Altered trafficking and stability of polycystins underlie polycystic kidney disease

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The most severe form of autosomal dominant polycystic kidney disease occurs in patients with mutations in the gene (PKD1) encoding polycystin-1 (PC1). PC1 is a complex polytopic membrane protein expressed in cilia that undergoes autoproteolytic cleavage at a G protein–coupled receptor proteolytic site (GPS). A quarter of PKD1 mutations are missense variants, though it is not clear how these mutations promote disease. Here, we established a cell-based system to evaluate these mutations and determined that GPS cleavage is required for PC1 trafficking to cilia. A common feature among a subset of pathogenic missense mutations is a resulting failure of PC1 to traffic to cilia regardless of GPS cleavage. The application of our system also identified a missense mutation in the gene encoding polycystin-2 (PC2) that prevented this protein from properly trafficking to cilia. Using a Pkd1-BAC recombineering approach, we developed murine models to study the effects of these mutations and confirmed that only the cleaved form of PC1 exits the ER and can rescue the embryonically lethal Pkd1-null mutation. Additionally, steady-state expression levels of the intramembranous COOH-terminal fragment of cleaved PC1 required an intact interaction with PC2. The results of this study demonstrate that PC1 trafficking and expression require GPS cleavage and PC2 interaction, respectively, and provide a framework for functional assays to categorize the effects of missense mutations in polycystins.

Introduction

The most common and severe form of autosomal dominant polycystic kidney disease (ADPKD) results from mutations in PKD1. PKD1 encodes human polycystin-1 (PC1), a large, complex, low-abundance polytopic membrane protein with structural features suggestive of a cell-surface receptor (1). PC1 is the common rate-limiting component of the ADPKD signaling pathway, and functional dosage of the gene also modulates the phenotypes in isolated autosomal dominant polycystic liver disease and autosomal recessive polycystic kidney disease (2). Mutations in PKD2, encoding human polycystin-2 (PC2), cause ADPKD with an identical, albeit milder, phenotypic spectrum compared with that of PKD1. PC2 is a member of the transient receptor potential (TRP) family of cation channels. Biochemical evidence shows that PC1 and PC2 interact directly (3, 4), and genetic and phenotypic evidence suggests that they act in a common pathway (2, 5–7). The prevailing hypothesis is that PC1 and PC2 form a receptor-channel complex with either mechanical or ligand stimuli as the upstream signals (8–11), although direct evidence for this functional interrelationship is lacking. Coassembly with PC1 has been proposed as a requirement for trafficking of PC2 to the cell surface (12), although PC2 can traffic to cilia independently of PC1 (13). There is also evidence for divergent functions for PC1 and PC2: e.g., PC2 is involved in left-right axis determination (14–16) and Ca\(^{2+}\) homeostasis in the ER (17, 18), whereas PC1 can be expressed in association with the lateral membrane (19–21), adherens junctions (22), and desmosomes (23).

Several lines of evidence have led to the emergence of the primary cilium as the central cellular compartment in the pathogenesis of cystic kidney diseases (24). PC1 and PC2 are expressed in the membrane overlying the primary cilium (25, 26). The majority of gene products associated with fibrocystic kidney diseases other than ADPKD are also expressed in the cilia-basal body complex (24, 27). A forward genetic screen in zebrafish based on the cystic pronephros phenotype identified a number of cilia-related proteins as well as PC2 (28). Fibrocystic disease–associated gene products, including PC2, are associated with other known ciliary phenotypes such as left-right axis defects (14–16). In addition, the role of cilia in cyst formation was tested prospectively by showing that disruption of cilia through selective inactivation of Kif3a or intraflagellar transport proteins in the kidney results in cyst formation (29–31). Recently, we have found that cyst formation following inactivation of PC1 or PC2 is markedly slowed by concomitant removal of structurally intact cilia, suggesting that intact cilia devoid of polycystins are the primary driving force in the progression of ADPKD (32). Despite these cogent arguments, direct evidence for the precise mechanism of PC1 and PC2 action in cilia has been elusive.

Human PC1 is composed of 4,302 amino acids with approximately 3,000 amino acid extracellular NH₃ termini, 11 transmembrane domains (33), and approximately 220 amino acid cytosolic COOH termini. The extracellular NH₃-terminal domain contains a distinct combination of protein motifs including immunoglobulin-
like PKD repeats (34–36), a receptor egg jelly (REJ) domain (37) that includes fibronectin type III repeats (38) and is part of a recently identified structural GAIN domain (39). The REJ/GAIN domain is required for autoproteolytic cleavage of PC1 at a G protein–coupled receptor proteolytic site (GPS) sequence, HL\_L\_T\_409 (40), that yields an extracellular NH\_2-terminal fragment (PC1-NTF) and an intramembranous COOH-terminal fragment (PC1-CTF) (39, 41, 42). PC1-NTF and PC1-CTF remain noncovalently associated with each other. The conservation of the GPS in all PC1 homologs (43) suggests functional importance; although at least 2 homologs, PKDREJ and SuREJ2, do not undergo GPS cleavage (44, 45).

Cysts in ADPKD are thought to occur primarily by a cellular recessive 2-hit mechanism, with the majority of clinically significant heterozygous germline mutations in PKD1 and PKD2 resulting in loss of function. Nonetheless, approximately 25% of the presumed pathogenic variants in PC1 are predicted to result in non synonymous amino acid substitution mutations (46). Increasing evidence shows that a significant proportion of these non synonymous substitution variants are functionally hypomorphic and have a milder clinical presentation compared with that of complete loss-of-function variants (47, 48). The pathogenic effects of such variants have proven difficult to assess experimentally in the absence of robust functional assays for PC1. In the current study, we examined the functional expression and cilia-trafficking properties of mutant forms of murine PC1 and human PC2 in vitro and of PC1 in vivo. We found that a subset of pathogenic amino acid substitution mutations in PC1 and PC2, as well as mutations affecting GPS cleavage in PC1, result in failure of polycystins to traffic to cilia. To extend these cell-based studies to mammalian tissues in vivo, we modified a Pkd1 BAC transgene that can fully rescue the embryonically lethal Pkd1\_cDNA phenotype with a mutation to abrogate GPS cleavage and showed that this mutation results in a complete loss-of-function allele that can no longer rescue any part of the Pkd1\_cDNA phenotype. We used the BAC transgenic models to establish that there are significant differences in the subcellular compartment distributions of PC1 and murine PC2 in tissues, supporting the hypothesis that these proteins may also have functions independent of each other. Finally, we examined PC1 expression in the absence of PC2 and found that interaction with PC2 is required to maintain steady-state expression levels of PC1-CTF. These studies highlight the role of defective polycystin trafficking and expression in all forms of ADPKD and suggest a role for therapies directed at these processes in a selected subset of ADPKD patients.

