Acceleration of polycystic kidney disease progression in cpk mice carrying a deletion in the homeodomain protein Cux1

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Acceleration of polycystic kidney disease progression in cpk mice carrying a deletion in the homeodomain protein Cux1. Am J Physiol Renal Physiol 295: F1725–F1734, 2008. First published October 1, 2008; doi:10.1152/ajprenal.90420.2008.—Polycystic kidney diseases (PKD) are inherited as autosomal dominant (ADPKD) or autosomal recessive (ARPKD) traits and are characterized by progressive enlargement of renal cysts. Aberrant cell proliferation is a key feature in the progression of PKD. Cux1 is a homeobox gene that is related to Drosophila cut and is the murine homolog of human CDP (CCAAT Displacement Protein). Cux1 represses the cyclin kinase inhibitors p21 and p27, and transgenic mice ectopically expressing Cux1 develop renal hyperplasia. However, Cux1transgenic mice do not develop PKD. Here, we show that a 246 amino acid deletion in Cux1 accelerates PKD progression in cpk mice. Cystic kidneys isolated from 10-day-old cpk/Cux1 double mutant mice were significantly larger than kidneys from 10-day-old cpk mice. Moreover, renal function was significantly reduced in the Cux1 mutant cpk mice, compared with cpk mice. The mutant Cux1 protein was ectopically expressed in cyst-lining cells, where expression corresponded to increased cell proliferation and apoptosis, and a decrease in expression of the cyclin kinase inhibitors p27 and p21. While the mutant Cux1 protein altered PKD progression, kidneys from mice carrying the mutant Cux1 protein alone were phenotypically normal, suggesting the Cux1 mutation modifies PKD progression in cpk mice. During cell cycle progression, Cux1 is proteolytically processed by a nuclear isoform of the cysteine protease cathepsin-L. Analysis of the deleted sequences reveals that a cathepsin-L processing site in Cux1 is deleted. Moreover, nuclear cathepsin-L is significantly reduced in both human ADPKD cells and in Pkd1 null kidneys, corresponding to increased levels of Cux1 protein in the cystic cells and kidneys. These results suggest a mechanism in which reduced Cux1 processing by cathepsin-L results in the accumulation of Cux1, downregulation of p21/p27, and increased cell proliferation in PKD.

p27; cyst formation; apoptosis; cellular polarity; multiorgan hyperplasia; p21; cell cycle; cell proliferation

POLYCYSTIC KIDNEY DISEASE (PKD) is a term applied to a group of inherited disorders characterized by the presence of renal cysts; however, multiple organs are usually affected. Human autosomal dominant PKD (ADPKD) results from mutations in one of two genes, PKD1 or PKD2, that encode polycystin-1 and polycystin-2 proteins, respectively (39, 58–60). Human autosomal recessive PKD (ARPKD) results from mutations within a single gene, polycystic kidney and hepatic disease 1 (PKHD1), encoding fibrocystin/polyductin (47, 66). Proteins that are mutated in human PKD (polycystin-1, polycystin-2, fibrocystin/polyductin), and in animal models of PKD (cystin, polaris), colocalize to the primary cilia (26, 46, 50, 69). This suggests that mutations in ciliary proteins affect common or overlapping signaling pathways resulting in PKD (43). The process of cyst formation in PKD is thought to involve various mechanisms including cell proliferation, fluid secretion, differentiation, abnormal basement membrane formation, matrix remodeling, apoptosis, and alteration in cellular polarity (10, 19, 22, 27). Growing evidence suggests that PKD is a developmental disorder (4, 7, 23, 26, 33, 34, 48, 67).

The murine transcription factor Cux1 is structurally related to the Drosophila homeodomain protein cut and contains four putative DNA binding domains (3 cut repeats, 1 homeodomain) (1, 2, 44, 45, 51, 64, 70). Drosophila cut is required for the proper development of the Malpighian tubules, the insect excretory and osmoregulatory organs (5, 6). Cux1 represses the expression of the cyclin kinase inhibitor (CKI) p21 in S phase and is part of the network controlling G1-S transition (12). Cux1 also represses the CKI p27, and ectopic expression of Cux1 in transgenic mice results in multiorgan hyperplasia from the aberrant downregulation of p27 (30). In the kidney, Cux1 expression is spatially and temporally regulated, with highest expression in the nephrogenic zone, where it is associated with cell proliferation (30, 64). During normal kidney development, p27 is absent from the nephrogenic zone, but is expressed in maturing glomeruli and tubules following the downregulation of Cux1 (11). Thus, Cux1 is a cell cycle-dependent transcription factor that promotes cell proliferation during the early stages of nephrogenesis by repressing p27 gene expression.

