immunoprecipitation (IP) with antibodies to the PC1 C-terminus (CT) or PC2 and detection with the PC1 NT antibody confirmed that the GPS/GAIN–cleaved products remained attached (Figure 1, B and D, Supplemental Figure 1E, and ref. 10). Redetection with the PC1 CT antibody PKS-A (51) identified both the C-terminal EndoH-sensitive (CTS) and C-terminal EndoH-resistant (CTR) GPS products (Supplemental Figure 1, C and D).

Interestingly, the normally rare full-length (FL; uncleaved) PC1, which was enriched by CT-associated IP (as well as the NT and CT glycoforms), coimmunoprecipitated with PC2, and was enriched in ADPKD may require somatic second hits. However, other data suggest a threshold/dosage model of cystogenesis in which cysts develop with some polycystin present due to stochastic and other factors, with the PKD severity related to the overall availability of functional polycystin (20, 49, 50).

Here we evaluate the processing, maturation, and localization of PC1, studying the endogenous protein wherever possible. The role that PC2 plays in this process is our focus, and we conclude that PC2 acts as a critical chaperone for PC1. Additionally, whole-animal studies reinforce the role of genetic interaction of Pkd1 and Pkd2 in the cystogenic process. These findings have important implications for understanding the pathogenesis of this disorder and suggest a high level of interplay between the 2 diseases.

Results

Endogenous PC1-PC2 complex in the ER before GPS/GAIN cleavage of PC1. We initially analyzed the glycosylation pattern of the human polycystin complex in a renal cortical tubule epithelial (RCTE) cell line using peptide-N-glycosidase F (PNGase F), to remove all sugars, and endoglycosidase H (EndoH), to excise high-mannose sugars typical of cis-Golgi-localized proteins. Consistent with previous findings from mouse tissue (20), PNGase F digestion showed that nearly all of human PC1 was cleaved at the GPS/GAIN site, and EndoH analysis indicated that approximately 50% of the PC1 N-terminal (NT) product was resistant (NTR; mature), and so likely surface localized, while the other approximately 50% was EndoH sensitive, suggesting ER localization (NTS) (Figure 1, A and D, and Supplemental Figure 1, A and B; supplemental material available online with this article; doi:10.1172/JCI76972DS1). All detectable PC2 was EndoH sensitive, consistent with an ER localization (13). Immunoprecipitation (IP) with antibodies to the PC1 C-terminus (CT) or PC2 and detection with the PC1 NT antibody confirmed that the GPS/GAIN–cleaved products remained attached (Figure 1, B and D, Supplemental Figure 1E, and ref. 10).

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cially seen under stringent (high-salt) conditions (Figure 1B and Supplemental Figure 1E). This indicates that complexing initiated in the ER before GPS/GAIN cleavage. Prolonged treatment with swainsonine, a small-molecule inhibitor of Golgi α-mannosidase II that blocks mature (post-ER) glycosylation (52), affected only PC1-NTR, not PC1-FL, PC1-NTS, or PC2 (Figure 1C and D, and Supplemental Figure 1F). Combined with the deglycosylation analyses, these data demonstrate that in the ER, PC2 complexes with PC1-FL and PC1-CTS, as well as PC1-NTS via PC1-CTS/PC1-NTS tethering, before PC1 maturation. Since PC2 was not affected by swainsonine and remained sensitive to EndoH, these data suggest that only PC1 passes through the Golgi, while PC2 is mostly retained in the ER.

Localization of PC1 glycoforms and PC2. Our results show that both ER and post-Golgi pools of PC1 coinmunoprecipitate with PC2, although PC2 retains ER-specific oligosaccharides. To determine whether mature PC1 and EndoH-sensitive PC2 were PM localized, we specifically labeled sialic acids, a marker of mature glycoproteins (53), on RCTE cell surfaces with 1 μM NaIO4 and alkylamine. This surface glycoprotein labeling and IP specifically precipitated mature PC1 (NTR) but not PC1-NTS (Figure 2A). Analysis of PC2 showed that a small fraction of this protein was also precipitated with the surface complex, despite being EndoH sensitive (Figure 2A). To further resolve the site of these endogenous proteins, we analyzed their fractionation pattern on OptiPrep gradients (2%–30%). Most detectable PC2 localized to ER enriched fractions (calnexin, STIM1), which was also the location of PC1-NTS (Figure 2B). PC1-NTR was enriched in fractions containing PM proteins (ORAI-1, SMO), which did not seem to contain PC2. PC1-NTR was also found within the primary cilias (Arl13b, SMO) enriched fraction, along with a low level of PC2. We conclude that the initial PC1-PC2 interaction occurs in the ER; however, most PC1 is trafficked alone through the Golgi to the PM. One explanation for the PC1-NTR and PC2 interaction and the surface localization data is that they complex while in different membranes, PC1-NTR in the PM and PC2 in the ER (54), similar to ORAI-1 (PM) and STIM1 (ER) at PM/ER junctions (55, 56). Indeed, a small amount of ORAI-1/STIM1 overlapped in fractions 12–14, which also contain both PC1 and PC2, albeit in relatively small amounts. Further analysis of the cilia enriched fraction showed only the PC1-NTR form of PC1 along with the EndoH-sensitive form of PC2 (Figure 2C). This is consistent with independent trafficking (bypassing the Golgi) of an EndoH-sensitive form of PC2 to cilia (19).