Results

Asparagine-linked glycosylation and apical expression of PC1. Full-length mouse PC1 (PC1-FL) with NH\_2- and COOH-terminal epitope tags (49) cleaved at the GPS site to yield the HA epitope-tagged PC1-CTF and the FLAG epitope-tagged PC1-NTF, which remained noncovalently associated with each other (ref. 41 and Figure 1A). We examined the asparagine-linked glycosylation (N-glycosylation) pattern of PC1. PC1-CTF showed both endoglycosidase H-resistant (endo H-resistant) and -sensitive forms, whereas the full-length, uncleaved PC1 protein PC1-FL was entirely endo H sensitive (Figure 1, B and C, and ref. 50). Endo H resistance defines the fraction of PC1-CTF that traffics past the middle Golgi apparatus to the cell surface. Digestion with peptide-N-glycosidase F (PNGase F) removes all N-glycan moieties. An absence of endo H resistance in PC1-FL shows that uncleaved PC1 is not present on the cell surface. Anti-FLAG immunoprecipitation (IP) of PC1-NTF only communoprecipitated the intracellular endo H-sensitive fraction of PC1-CTF (Figure 1C). The failure to detect the cell-surface pool of PC1-NTF was likely due to shedding of the surface-expressed PC1-NTF into the culture medium (ref. 41 and Figure 1D). We further examined the surface location of PC1 biochemically by selective biotinylation of the apical and basolateral compartments in nonpermeabilized, polarized LLC-PK1 cells grown on filter inserts (51). We found that PC1-CTF was strongly biotinylated on the apical surface of cells, but was virtually absent from the basolateral surface (Figure 1E). uncleaved PC1-FL was not detected by cell-surface biotinylatión in live cells (Figure 1E). We next determined whether the absence of PC1-FL from the cell surface was indicative of very efficient cleavage of PC1 during trafficking or an intrinsic property of uncleaved PC1. The cleavage-deficient mutant PC1\_L\_409H/H\_410 bearing an amino acid substitution at the consensus G\_409\_L\_T\_410 of the mouse sequence remained completely endo H sensitive, indicating that the uncleaved protein cannot reach the cell surface (Figure 1F). The fact that PC1-CTF acquired endo H resistance suggests that its path to the cell surface passes through the Golgi apparatus. The biotinylation pattern in polarized epithelial cells shows that in the steady state, PC1-CTF is preferentially expressed on the apical surface in at least some cell types.

Truncation mutations and cilia trafficking of PC1. The site of expression of PC1 on the apical surface most relevant to the pathogenesis of ADPKD is the primary cilium. We investigated the structural elements of PC1 that are critical to cilial trafficking. We hypothesized that defective trafficking of PC1 to cilia would serve as an in vitro functional test for a subset of putative pathogenic variants in Pkd1. For the purposes of this study, all human PC1 sequence variants were introduced into the equivalent codon in mouse Pkd1 cDNA to achieve the same amino acid substitution. The variants are described using mouse sequence nomenclature and mouse-to-human amino acid equivalences, and the pathogenic potential for all variants are presented in Supplemental Table 1 (supplemental material available online with this article; doi:10.1172/JCI67273DS1). PC1 was detected in cilia by immunofluorescence (IF) cell staining using COOH- or NH\_2-terminal epitopes independently in a variety of cell lines including previously reported mouse embryonic fibroblast (MEFs) (2), LLC-PK1, IMCD3 and MDCK cells (data not shown). The HA and FLAG epitopes colocalized in cilia, indicating that both PC1-CTF and PC1-NTF appeared in cilia together (Figure 2A). We observed PC1 expression along the entire axoneme of the cilia, and PC1 did not colocalize with basal bodies marked by γ\_tubulin (Figure 2, A and B). The cleavage-deficient PC1\_L\_409H/H\_410 was not detectable in cilia axonemes or at the basal bodies despite robust expression in the cell body, showing that GPS cleavage-deficient PC1 carrying this specific GPS motif variant did not traffic to cilia (Figure 2C). These findings imply that the HA and FLAG epitopes are detecting cleaved PC1-CTF and PC1-NTF, respectively, as the only forms of PC1 in cilia.

Between 65% and 75% of pathogenic mutations in PKD1 are either nonsense, splice site, or insertion/deletion changes that are predicted to result in premature termination of the PC1 pep-
Figure 1. N-glycosylation and surface expression of PC1. (A) LLC-PK1, cell lysates expressing PC1 with NH2-terminal triple-FLAG and COOH-terminal triple-HA epitope tags underwent IP with either nonimmune IgG (IgG) or anti-HA (HA) and IB with anti-HA (left) and anti-FLAG (right). The 2 panels are from a single gel and show PC1-NTF (NTF), PC1-CTF (CTF), and uncleaved full-length PC1 (FL). (B) PC1 IP followed by digestion with endo H, PNGase F (PNG F), or reaction buffer (Control). Panels at right are magnifications of the left panel (original magnification, ×2). PC1-CTF shows endo H–resistant (+) and –sensitive (−) products. PC1-FL shows complete endo H sensitivity. (C) IP of PC1 by anti-FLAG coimmunoprecipitated the endo H–sensitive fraction of PC1-CTF, indicative of the intracellular PC1-NTF/PC1-CTF pool. (D) Shedding of PC1-NTF into the culture medium may explain the absence of a detectable cell-surface PC1-NTF/PC1-CTF complex. Medium was either run directly (No IP) or subjected to IP prior to IB. Lysate, total cell lysate. (E) LLC-PK1, cells grown on semipermeable supports selectively labeled with biotin on the apical or basal surface. Total lysates (left panel) and streptavidin-precipitated biotinylated proteins (right panel) were run on a single gel and analyzed by IB with anti-HA (upper 2 panels) and anti–Na-K-ATPase (lower panel). PC1-CTF was biotinylated on the apical surface. Na-K-ATPase served as a positive control for the basal surface. (F) GPS cleavage–deficient PC1L3040H was entirely endo H sensitive, suggesting that it did not traffic past the middle Golgi apparatus in cells; the 2 panels show noncontiguous lanes on a single gel.
ated a series of human pathogenic missense variants in an effort to categorize their functional consequences by in vitro assays of cilia trafficking. We first determined whether naturally occurring mutations that impair GPS cleavage also abrogate cilia trafficking of PC1. A previous study had shown that 2 human mutations remote from the actual G protein–coupled proteolytic cleavage sites, PC1E2771K and PC1F2853S, respectively corresponding to mouse codons PC1E2766 and PC1F2848, abrogate GPS cleavage (41). These mutations, originally referred to as REJ mutations, occur in the GAIN domain recently defined by structural studies as being required for autoproteolytic cleavage at the GPS (39). For the current study, we selected another human mutation, PC1L2816P, corresponding to mouse PC1L2811P, which is located in the GAIN domain between PC1E2771K and PC1F2853S and is considered likely to be pathogenic (Supplemental Table 1). PC1L2811P did not cleave at the GPS (Figure 3A) and did not localize to cilia (Figure 3B). As a positive control for the assay technique, the variant PC1C2085S, that changes a nonconserved residue in the fifteenth PKD domain in the mouse sequence (PC1C2085) to the equivalent amino acid from the human sequence (PC1R2089), was cleaved normally at the GPS (Figure 3A) and trafficked normally to cilia (Figure 3C). The finding that 2 independent mutations, PC1L3040H and PC1L3040N, which result in loss of GPS cleavage, also failed to traffic to cilia is consistent with the hypothesis that GPS cleavage is required for trafficking of PC1 to cilia.

We next investigated whether cilia-trafficking defects were a feature of amino acid substitution mutations in the extracellular domain of PC1, irrespective of their effects on GPS cleavage. We selected a pair of human mutations, PC1L2100C and PC1V690D, outside the GAIN domain region that were predicted to be pathogenic (Supplemental Table 1). PC1C2085G alters a highly conserved cysteine in the WSC domain. PC1V690D is in the interdomain region between the C-lectin and PKD domains. We produced the corresponding mouse variants, PC1C2085G and PC1V690D. PC1C2085G and PC1V690D cleaved normally at the GPS (Figure 3A). Despite normal GPS cleavage and robust expression in the cell body, PC1C2085G and PC1V690D failed to traffic to cilia (Figure 3, D and E, and Supplemental Figure I). These data show that pathogenic amino acid substitution mutations in PC1 can abrogate cilia trafficking independently of GPS cleavage and support the hypothesis that cilia trafficking is an effective in vitro surrogate assay for a subset of pathogenic PC1 mutations.

We next determined whether amino acid substitution mutations affect other functions of PC1 besides GPS cleavage and trafficking to cilia. PKD domains were first identified in the extracellular region of PC1 (34–36) and were proposed to have possible ligand-binding functions (54). The PKD domains have a β-sandwich fold, also called an Ig-like fold, among which a WDFGDG motif is the most conserved sequence (54). We examined a putative “likely” pathogenic human mutation, PC1G1166S (Supplemental Table 1), that affected the WDFGDG1166S motif in the fifth predicted PKD domain (54). The mouse-equivalent PC1G1166S variant is a stable protein product that was cleaved at the GPS (Figure 3A) and was expressed abundantly in cilia (Figure 3F). If this variant is indeed pathogenic, as suggested by sequence conservation and structural data (54), these findings suggest that functions other than GPS cleavage and cilia trafficking, perhaps involving ligand interactions, may also be disrupted in pathogenic amino acid substitution mutations in PC1.

Missense mutations and cilia trafficking of PC2. Although missense variants are less common in PKD2 (~6%–14%), we took this opportunity to evaluate whether cilia-trafficking mutations also play a role in ADPKD resulting from mutations in PKD2. We evaluated PC2W414G, a mutation in the highly conserved polycystin domain (55) common to both PC1 and PC2 and located in the large extracellular loop between the first and second transmembrane segments of PC2 and the sixth and seventh transmembrane segments of PC1 (43). Single-channel recordings using bilayers made with ER-enriched vesicles from LLC-PK1 cells stably expressing PC2W414G showed that the mutation did not abrogate channel activity of PC2 in the ER (Figure 4, A and B). The calcium concentration on the cytoplasmic side needed to maintain the open probability near approximately 50% was the same for both the WT and PC2W414G channel, suggesting that there were no obvious differences in the open probability between the WT and mutant channels. At higher holding potentials, the currents through PC2W414G showed a lower conductance than did the currents through the WT channel, but at the lower holding potentials, which were more representative of the ER membrane, the channel conductances were similar for the WT and PC2W414G mutant. In aggregate, it is unlikely that the differences in channel activity account for the pathogenicity of PC2W414G.

PC2W414G did not traffic to cilia despite robust expression in cells, suggesting this to be the more likely mechanism for its pathogenesis (Figure 4C). As a positive control, a channel-dead pathogenic missense variant, PC2D1517F (17, 56), trafficked to cilia in the same cell type (Figure 4D). We further confirmed the specificity of the cilia-trafficking defect in PC2W414G by introducing the mutation into the truncated PC2C2200X backbone that normally shows particularly robust trafficking to cilia (Figure 4E and ref. 13). The truncated protein containing the point mutation PC2W414G2200X did not traffic to cilia (Figure 4F). The data suggest that cilia trafficking mutants occur in PC2, that the conserved extracellular domain unique to polycystins is important in trafficking of PC2,
that cilia location is critical to PC2 function in ADPKD, and that channel activity in the ER without cilia location is not sufficient to avoid polycystic disease.

**Bacterial artificial chromosome transgenic models of PC1 expression.** PC1 is a complex, low-abundance protein that has been difficult to evaluate by in vivo studies, yet such studies are essential for validating and extending findings based on cell line and other in vitro approaches. We therefore established and validated a model system based on modification (“recombineering”) of bacterial artificial chromosomes (BACs) by homologous recombination in *E. coli*, followed by the generation of transgenic mouse lines expressing these modified BACs to evaluate the properties of PC1 in vivo (2, 57–59). This approach allows for targeting of a single allele more than once in a manner that is not available in knockin models, while still providing high fidelity to native gene expression patterns in a copy number–dependent, integration site–independent manner. We initially modified the mouse BAC clone RPCI22-287A3 containing *Pkd1* by inserting a triple-FLAG epitope after the NH2-terminal leader sequence and a triple-HA epitope before the stop codon in the same locations as in the *Pkd1* cDNA (2, 49).