To explore the localization of the polycystins by immunofluorescence (IF), we exogenously expressed N-terminally tagged proteins, mCherry-PC1 and GFP-PC2, in the RCTE cell line (Figure 3A). Surface prefixation labeling of PC1 and confocal analysis with an mCherry antibody confirmed that a fraction of PC1 was PM localized (Figure 3B). Surface PC1 signal occasionally colocalized with PC2 in specific punctae but did not universally overlap, consistent with separate PM and ER localizations (55). Total PC1 signal overlapped with PC2 in the ER (Figure 3B and Supplemental Figure 2A). Localization of surface-labeled and total mCherry-PC1 with GFP-PC2 induced to develop cilia showed both proteins colocalized on cilia in about 60% of ciliated cells (Figure 3C and Supplemental Figure 2A, B and C). Together, these data indicate that PC2 facilitates ER exit and maturation of PC1.

PC2 is required for endogenous PC1 maturation. Consistent with the exogenous expression data, analysis of mouse embryonic fibroblasts (MEFs) from Pkd2−/− mice showed that PC1-NTR was completely lost and PC1-NTS increased, sometimes substantially, when PC2 was absent (Figure 4A, A and B). An increased level of PC1-FL was also seen in Pkd2−/− mice, compared with Pkd2+/− and WT mice. Next, we tested the effect of PC2 loss on PC1 secretion. Only the PC1-NTR glycoform has previously been shown secreted on ELVs in urine and from cells in culture (14, 20). Analysis of total media protein from cultured MEFs showed no PC1 secreted from Pkd2−/− cells (Supplemental Figure 4); therefore, the absence of PC1-NTR when PC2 is lost is not due to increased secretion of this product. Previous exogenous expression studies suggested that PC2 trafficking to cilia independently of PC1 (19, 35); however, when we analyzed Pkd1−/− MEFs, PC2 was not detected on cilia (Figure 4C and D). Although our data demonstrated that endogenous PC1 maturation and, hence, PM and cilia localization require PC2, confirmation of these findings using IF was not possible because of the lack of IF detection of endogenous PC1 with available PC1 antibodies. These data show that endogenous PC1 maturation and secretion require PC2, and that in MEFs PC2 cilia localization depends on PC1.

PC1 maturation is regulated by the dosage of PC2. Next we explored whether there was a corresponding reduction in the level of PC1-NTR if PC2 was reduced but not completely eliminated. Interestingly, compared with the expected 50% reduction of PC1-NTR (and PC1-NTS) observed in Pkd1−/− MEFs, Pkd2−/− kidneys and MEFs showed a modest (~25%) but significant reduction of mature PC1 (NTR) (Figure 4A and Figure 5A and C). We previously reported in the hypomorphic Pkd1R3277C/R3277C (Pkd1RC/RC) mouse model that PC1-NTR in ELVs was reduced to approximately 40% of WT (20), and quantification here in MEFs showed a consistent decrease to approximately 35% (Figure 5A–C, with
Figure 2. Subcellular localization of PC1 glycoforms. (A) Labeling of RCTE cell surface proteins using alkoxyamine biotin (Alk. Biotin) and IP with neutravidin shows that PC1-NTR was the sole PC1 glycoform localized to the cell surface. PC2 was only detected with neutravidin IP after prolonged exposure and remained sensitive to EndoH digestion; compare level of input and surface protein. EGFR was used as a PM protein control. Representative blots are shown from 3 independent experiments. IF of streptavidin-488–labeled Alk. Biotin–treated and untreated cells shows efficient surface glycoprotein labeling. Scale bar: 20 μm. (B) Density gradient fractionation of RCTE cells shows that a portion of PC1-NTR cofractionated with markers of PM (ORAI/SMO) and cilia (Arl13b/SMO), while most detectable PC2 was distributed in fractions overlapping with ER proteins, calnexin, and STIM1, with minor overlap with the cilia fraction. The relative signal intensity is plotted below. Gradient samples were loaded on 2 different SDS-PAGE gels that were run simultaneously and transferred onto the same membrane for detection. Representative blots are shown from 3 independent experiments. (C) Coimmunoprecipitation of PC2 with PC1 from gradient fraction 12 (cilia enriched) followed by deglycosylation using EndoH (+E) or PNGase F (+P) or no enzyme (Un). Only the EndoH-resistant PC1-NTR glycoform along with EndoH-sensitive PC2 cofractionated with Arl13b.
did not reach statistical significance from Pkd1RC/RC alone (Figure 5C). To better quantify the reduction, we measured the PC1-NTR/NTS ratio, thus providing an internal control. That analysis (Figure 5D) showed a significant difference in the PC1-NTR/NTS ratio when the Pkd2+/– allele was introduced. Taking these results together, we determined that a 50% reduction of PC2 (Pkd2+/–) resulted in a nonequimolar reduction of PC1-NTR by about 25% in the WT and Pkd1RC/RC setting.