BAC clone RPCI22-287A3 contains 7 other genes in their entirety as well as parts of 2 additional genes (Supplemental Table 2). Seven transgenic founder lines produced with this modified *Pkd1F/H-BAC* transgene gave germline transmission; 4 of these lines were analyzed further (Figure 5 and Supplemental Tables 3 and 4).

BACs typically yield low copy number transgenic insertions, and our founders had 1–8 copies of the BAC transgene as determined by quantitative PCR (qPCR) (Supplemental Table 3). We found that PC1 protein expression determined by semiquantitative immunoblotting (IB) was approximately 2- to 4-fold higher relative to PC1 expressed in the single-copy founder Tg14 (Figure 5, A and B, and Supplemental Table 3). PC1 expression correlated with the transgene copy number in 3 of the 4 founders. It is likely that the 8 “copies” of the BAC detected by qPCR in Tg8 included some BAC inserts that were disrupted in the integration process, so that only a subset of the BAC inserts actually expressed PC1. The distinct nature of the respective modifications in the *Pkd1H/BAC* transgene and the native *Pkd1*-null allele enabled us to distinguish null, WT, and BAC alleles for purposes of genotyping (Supplemental Figure 2). All 4 founder lines were able to rescue the embryonic lethality when combined with the *Pkd1–/–* background (Supplemental Table 4 and ref. 2), indicating that the BAC transgene was effective in producing a functional protein independently of the integration site and copy number and that the epitope tags did not impact in vivo function of PC1. High-copy number *Pkd1* transgenes have been associated with kidney cyst formation (60, 61). We did not observe cyst formation in our low-copy number BAC transgenic models, nor were any other abnormal pheno...
The Pkd1F/H-BAC transgene expressed readily detectable epitope–tagged PC1 in kidney tissue (Figure 5, A and B, and ref. 2). We observed that the PC1-CTF appeared as 2 bands of differing migration (Figure 5C), consistent with the previously reported alternative splicing of exon 31 in the rodent, but not human, transcripts (62). The presence of the dual-epitope tags allowed simultaneous detection of PC1-CTF and PC1-NTF in tissue (Figure 5, A and B). All 4 transgenic lines showed that PC1 exists primarily as a cleaved protein in vivo (Figure 5, A, B, and D). We found that PC1 from the Pkd1F/H-BAC transgene was expressed most strongly in membrane–enriched fractions from lung tissue, while kidney and heart showed moderate expression (Figure 5D). PC1 expression in other tissues was difficult to detect by IB of membrane–enriched fractions (Figure 5D, left panel); expression in these tissues was detectable by IP followed by IB and longer exposures (Figure 5D, right panel). It is also notable that PC1 expression has been reported to be downregulated soon after birth (63, 64). Consistent with this, we found that detection of PC1 expression was markedly reduced in mice after 2 weeks of age (data not shown). The patterns of expression we observed were reproduced among several transgenic lines (data not shown), showing that BAC transgenic expression of PC1 is indeed faithful to the native protein pattern of expression.

The BAC transgenic lines showed that the overall abundance of PC1 in all tissues was very low. Even using anti-epitope antibodies required either enrichment of membrane fractions in tissue lysates (Figure 5, A–D) or IP followed by IB (Figure 5D) to reliably detect the protein in native tissues. As a consequence of this low abundance, we were unable to reliably detect PC1 in native tissue immunohistochemistry. We therefore prepared MEFs from Pkd1F/H-BAC transgenic and nontransgenic mice and confirmed expression and GPS cleavage of PC1 in the MEFs (Figure 5E). We were able to detect the epitope–tagged PC1 in cilia of MEFs, confirming expression of the transgene–encoded protein in cilia (Figure 5F and ref. 2). The data support the conclusion that modified BAC transgenic lines are an effective model for studying PC1 in vivo.

Effects of PC1 mutations in native tissues. The dual-epitope–tagged Pkd1F/H-BAC transgene can serve as a platform for in vivo structure function studies of mutations in Pkd1. To establish this model, we introduced the PC1<sup>L3040H</sup> GPS mutation into the
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Figure 5. PC1 expression in Pkd1\textsuperscript{fl/fl}-BAC transgenic mouse lines. (A and B) Membrane-enriched protein fractions prepared from kidneys of 2-week-old mice descended from 4 independent transgenic founders (Tg248, Tg276, Tg8, and TgH; transgene copy number is in parentheses) analyzed by IB with anti-HA (A) and anti-FLAG (B). WT, nontransgenic littermate; PC1, cell lysate overexpressing PC1. Tubulin served as a loading control. Panels represent noncontiguous lanes on a single gel. (C) Evidence of alternative splicing of PC1 expressed from the mouse Pkd1\textsuperscript{fl/fl}-BAC transgene. Kidney tissue lysates from Tg248 underwent IP with anti-HA and were treated with PNGase F or buffer without enzyme (C) prior to IB with anti-HA. Arrows indicate CTF forms with differing migration after PNGase F. (D) Membrane-enriched protein fractions from the indicated organs of a 2-week-old Tg248 mouse underwent IB using anti-HA to show the relative tissue expression of transgenically expressed PC1 (left panel). IP followed by IB of PCK1 using anti-HA (right panel) shows detectable levels of PC1 in organs in which IB alone was not sufficiently sensitive. Panels represent noncontiguous lanes on a single gel. (E) Pkd1\textsuperscript{fl/fl}-BAC kidney tissue (Pkd1\textsuperscript{fl/fl} kidney), MEFs (Pkd1\textsuperscript{fl/fl} MEFs), and nontransgenic (WT MEFs) cell lysates subjected to IB with anti-HA show expression and GPS cleavage of PC1 in MEFs. (F) PC1 from the Pkd1\textsuperscript{fl/fl}-BAC was expressed in cilia of MEFs from Tg248 mice. Green, anti-HA; red, anti–acetylated α-tubulin. The rightmost panels in F represent a merge of the images at left. Scale bars: 5 μm.