a corresponding increase in PC1-NTS. Consistent with Pkd1RC being partially a GPS/GAIN cleavage mutant, more PC1-FL was also seen with this model (ref. 20 and Figure 5, A and B). Addition of the Pkd2+/– genotype to these animals resulted in an additional modest depletion of PC1-NTR (to ~30% of WT), a 22% reduction compared with Pkd1RC/RC (Figure 5, A–C). However, we found it difficult to accurately measure PC1-NTR in those MEFs because of the low abundance of this PC1 glycoform, and the 22% reduction did not reach statistical significance from Pkd1RC/RC alone (Figure 5C). To better quantify the reduction, we measured the PC1-NTR/NTS ratio, thus providing an internal control. That analysis (Figure 5D) showed a significant difference in the PC1-NTR/NTS ratio when the Pkd2+/– allele was introduced. Taking these results together, we determined that a 50% reduction of PC2 (Pkd2+/–) resulted in a nonequimolar reduction of PC1-NTR by about 25% in the WT and Pkd1RC/RC setting.
Reduced PC2 dosage worsened PC1-dependent PKD severity. Previous studies of \( \text{Pkd}^{1-/-}\ \text{Pkd}^{2+/-}\) mice showed that they developed very mild cystic disease (with single heterozygotes developing practically no cysts) (46). In this bilinear combination we estimated a PC1-NTR level of about 40% of WT (Figure 5A). Analysis of the \( \text{Pkd}^{1\text{RC/RC}}\) model showed the presence of only the mutant PC1 protein, and reduction of PC1-NTR by about 65% resulted in more robust cyst development (20). We therefore analyzed the consequences of addition of the \( \text{Pkd}^{2+/-}\) allele to the \( \text{Pkd}^{1\text{RC/RC}}\) background (PC1-NTR reduction to ~30% of WT; Figure 5C) for the cystic phenotype in 4-month-old C57BL/6 inbred animals (C57BL/6 inbred \( \text{Pkd}^{1\text{RC/RC}}\) mice have a milder phenotype than do mice in the outbred state [ref. 57]). Percentage of kidney weight/body weight (%KW/BW) and cystic index (percentage cystic area) showed a significant increase in renal disease severity in the bilinear mice (Figure 6, A–C, and Supplemental Table 1), although they were much less severe than in the \( \text{Pkd}^{1\text{RC/–}}\) model, where PC1-NTR is estimated to be reduced to about 20% of WT (20). Neither blood urea nitrogen nor serum creatinine was significantly elevated in these animals with relatively early-stage disease (Figure 6D and Supplemental Table 1). To further test whether the level of PC2 influenced disease severity, the \( \text{Pkd}^{1\text{BC/RC}}\\text{Pkd}^{2\text{WS25/+}}\) model was bred with mice with the hypermutable \( \text{Pkd}^{2\text{WS25/–}}\) allele (58), further lowering PC2 levels in the total kidney and likely removing all PC2 in some cysts in the \( \text{Pkd}^{2\text{WS25/–}}\) model. The \( \text{Pkd}^{1\text{BC/RC}}\\text{Pkd}^{2\text{WS25/+}}\) model was, as expected, intermediate between the \( \text{Pkd}^{1\text{BC/RC}}\) and \( \text{Pkd}^{1\text{BC/RC}}\\text{Pkd}^{2+/-}\) genotypes, while the \( \text{Pkd}^{1\text{BC/RC}}\\text{Pkd}^{2\text{WS25/–}}\) combination resulted in rapidly progressive disease, significantly more severe than all other genotypes (Figure 6, A–D, and Supplemental Table 1). These results suggest that below a PC1-NTR threshold, reduction in the level of PC2 by \( \text{Pkd}^{2} \) mutation results in more severe disease and the severity is correlated with the level of PC1-NTR.