genomic Pkd1 sequence in Pkd1\textsuperscript{fl/fl}-BAC and generated transgenic mice with the modified Pkd1\textsuperscript{L3040H}-BAC transgene. We obtained 3 transgenic founders with germline transmission: 2 lines (Tg46\textsuperscript{L3040H} and Tg9\textsuperscript{L3040H}) had 1 copy of the BAC transgene by genomic qPCR, whereas the third line (Tg7\textsuperscript{L3040H}) had 3 copies (Supplemental Table 5). All 3 transgenic lines showed normal histology at 6 months on a WT background, with no evidence of microscopic cysts in the kidney, liver, or pancreas (data not shown). PC1 expressed from Pkd1\textsuperscript{L3040H}-BAC appeared as a single band migrating as PC1-FL without evidence of cleavage at the GPS (Figure 6A). The tissue pattern of expression was similar to that of WT Pkd1\textsuperscript{fl/fl}-BAC (Figure 6B). PC1\textsuperscript{L3040H} retained the capacity to interact with PC2, as evidenced by cell-based cotransfection, co-IP studies (Supplemental Figure 4). None of the Pkd1\textsuperscript{L3040H}-BAC transgenic lines were able to rescue the embryonic lethality of the Pkd1\textsuperscript{-/-} mice (Figure 6C and Supplemental Table 5), indicating a complete loss-of-function allele based on a single codon change in the entire BAC insert sequence that included Pkd1 and the other “passenger” genes. We evaluated the ability of Pkd1\textsuperscript{L3040H}-BAC transgenic lines to specifically rescue the cystic phenotype following kidney-selective conditional inactivation of Pkd1. Pkd1\textsuperscript{L3040H}-BAC (Tg46\textsuperscript{L3040H} and Tg7\textsuperscript{L3040H}) did not ameliorate the postnatal kidney cystic phenotype in Pkd1\textsuperscript{-/-} Ksp-Cre mice, which developed cysts in the medullary thick ascending loop of Henle (mTAL), the distal convoluted tubule (DCT), and the collecting duct (Figure 6D and ref. 65). We found that the same was true in Pkd1\textsuperscript{fl/fl} Pkhd1-Cre mice, in which the cysts were confined to the collecting duct (Figure 6E). IP followed by IB of cystic tissue lysates from both models documented expression of the PCK1\textsuperscript{L3040H} protein (Figure 6, F and G). In aggregate, these data indicate complete loss of PC1 function in GPS cleavage–deficient Pkd1\textsuperscript{L3040H} in vivo.

Aberrant trafficking and distinct pools of polycystins in native tissues. We took advantage of the epitope-tagged BAC transgenic models to examine the trafficking properties of WT PC1 and the mutant Pkd1\textsuperscript{L3040H} in vivo. PC1-CTF from WT Pkd1\textsuperscript{fl/fl}-BAC transgenic brain tissue showed endo H–resistant and –sensitive forms in vivo (Figure 7A). Uncleaved PC1-FL and PC1\textsuperscript{L3040H} were completely sensitive to endo H (data not shown). For tissue fractionation studies, we used lung tissue, because it showed the most robust expression of both forms of PC1 by IB (Figure 5D and Fig-
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The form, PC1L3040H. In tissue, full-length, uncleaved WT PC1 or cleavage-deficient mutant PC1L3040H remained in the ER compartment, while the cleaved PC1 products trafficked to the cell surface. We had previously shown that endogenous PC2 distributes primarily in fractions associated with ER membranes in native kidney tissues (17). We took advantage of the linear-gradient fractionation of tissue from the Pkd1-BAC transgenic mice to examine the relative distributions of PC1 and PC2 in tissue (Figure 7, B and C). The fractions in which PC2 was most abundant were largely devoid of PC1-CTF and PC1-NTF; conversely, PC1-CTF and PC1-NTF appeared predominantly in fractions that contained only a minority of tissue PC2 (Figure 7B). The tissue distribution of PC2 did not change with PC1L3040H expression (Figure 7C), consistent with our previous finding that PC2 traffics independently of PC1 (13). The relatively disparate tissue distribution of PC1 and PC2 supports previous data that PC2 functions as an ER channel independently of PC1 (17, 18) and that PC2 has functions independent of PC1 (15, 21).

We fractionated lung tissue lysates on iodixanol-based linear density gradients and compared the distribution of PC1 and PC1L3040H (Figure 7, B and C). We found that WT PC1-NTF and PC1-CTF mainly distributed in the lighter membrane fractions at the top of the gradient that were also enriched in NaK-ATPase, a plasma membrane protein (Figure 7B). Uncleaved PC1-FL, as well as a small amount of PC1-NTF, but not PC1-CTF, appeared in fractions enriched for the ER membrane protein calnexin (Figure 7B). Fractionation of tissue from PC1L3040H transgenic mice showed that the full-length, uncleaved protein was retained entirely in fractions marked by the ER protein calnexin (Figure 7C). PC1L3040H was absent from fractions most enriched for NaK-ATPase (Figure 7C). We confirmed that the product detected by anti-FLAG in the PC1L3040H transgenic line was a full-length protein by running it alongside fractions from the WT PC1 gradient (Figure 7D). The data show in vivo fractionation of functional PC1 and the disrupted subcellular distribution in tissue of a loss-of-function mutant form, PC1L3040H. In tissue, full-length, uncleaved WT PC1 or cleavage-deficient mutant PC1L3040H remained in the ER compartment, while the cleaved PC1 products trafficked to the cell surface.

We had previously shown that endogenous PC2 distributes primarily in fractions associated with ER membranes in native kidney tissues (17). We took advantage of the linear-gradient fractionation of tissue from the Pkd1-BAC transgenic mice to examine the relative distributions of PC1 and PC2 in tissue (Figure 7, B and C). The fractions in which PC2 was most abundant were largely devoid of PC1-CTF and PC1-NTF; conversely, PC1-CTF and PC1-NTF appeared predominantly in fractions that contained only a minority of tissue PC2 (Figure 7B). The tissue distribution of PC2 did not change with PC1L3040H expression (Figure 7C), consistent with our previous finding that PC2 traffics independently of PC1 (13). The relatively disparate tissue distribution of PC1 and PC2 supports previous data that PC2 functions as an ER channel independently of PC1 (17, 18) and that PC2 has functions independent of PC1 (15, 21).
level overexpression systems using heterologous promoters, we established conditionally immortalized renal epithelial cell lines from mTAL of Pkd2fl/fl Pkd1F/H-BAC mice carrying the ImmortoMouse (H-2Kb­tsA58) transgene (Figure 8). These parental cells underwent transient expression of adenovirus­expressed Cre recombinase (adeno­Cre) followed by cloning using limiting dilution. Cells infected with adeno­Cre yielded Pkd2 –/– Pkd1 F/ H-BAC cells that did not express PC2, while cells not infected with adeno­Cre gave Pkd2 fl/fl Pkd1 F/ H-BAC cells that retained normal PC2 expression (Figure 8, A and B). Cells grown for 7 to 10 days under non­permissive conditions exhibited epithelial proper­ties including lateral ZO-1 and E­cadherin staining (Figure 8A and data not shown). Since these cells all carry the same 3­copy Pkd1F/H-BAC transgenic inser­tion, expression of the epitope­tagged PC1 protein is expected to be constant between cell lines. We found that the steady­state expression levels of PC1-CTF in Pkd2–/– cells were markedly reduced compared with those in Pkd2fl/fl cells that still express PC2 (Figure 8C). This did not reflect a global decrease in PC1 levels, as there was no change in the steady­state expression of PC1-FL (Figure 8, C and E). This also did not reflect a decreased efficiency of cleavage at the GPS, as we observed no change in PC1-NTF steady­state expression levels between Pkd2 fl/fl and Pkd2 –/– cells (Figure 8D), nor was there a reciprocal increase in PC1-FL in Pkd2 –/– cells (Figure 8, C and E). These results suggest that PC2 is not required for GPS cleavage of PC1. To determine whether the decrease in steady­state levels of PC1-CTF was the result of hyperubiquitination and degradation via the proteasome, we treated Pkd2 –/– cells with the irreversible proteasome inhibitor carfilzomib (Supplemental Figure 5). As a positive control, HIF1α levels were stabilized in the presence of carfilzomib. We observed that PC1-CTF expression levels are markedly reduced in the absence of PC2. Finally, we sought to examine the unresolved question of the impact of inactivation of Pkd2 on PC1 expression. Our observation that overexpression of WT PC2, but not the truncated form PC2L703X, increased the steady­state levels of PC1-CTF (Supplemental Figure 4) led us to question what effect the absence of PC2, as occurs in cyst cells in patients, has on PC1. To test this directly and to avoid potentially artefactual results due to high-

66). The data further suggest that while only a minor proportion of PC2 appears in the same fractions with the PC1-CTF and PC1-NTF, this subfraction may represent the proposed PCI-PC2 functional complex (3, 4, 67) that is important in the pathogenesis of PKD.

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domain, was able to fully rescue PC1-CTF expression in Pkd2–/– cells (Figure 8F). These data suggest that PC2 interaction is sufficient for stabilizing PC1-CTF, and this latter function does not require the channel activity of PC2. It is noteworthy that the majority (~85%) of PC2 mutations in patients are truncating and expected to abrogate interaction with PC1. These data pose the novel possibility that the pathogenic effect from truncating mutations in PC2 includes a reduced stability of PC1-CTF below a critical threshold and that cyst formation in PKD2 patients results, at least in part, from a reduced functional dosage of PC1, akin to the mechanism for cyst formation in isolated human polycystic liver diseases (2). The pathogenicity of PC2D511V in patients further suggests that the channel activity of PC2 is required for functional integrity of the PC1-PC2 complex independently of PC1-CTF levels in preventing ADPKD.

Discussion

Recent data have established the role of PC1 dosage as the rate-limiting determinant of ADPKD and ADPLD severity (2, 47, 48, 52). The data presented here extend these findings by showing that PC2 is necessary to maintain the functional integrity of the PC1-PC2 complex both independently and via its interaction with PC1-CTF. This interaction is required for maintaining the stability of PC1-CTF. A channel-dead pathogenic point mutant, PC2D511V, which retains the PC1 interaction domain, was able to fully rescue PCI-CTF expression in Pkd2–/– cells (Figure 8F). These data suggest that PC2 interaction is sufficient for stabilizing PCI-CTF, and this latter function does not require the channel activity of PC2. It is noteworthy that the majority (~85%) of PC2 mutations in patients are truncating and expected to abrogate interaction with PC1. These data pose the novel possibility that the pathogenic effect from truncating mutations in PC2 includes a reduced stability of PC1-CTF below a critical threshold and that cyst formation in PKD2 patients results, at least in part, from a reduced functional dosage of PC1, akin to the mechanism for cyst formation in isolated human polycystic liver diseases (2). The pathogenicity of PC2D511V in patients further suggests that the channel activity of PC2 is required for functional integrity of the PC1-PC2 complex independently of PC1-CTF levels in preventing ADPKD.
Aside from conformational changes, we also found that part of the COOH terminus region of PC1 was necessary for trafficking out of the ER and into cilia. The pathogenic truncation mutant PCI^{K4204X} entered cilia, indicating that the coiled-coil domain (residues 4,214–4,248) thought to mediate interaction with PC2 (53, 74) is not required for trafficking PCI to cilia. Complete deletion of the COOH terminus in PCI^{R4100X} shows a novel functional requirement of the region between PCI^{V4100} and PCI^{R4204} for PCI trafficking. While it is possible that this region is required to maintain conformational integrity to allow trafficking much the way point mutations do, it is more likely that this region of the COOH terminus is required for interaction with factors that are critical for PCI egress from the ER. Not all point mutations in PCI affect cilia trafficking. A mutation in a conserved residue in one PKD domain, PCI^{G1160S}, remains competent in GPS cleavage, cilia trafficking, and interaction with PC2, but still lacks normal function. Mutations in PKD domains may be part of a class of variants deficient in PC1 functions, perhaps related to ligand interaction, which are required once the protein is in cilia. Experimentally, this type of mutation may serve as an appropriate entry point for discovery of the cilia-specific functions of PC1 (75, 76) — i.e., if a cellular or physiological response is attributed to PCI specifically in cilia, then expression of these mutants in place of WT protein would be expected to result in loss of the specific response.