Defining the C-tail region that is critical for PC1 maturation. Previous studies found that the PC1-PC2 interaction depends on the cytoplasmic CT of PC1 (31). To test whether the PC1-CT is required for GPS/GAIN cleavage (17) and maturation of PC1, we analyzed glycosylation of endogenous PC1 truncation mutants. MEFs from a lethal \( \text{Pkd}^{1}\) knock out with a frameshift at exon 31 (\( \text{Pkd}^{1\text{del31/del31}}\)), truncating PC1 in the 2nd cytoplasmic loop, revealed a normal PC1-NTS product, indicating that this truncated product is folded, inserted into the membrane, and GPS/GAIN cleaved, although a truncated, GPS/GAIN noncleaved product (tFL) is evident (Figure 7, A and B). However, no mature, PC1-NTR product was detected (Figure 7B). IP with PC2 confirmed that the truncated PC1 did not interact with PC2 (Figure 7C). Two additional truncating mutations in the extracellular part of PC1, the heterozygous null mouse model \( \text{Pkd}^{1\text{del17/+}}\) (WT/del17; Figure 7, D and E) (59) and a translocation in exon 15 in PKD1 patient 77-2 (WT/tr15; Figure 7, F and G) (60), resulted in the truncated PC1-NT (tNT) product remaining EndoH sensitive, with no mature product detected.

Given these results and previous studies (31, 32), we tested whether mutants including or excluding the PC1-PC2 interaction domain (PC1-CC) matured. PC2 was cotransfected with PC1 constructs containing nonsense mutations before (p.E421X),...
within (p.E4220X, p.Q4247X), or after (p.G4257X) the coiled-coil domain. Analysis with deglycosylation showed a clear PC1-NTR product if the deletion occurred after or at the end of the coiled-coil domain and no product if the coiled-coil domain was completely deleted (Supplemental Figure 5, A and B). These data suggest that loss of the PC1 coiled-coil domain, the site of complexing with PC2, regulates PC1 maturation.

Deletion of CC2 and missense mutations in PC2 influence PC1 maturation. To obtain more specific information about how PC2 determined the maturation of PC1, the glycosylation pattern of exogenous PC1 was analyzed when variously mutated forms of PC2 were coexpressed. As expected, loss of the entire C-tail (CT; PC2-p.L703X) completely prevented PC1 maturation, but interestingly, deletion of the proximal coiled coil and the Ca\(^{2+}\) binding domain of the EF hand (delEF+CC1), previously found to be important for PC2 oligomerization and function (34), had no effect on PC1 maturation (Figure 8, A–D, and Supplemental Figure 6). In contrast, truncation of PC2 after the ER-retention motif and before CC2 (p.S835X), or in-frame deletion of the CC2 (delCC2), disrupted PC1 maturation, consistent with the suggested role of CC2 in complexing with PC1 (34). Analysis of PC2 on a non-denaturing gel showed monomers when CC2 was deleted (but not CC1), which were not seen in the WT, supporting previous findings that CC2 but not CC1 promotes PC2 dimerization (ref. 34 and Supplemental Figure 7). These results indicate that PC2-CC2 is necessary for efficient PC1 maturation.

Although most PKD2 patients harbor truncating mutations, about 10% have missense changes (61). To determine whether
Discussion

Here we show that the maturation of PC1 resulting in the development of the critical PC1-NTR product is regulated by PC2 and, hence, that PC2 plays a central chaperone-like role in PC1 processing. The strengths of the study are that we have largely analyzed endogenous PC1 and PC2 in cells, tissue, and whole animals in the WT setting and in the context of specific mutations that remove, reduce, or alter one or both ADPKD proteins. The importance of interplay between PC1 and PC2 manifests as significantly more severe disease in bigenic models, emphasizing the importance of the interaction to understanding the cystogenic process.

missense PC2 mutations alter PC1 maturation, we generated 4 of the most common or best-characterized substitutions: p.R322Q, p.R322W, p.W414G, and p.D511V (ADPKD Mutation Database: PKDB; http://pkdb.mayo.edu). Interestingly, the data differed between mutations, with some, like p.W414G, completely eliminating PC1 maturation, while the apparent channel-dead mutant p.D511V (6) still facilitated some PC1-NTR generation; none seemed to effect PC2 oligomerization (Figure 8, C and D, and Supplemental Figure 6). IP of doubly transfected cells with PC2 (GFP) showed that co-IP with PC1 was greatly reduced if CC2 was deleted, but PC2 with missense mutations still complexed with PC1 (Supplemental Figure 7).

Figure 6. Pkd2 depletion aggravates the Pkd1RC/RC cystic phenotype. (A) Masson trichrome–stained kidney cross sections of 4-month-old mice of the Pkd1RC/RC genotype with the addition of Pkd2WS25/+, Pkd2+/−, or Pkd2WS25/− mice with the Pkd1RC/− genotype. PKD severity and fibrosis worsened in bigenic mice, with evidently more severe disease in the Pkd1RC/RC Pkd2+/− genotype, corresponding to PC1-NTR levels of about 30% (Figure 5C). However, the Pkd1RC/RC Pkd2WS25/− combination resulted in the most severe disease. Scale bar: 1 mm. (B–D) Graphical representations of %KW/BW (B), cystic index (C), and blood urea nitrogen (D) of the various genotypes quantify the increased disease severity with Pkd1/Pkd2 combined phenotypes (see Supplemental Table 1 for details). Error bars depict ± SD. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 using a 2-way ANOVA with Student’s t test. F, female; M, male. *Note that 5 of 10 Pkd1RC/RC Pkd2WS25/− animals died before 4 months (F: P42, P74; M: P38, P51, P79).
Previous analysis of the hypomorphic Pkd1: p.R3277C allele showed that the pathomechanism was related to a lower level of  PC1-NTR, due to reduced GPS/GAIN cleavage and defective protein folding (20). Here we show that PC1 truncation within the transmembrane region results in a stable mutant protein, integrated into the membrane and cleaved at the GPS/GAIN domain. Truncation before the first transmembrane region resulted in a stable, sometimes greatly overexpressed, protein, which in the case of the tr15 mutant product is overexpressed at the mRNA level (60). Overexpressed truncated protein may be due to loss of the PEST sequence in the PC1-CT (32), although not all truncated PC1 proteins are overexpressed (del31). None of the truncated proteins generate mature PC1 products, likely because of their inability to interact with PC2; therefore, we predict that PC1 truncation mutants preceding the coiled-coil domain do not mature and fail to reach the cell surface.

Our data indicate that PC1 and PC2 initially interact in the ER before GPS/GAIN cleavage and that this interaction is central to the subsequent maturation of PC1, including exiting from the ER and cell surface localization. In contrast to previous studies (17), efficient GPS/GAIN cleavage occurred when no PC2 was present, and truncated PC1 was still efficiently cleaved even without the ability to interact with PC2. We corroborate that PC2 influences cell surface localization of PC1 (17), and show an absolute requirement of that interaction for PC1 surface localization. Some, but not all, PC2 pathogenic missense mutations failed to facilitate PC1 maturation, even though the missense mutants were able to interact with PC1 and form PC2 oligomers. Hence, further analysis is required to fully understand their pathomechanism.

Our studies provide insight into the subcellular localizations of specific PC1 and PC2 glycoforms. All detectable PC2 appeared to be EndoH sensitive (even after enrichment by PC1 IP) and mostly ER resident (13). However, we describe a corresponding ER-resident form of PC1 (NTR) that complexes with PC2. This may be an intermediate form associated with the maturation of PC1, but suggest an ER-localized form of the polycystin complex. Our studies show that PC1-NTR was secreted in total media, consistent with the PC1 secreted on ELVs in urine and from cultured cells being PC1-NTR (14, 20). We also found that secretion of PC1 was PC2 dependent. We confirmed that exogenously expressed PC1 and PC2 (26, 27), and endogenous PC2, localize to cilia and showed that PC1-NTR accumulated in a cilia enriched fraction -Golgi, independent of PC1