While amino acid substitution variants are less common in PC2 and therefore less likely to be targets for therapy, they can nonetheless be instructive in discerning function for the polycystins. The pathogenic missense variant PC2^{D511V} has complete loss of channel activity (17) but traffics to cilia, whereas a variant in a highly conserved residue, PC2^{W414G}, which has largely preserved channel function, does not traffic to cilia. These data provide support for the hypothesis that both cilia location and channel activity in cilia are required for polycystin function (15, 16). On the other hand, recent data directly examining ciliary calcium suggest that calcium concentrations in cilia are significantly higher than those in the cell body (75, 76). To put all of these findings together, the hypothesized role of ciliary polycystins in global cellular calcium responses may require significant revision. The aggregate information available suggests a hypothesis that the ciliary calcium channel function of polycystins has very local effects, possibly acting only within the polycystin protein complex. Nonetheless, our findings offer the first opportunity to categorize groups of missense mutations in polycystins into functionally distinct subtypes. The groups of variants for both polycystins that do not traffic to cilia may be appropriate to target with therapies aimed at countering protein-folding defects (77) and raise the prospect that such therapies may be applicable to selected patients with ADPKD.

ADPKD is a disease of 3D organ structure manifest in the complex cellular milieu of the adult kidney. As a consequence, the relevance of mechanistic discoveries based on in vitro cell culture models requires validation with in vivo models. To this end, we developed BAC-recombineering transgenic models for Pkd1 (2). BAC transgenic models have practical advantages over conventional knockin models, including the ability to target the same allele multiple times, the speed with which mutant mouse lines can be obtained, and the reduced cost in doing so. The emergence of genome-editing technologies, specifically the CRISPR/
Cas9 system, may offer similar advantages for future studies. Previous reports of Pkd1-BAC transgenic models showed cystic phenotypes that were the result of high transgene copy numbers (60, 78) and the use of heterologous promoters (61). Our WT Pkd1+/+–BAC founders were low copy numbers and achieved complete functional rescue of the Pkd1+ allele. The mutant Pkd1T3041V-BAC model was true to the in vitro data, producing uncleaved PC1 retained in the ER and resulting in a complete loss-of-function allele in vivo. The finding that the entire functionality of the BAC transgene in multiple founders could be eliminated by a single codon change in only 1 gene, Pkd1, eliminated any possibility that the other genes contained in the BAC had any contribution to the observed phenotypic effects in vivo. Our result with a GUS mutant is somewhat different from that of a knockin mouse model with a mutation in an adjacent codon, Pkd1T3041V (Pkd1+/+), which had postnatal survival up to 2 to 6 weeks with polycystic kidneys (73). These phenotypic differences highlight the fact that the specific nature of the genomic alteration and associated amino acid substitution is likely to be a key determinant of the degree of functional inactivation of polycystins.

These in vivo models also allowed us to compare the fractionated tissue distribution of PC1 and PC2. While both proteins are thought to function together in cilia in preventing cyst formation in the kidney, there is ample evidence that they also function independently in various tissues and subcellular locations. PC2 has a role in left-right axis determination, whereas PC1 does not (14, 66). PC2 is abundantly expressed in the ER, where it has channel activity, but that role appears to be independent of PC1 (17, 18). Our WT Pkd1 transgenic lines provided direct evidence that PC1 and PC2 distributed in distinct but overlapping density fractions. The majority of cleaved WT PC1 resides in presumed plasma membrane–associated fractions that contained a minute fraction of total tissue PC2. The existence of the bulk of PC2 protein in ER-associated tissue fractions devoid of cleaved PC1 lends strong support to the hypothesis that PC2 has cellular functions in the ER that are independent of PC1. Whether PC2 function independent of PC1 has a role in ADPKD remains to be determined.

Finally, we took advantage of our in vivo and in vitro models to examine the interrelationship between PC1 and PC2 expression. Surprisingly, we found that PC1-CTF required interaction with PC2 to maintain steady-state expression levels. The loss of PC1-CTF in the absence of PC2 was not the result of proteasomal degradation. Although not experimentally verified, there have been reports of a PEST domain in the COOH terminus of PC1 (4), which raises the possibility that the reduced steady-state levels of PC1-CTF in the absence of PC2 are the result of increased degradation by calpain protease (79). In this regard, it is notable that PC1 truncation mutants, in which we eliminated the possible PEST domain and the coiled-coil PC2 interaction domain, nonetheless had sufficient stability to traffic to cilia. Interpretation of these findings must be tempered, however, by the fact that these cells highly overexpressed Pkd1 cDNA from a heterologous promoter and cannot be directly compared with a 3-copy BAC transgene in the Pkd2-/- cells. A recent study has suggested that PC1-NTF can exist stably without PC1-CTF, but PC1-CTF is critically required for PC1-NTF trafficking (50). In keeping with this, we did not find a profound reduction in PC1-NTF steady-state levels in the absence of PC2. Our data suggest the possibility that one of the pathogenic effects of truncation mutations in PC2 is a markedly decreased steady-state level of PC1-CTF protein, which in turn is critically required for PC1 function. The finding that the channel-dead pathogenic variant PC2D211V (17) that traffics to cilia in LLC-PK, cells (80) and can rescue the stability of PC1-CTF nonetheless results in human ADPKD (56) suggests that both PC2 interaction with PC1 and PC2 channel activity are independently required for preventing ADPKD.

In aggregate, these studies highlight 5 important findings in the pathogenesis of ADPKD: (a) a subset of pathogenic nonsynonymous substitution mutations results in impaired trafficking of PC1 and PC2 to cilia; (b) mutations that impair GPS cleavage also impair cilia trafficking; (c) cilia-trafficking studies can serve as in vitro assays for PC1 (and PC2) function; (d) the bulk of PC2 resides in the ER without associated PC1 in vivo; (e) truncation mutations in PC2 result in markedly reduced PC1-CTF protein steady-state expression levels. We propose that cilia-trafficking mutant forms of PC1 may be amenable to therapies directed at assisting protein folding with a readout of successful localization to cilia in vitro as the first step to identifying candidate therapeutic agents. In addition, understanding the molecular basis for the stabilization of PC1-CTF by PC2 interaction could lead to the discovery of agents that increase steady-state levels of PC1 as a therapy for hypomorphic PC1 variants.

**Methods**

*cDNA constructs, antibodies, and cell culture.* The full-length cDNA of mouse Pkd1 was cloned, sequenced, verified, and modified with NH₂-terminal triple–FLAG and COOH-terminal triple–HA epitope tags as described previously (49). Mutant Pkd1 cDNA constructs were generated on this backbone by site-directed mutagenesis using the QuickChange kit (Stratagene). All mutations were confirmed by direct sequencing. LLC-PK, cells stably expressing WT and mutant Pkd1 constructs were established as described previously (51). Briefly, cells were transfected with plasmid DNA using Lipofectamine 2000 (Invitrogen) and split at a 1:10 dilution after 24 to 48 hours. Selection with G418 (400–600 μg/ml) was begun after overnight culture. Selected cell clones were characterized by IF cell staining and IB of 50 to 100 μg of protein per sample using anti–HA and anti–FLAG antibodies.