- Figure 7. Analysis of maturation of endogenous PC1 truncation mutants. (A) Diagram showing approximate locations of the truncations in PC1 and the locations of the REJ domain, PKD repeats, and CT-CC. (B) Glycosylation analysis of MEFs isolated from WT and Pkd1<sup>-/-</sup>/WT embryos detected with the PC1 NT antibody. The del31 mutation truncated PC1 after the GPS/GAIN cleavage site, and since the truncated protein was cleaved, an NTS as well as a truncated FL (tFL) product were observed. EndoH analysis shows that PC1-NTS, but not PC1-NTR, was generated. Representative blots are shown from 3 independent experiments. (C) IP of endogenous PC2 (H280) in WT and Pkd1<sup>-/-/-</sup>embryos shows that PC2 did not communoprecipitate with the del31 mutant PC1. Lysate control is shown above. Representative blots are shown from 3 independent experiments. (D–G) Pkd1<sup>-/-/-</sup> (WT/del17) adult mouse kidney (D and E) and human fibroblasts from a female ADPKD patient with an extracellular truncation due to a translocation in exon 15 (WT/tr15; 77-2) (F and G), disrupting PC1 extracellularly in the REJ domain or PKD1 repeats, respectively. (D, F, and G) Glycosylation analysis comparing untreated (Un), EndoH-digested (+E), and PNGase F–digested (+P) protein. In all cases, only the EndoH-sensitive truncated product (PC1-tNTS) was seen, with no PC1-tNTR glycoform. In del17 and tr15, the PC1 truncated product was expressed at a much higher level than that detected WT, and so longer exposures are shown (E and G) to visualize the WT allele/products. Representative blots are shown from 3 independent experiments.
and here we show that the PC2 reduction in PKD2 results in a non-equimolar reduction of PC1-NTR, which likely modifies the PKD2 phenotype. The bigenic mouse studies show that addition of a single \( Pkd2 \) mutant allele (null or WS25) had a significant impact on the \( Pkd1RC/RC \) phenotype, with corresponding changes seen with a single \( Pkd1RC \) allele on the \( Pkd2WS25/– \) background. Once below a critical PC1-NTR threshold, 35%–40% of WT in mice (and probably ~50% in humans), small additional reductions are associated with significant phenotypic changes (20). Of interest is the significantly different disease severity between \( Pkd1+/– \ Pkd2+/– \) and \( Pkd1 RC/RC \). PC1-NTR localizes to the PM (17). An additional study published after the submission of this manuscript also localized PC1-NTR to the PM (63), and suggested a PC1-NT product not tethered to PC1-CT. Our results suggest that the PC1-PC2 complex formation is required for ER exit of both proteins and imply that PC2 plays a role in folding/quality control of PC1. This is consistent with the observation that PC1 structure is altered upon PC2 binding (64).

The major conclusion from this study is that PC2 acts as an important chaperone for PC1 maturation and localization. In PKD1, the level of PC1-NTR is critical for disease severity (20), and here we show that the PC2 reduction in PKD2 results in a non-equimolar reduction of PC1-NTR, which likely modifies the PKD2 phenotype. The bigenic mouse studies show that addition of a single \( Pkd2 \) mutant allele (null or WS25) had a significant impact on the \( Pkd1RC/RC \) phenotype, with corresponding changes seen with a single \( Pkd1RC \) allele on the \( Pkd2WS25/– \) background. Once below a critical PC1-NTR threshold, 35%–40% of WT in mice (and probably ~50% in humans), small additional reductions are associated with significant phenotypic changes (20). Of interest is the significantly different disease severity between \( Pkd1^{+/–} \ Pkd2^{+/–} \) and \( Pkd1^{RC/RC} \).
mice. This may be because in this combination no WT Pkd1 is present, although the level of PCI-NTR (5% reduced) may be most significant (46, 57). A combined effect of both loci is consistent with the observations of Pkd1 somatic mutations in Pkd2 patient cysts (65), and vice versa (66), likely acting by lowering PCI-NTR below a cystogenic threshold and so favoring cyst development.

Overall our studies emphasize that PCI-NTR is the cell surface glycoform of PCI, that maturation of PCI-NTR requires the presence of PC2 in a dose-dependent manner, and that the renal disease severity in Pkd1/Pkd2 mice. This may be because in this combination no WT Pkd1 is present, although the level of PCI-NTR (5% reduced) may be most significant (46, 57). A combined effect of both loci is consistent with the observations of Pkd1 somatic mutations in Pkd2 patient cysts (65), and vice versa (66), likely acting by lowering PCI-NTR below a cystogenic threshold and so favoring cyst development.

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pellet solubilized in IP buffer or high-salt (500 mM NaCl) IP buffer. Samples were precleared with blank A/G agarose for 2 hours. Antibodies to PCI CT (goat anti-PKD1, Everest Biotech; rabbit BD3, Genzyme) and PC2 (H280 or YCE2; Santa Cruz Biotechnology Inc.) were added overnight. Protein/antibody mixture was then incubated with 50 μl of packed washed A/G agarose (Thermo Scientific) for 2 hours. The agarose was washed 3 times in IP buffer or high-salt (500 mM NaCl) IP buffer and once in ice-cold H2O, and the protein eluted in either LDS plus TCEP or agarose was split into 3 equal parts (untreated, EndoH, and PNGase F) and subjected to deglycosylation analyses. Twenty-five micrograms of input and 100% of the IP were loaded on SDS-PAGE.

Transfection of renal epithelial cells. RCTE (68) and MDCK (ATCC) cells were split 1:2 the day before electroporation and transfected at approximately 80% confluence. Transfection was performed using the A-020 (RCTE) and A-023 (MDCK) programs of the Amaza Nucleofector I (Lonzza) following the manufacturer’s directions. Electroporation buffer (20 mM HEPES, 135 mM KCl, 2 mM MgCl2, 0.5% Ficoll 400, pH 7.6) was supplemented with 1:100 volume 200 mM ATP/500 mM glutathione immediately after electroporation. Cells were then recovered at 35°C overnight to promote folding and expression of the large, mCherry-PCI, protein, then washed and returned to 37°C. Stable mCherry-PCI and GFP-PC2 expression was achieved by growing cells for 7 days after transfection in cell culture media supplemented with G418 (400 μg/ml).

Confocal microscopy, immunofluorescence, and surface PCI labeling. RCTE cells were grown on glass cover slips, washed once with DPBS, fixed in 3.5% paraformaldehyde (PF) for 30 minutes, and permeabilized with 0.1% Triton in DPBS (pH 7.5). Cells were washed again in PBS and incubated in blocking buffer (10% normal goat serum, 1% PBS and incubated in blocking buffer (10% normal goat serum, 1% BSA, 0.1% Tween in PBS [pH 7.5]) for 30 minutes. After 3 PBS washes, primary antibodies were added in IF buffer (1% BSA, PBS [pH 7.5], 0.1% Tween) for 2 hours at room temperature or overnight at 4°C with gentle agitation. After 3 PBS washes, conjugated secondary antibodies (Alexa Fluor; Invitrogen) were added for 1 hour. DAPI was added for 1 minute to stain cell nuclei.

Surface labeling of mCherry-PC1 was performed as follows. Transfected RCTE cells were cooled at 4°C for 15 minutes and washed once in ice-cold PBS, and prechilled mCherry antibody (BioVision), diluted in 0.5% BSA in PBS, was added for 30 minutes at 4°C. Cells were then fixed in 3.5% PF and conjugated secondary antibody added for 30 minutes in IF buffer. Confocal microscopy was performed using a Zeiss Axiovert equipped with Apotome. High-quality confocal imaging was performed using a Zeiss laser-scanning (LSM780) microscope.

pH-shift/SDS immunofluorescence. The fixation/antigen retrieval method of pH-shift/SDS immunofluorescence was performed to visualize endogenous PC2 in MEFs (Figure 4C). Cells were grown to 100% confluence and serum starved for 48 hours, fixed in 3% PF (pH 7.5) for 15 minutes and 4% PF (pH 11) in 100 mM borate buffer for 15 minutes, and then permeabilized in 1% SDS for 5 minutes, to partially denature the protein. Subsequent antibody staining was performed as described above with the PC2 antibody (H280) staining performed overnight at a 1:200 dilution at 4°C. Antibody specificity was ensured by comparison between WT and Pkd2-null MEFs, in which no staining was observed.

Mouse genetics and phenotype studies. The following mouse lines were used: Pkd1 del2 (Pkd1-/-) (69) (a gift from Shigeo Horie, Tokyo University, Tokyo, Japan); Pkd1<sup>1221KfsA</sup> (Pkd1<sup>BRC/RC</sup>) (20), Pkd2<sup>243R<sub>43</sub></sup> (Pkd2<sup>-/-</sup>) (45), and Pkd2<sup>243R<sub>43</sub></sup> (Pkd2<sup>-/-</sup>) (58) (gifts from Stefan Somlo, Yale University, New Haven, CT); and Pkd1 del17 (59) (a gift from Richard Sandford, University of Cambridge, Cambridge, United Kingdom). Pkd1 del31 is a newly described Pkd1-null model where exons 31-34 are deleted and replaced with a selection cassette, mimicking the mutation in patient OX114 (P98) (60). All lines were fully inbred into the C57BL/6 strain.