Rabbit polyclonal antisera for PC2 COOH terminus (YCC2) and NH₂ terminus (YCB9) have been described previously (51). Other antibodies used were from the following sources: mouse monoclonal anti–Myo (clone 9E10, 1667149; Roche); rabbit polyclonal anti–Myo-My (2272; Cell Signaling Technology); anti–c–Myo–conjugated agarose (A7470; Sigma-Aldrich); rat monoclonal anti–HA (clone 3F10, 1847723; Roche); rabbit polyclonal anti–HA (715500; Zymed); mouse monoclonal anti–FLAG M2–conjugated agarose (A2220; Sigma-Aldrich); rabbit polyclonal anti–FLAG (F7425; Sigma-Aldrich); mouse monoclonal anti–acetylated α-tubulin (T6793; Sigma-Aldrich); mouse monoclonal anti–γ-tubulin (T6557; Sigma-Aldrich); mouse monoclonal anti–β-tubulin Cy3 conjugate (C4585; Sigma-Aldrich); mouse monoclonal anti–HA FITC conjugate (H7411; Sigma-Aldrich); mouse monoclonal α-subunit of NaK-ATPase clone 6H (gift of Michael Caplan, Yale University, New Haven, Connecticut, USA); and rabbit polyclonal anti–calnexin (SPA-860; Stressgen).

**Immunocytochemistry, IP, and IB.** IF cell staining was performed as described previously (49, 51). Briefly, cells grown on coverslips or
semipermeable filter inserts were fixed with 2.5% paraformaldehyde and permeabilized with 0.3% Triton X-100 and 0.1% BSA in PBS. Blocking was carried out using 3% BSA, and primary and secondary antibodies were applied at empirically determined dilutions. Cells were examined either by wide-field fluorescence (TE2000U; Nikon) with MetaMorph acquisition software or by confocal microscopy (LSM 510; Zeiss). IP experiments were performed as described previously, with minor modifications (49, 51). Cells were lysed with glycerol lysis buffer (12), and lysates were pre cleared by incubation with protein A for 30 minutes, followed by IP starting with 500 to 1,000 μg of protein using anti-HA agarose beads (Sigma-Alrich) at 4°C overnight. The beads were washed with lysis buffer 5 times, followed by SDS-PAGE on 3% to 8%-gradient gels and IB as described previously (49, 51).

Glycosylation analysis and cell-surface biotinylation. Glycosylation analysis was performed as described previously (49, 51). PC1 was subjected to IP using either FLAG or HA antibodies and incubated with either endo H, PNGase F, or reaction buffer alone (New England Biolabs) at 37°C for 1 hour followed by IB. Biotinylation of cell-surface proteins was performed using EZ-Link NHS-SS-Biotin (Pierce Biotechnology) as described previously (49, 51). N-hydroxysuccinimide-SS-biotin was applied to living cultured cells stably expressing PCI for 30 minutes at 4°C. Biotinylated proteins were recovered using streptavidin agarose beads and detected by IB.

BAC modification. The mouse BAC clone RP12-287A3 containing Pkd1 was modified by homologous recombination in E. coli using the method of Lalioti and Heath (57, 58) in the Mouse Genetics and Cell Line Core (George M. O’Brien Kidney Center, Yale School of Medicine, New Haven, Connecticut, USA). Pkd1fl/fl-BAC and Pkd1L3040H-BAC were generated by sequential BAC modifications to introduce 3 copies each of HA and FLAG epitopes and then the Pkd1L3040H mutation. In general, for each modification step, approximately 1.0- to 1.3-kb segments of genomic DNA spanning the sites for the desired variants were amplified by PCR, subcloned into Zero Blunt TOPO (Invitrogen), and sequenced in both directions. In-frame triple-HA and triple-FLAG sequences and the variant encoding the L3040H were cloned into the shuttle vector PKO4F-Kan (57, 58) containing kanamycin resistance for positive selection and 2 negative selection cassettes, and transformed with pK04F-Kan containing the targeting sequence and confirmed by sequencing. These targeting constructs were electrocompetent E. coli DH10B bacteria (which blocks growth on sucrose). DH10B bacteria were transformed with pK04F-Kan containing the targeting sequence and confirmed by sequencing. Confirmed cointegrants underwent resolution (removal of the reduplicated region) by a second round of transformation with the pDF25-Tet containing tetracycline resistance. Kidney tubule epithelial cell lines were produced as described previously (2, 82). Resultant Pkd1L3040H-BAC+ paren-
tal” cell lines were converted to Pkd2+/Pkd1fl/fl-BAC-null cell lines ex vivo by infection with 1 to 10 PFU/cell of adenov-Cre (0100062001; Eton Bioscience), followed by cloning using limiting dilution. Uninfected cells were used as controls. The Pkd2+/Pkd1fl/fl-BAC cell lines used were SF3a and SF3b; the Pkd2fl/fl-Pkd1fl/fl-BAC cell line used was SF4. Prior to the experiments, cells were allowed to differentiate following silencing of the SV40 large T antigen under nonpermissive conditions (37°C, without IFN-γ) for 7 to 21 days.

Reexpression of WT or mutant Pkd2 carrying the Myc tag into the Pkd2+/Pkd1fl/fl-BAC cells. Target gene cDNA was cloned into the pLVX-IRES-puro lentiviral expression vector (Clontech), followed by cotransfection with the packaging system (pMDLg/pRRE plus pRSV-Rev plus pMD2.G; Addgene) into 293T cells using the calcium phosphate transfection method. After incubation of the cells in a 37°C CO₂ incubator for 40 to 48 hours, the culture medium supernatant was collected, filtered, and concentrated by centrifugation. The viruses were resuspended in culture medium and used to infect the Pkd2+/Pkd1fl/fl-BAC cells. The infected cells were further selected for 3 to 5 days by puromycin selection at 1 to 4 μg/ml. Target gene expression was verified by IB and IF cell staining.

Statistics. Statistical significance was determined by 2-tailed Student’s t test, with a P value of less than 0.05 considered statistically significant.

Study approval. All animal studies were reviewed and approved by the IACUC of Yale University.

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