For the phenotypic analysis, the animals were sacrificed at 4 months by CO2 exposure and the kidneys of the animals recorded. Subsequently, blood was collected via cardiac puncture and kidneys harvested and weighed. The left kidney was flash frozen and the right kidney fixed in 4% PF. All tissues were paraffin embedded and stained (H&E, Masson trichrome) by the Animal Histology Core (Mayo Clinic) and examined for abnormal pathologies. Cystic indices were calculated and averaged from 3 cross sections per kidney using MetaMorph software. Blood urea nitrogen was as analyzed from plasma using pHOx Ultra (Nova Biomedical), and plasma creatinine levels were measured following the BioAssay Systems protocol.

Antibodies used. The following antibodies were used: PCI NT IgG1, 7e12 (70) (WB 1/1,000); PCI CT-Rb, BD3 (a gift of Oxana Beskrovnaya, Genzyme, Framingham, Massachusetts, USA) (IP 1/250); PCI CT-Gt, EB08670 (Everest Biotech) (IP 1/250); PCI CT-Rb, PKS-A (71); PC2-Rb, H280 (Santa Cruz Biotechnology Inc.) (WB 1/5,000); PC2 IgG2a, YCE2 (Santa Cruz) (WB 1/2,000, IF 1/500); EGFR-Rb (BD Transduction Laboratories) (WB 1/1,000); acetylated α-tubulin, IgG2b (Invitrogen) (IF 1/5,000); anti-Tag(CGY)FP (Evrogen Biotech) (1/250); PC1 NT Rb, BD3 (a gift of Oxana Beskrovnaya, Novus Biologicals) (WB 1/5,000); calnexin Rb (Novus Biologicals) (1/250 IF, 1/1,000 WB); ORAI-1 Rb (H-46; Santa Cruz Biotechnology Inc.) (1/1,000 WB); AdI3b (77111-1-AP; Proteintech) (1/1,000 WB); and STIM1 IgG2a (M01; Abnova) (1/2,000 WB).

Cells. RCTE (PKD1<sup>-/-</sup>) and 9-12 (PKD1<sup>Y</sup>-/-)<sup>Y</sup>) cells (provided by Mahmoud Loghman-Adham, Baxter Healthcare Corporation) were previously described (68). WT, Pkd1<sup>-/-</sup>, Pkd1<sup>W</sup>-/-, Pkd2<sup>-/-</sup>, Pkd2<sup>Y</sup>-/-, Pkd2<sup>W</sup>-/-, Pkd2<sup>Y</sup>-/-/RC, Pkd2<sup>W</sup>-/-/RC, and Pkd2<sup>W</sup>-/-/RC, and Pkd2<sup>W</sup>-/-/RC MEFs were generated from E13.5 mouse embryos following standard techniques. Briefly, embryos were washed twice in PBS, decapitated, and eviscerated. The spinal column was minced using sterile razor blades and trypsinized. A single-cell suspension was ensured by continuous mixing before P0 cells were plated in monolayer culture. Dermal or renal primary fibroblasts were isolated from the PKD1 patient by skin biopsy or at nephrectomy and expanded by standard methods.

Statistics. All analyses were performed using JMP Pro9. For the murine studies, analyses were performed by 2-way ANOVA and least-squares means (LSMEANS) Student’s t post hoc test. A P value below the α level of 0.05 was considered significant. A 2-tailed Student’s t test was used to compare means and SDs between densitometry-derived NTR and NTS relative band intensities. For cilia quantification, a 2-tailed Fisher’s exact test was performed on data collected in contingency tables.

Study approval. The Mayo Clinic Institutional Animal Care and Utilization Committee approved the use of all murine models and experimental protocols within this study.

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Shigeo Horie for the Pkd1 del2 (null) model, Stefan Somlo for the Pkd2 del2 (null) and WS25 models, and Mahmoud Loghman-Adham for the RCTE and 9-12 cells. In addition, we thank William Wood, Jackie Sloane-Stanley, and Jackie Sharpe (Weatherall Institute of Molecular Medicine, University of Oxford, Oxford, United Kingdom) for assistance in generating the Pkd1 del31 model and Mathieu Mateo for helpful discussions and methods. The study was supported by NIDDK grant ROI-DK058816 (to P.C. Harris), the NIDDK-funded Mayo Translational PKD Center (DK090728), Mayo Graduate School (to V.G. Gainullin), and an American Society of Nephrology, Ben J. Lipps Research Fellowship (to K. Hopp).

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