In addition to the full-length isoform, called Cux1 (p200), there are several truncated isoforms. A nuclear isoform of the cysteine protease cathepsin-L has been identified that proteolytically processes Cux1 (p200) in S-phase (21). While the full-length Cux1 protein (p200) contains three cut repeats and the homeodomain, the proteolytically processed Cux1 (p110 or p90) contains only cut repeats 2 and 3, together with the homeodomain (21). Cux1 (p200) exhibits transient DNA binding activity and functions as a transcriptional repressor, whereas p110 stably binds DNA and can function as a transcriptional activator (40). Other Cux1 isoforms result from

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alternate splicing (CASP) (31), alternate promoter use (p75) (20), or proteolytic processing (p150) (37). Another Cux1 transcript, found exclusively in testis, encodes a 55-kDa protein; however, it is not known whether it originates by alternate splicing or an alternate promoter (65).

Cux1 (p200) is highly expressed in cystic kidneys isolated from both Pkd1 null and cpk mice, murine models of ADPKD and ARPKD, respectively (53). In cystic kidneys from Pkd1 null mice, Cux1 is highly expressed in both cyst-lining cells and in normal appearing tubule epithelium where expression of Cux1 is associated with increased cell proliferation. In cystic kidneys from cpk mice, Cux1 is not abnormally expressed until late stages of cystogenesis where expression of Cux1 is associated with increased apoptosis in cyst-lining cells. However, CMV/Cux1 transgenic mice do not develop cystic kidney disease. However, it was recently shown that transgenic mice ectopically expressing the p75 isoform of Cux1 develop renal abnormalities including renal tubule hyperplasia and cystic dilations with long latency (9).

In the present study, we generated Cys1cpk mice carrying a mutated allele of Cux1 to determine whether changes in Cux1 affect the progression of disease. The mutated Cux1 allele, called Cux1tm1Ejn, carries an in-frame deletion of Cux1, encompassing exons 15 and 16 (63). The Cux1tm1Ejn mice display wavy hair and curly vibrissae, and females are unable to support pups because of lactation defects. However, the kidneys of these mice are phenotypically normal. The null mice heterozygous for both Cux1tm1Ejn and Cys1cpk were crossed with female mice heterozygous for both Cux1tm1Ejn and Cys1cpk to generate double homozygous Cux1tm1Ejn/Cys1cpk mice. All Cux1tm1Ejn, Cys1cpk, and Cux1tm1Ejn/Cys1cpk mice analyzed were on the same B6129SF1/J mixed genetic background. All protocols were approved by the University of Kansas Medical Center Animal Care and Use Committee.

The University of Kansas Medical Center is fully accredited by the American Association of the Accreditation of Laboratory Animal Care.

Characterization of cystic phenotype. Cystic kidneys were collected from 3- and 10-day-old cpk and Cux1tm1Ejn/Cys1cpk mice. The weight of both cystic kidneys (KW) was divided by body weight (BW) and calculated to determine KW as a percent BW. Kidney length measurements of bilateral kidneys were performed and divided by crown-rump measured in centimeters (cm). Cystic kidney midagittal sections (5 μm) were utilized to stage cysts, as described previously (61). Cysts were considered early stage if there were less than 50 cyst-lining epithelial cells, intermediate if there were 51–200 cyst-lining epithelial cells, and advanced stage if greater than 200 cyst-lining cells.

Serum chemistry. Blood was collected by intracardiac puncture and immediately centrifuged at 2,000 g for serum collection. Blood urea nitrogen (BUN) was determined using an autoanalyzer (Physicians Reference Laboratory, LLC, Overland Park, KS).

Immunohistochemistry. Immunohistochemistry was performed as previously described (53). Kidney sections were immersion fixed in

Fig. 1. Increased kidney size and decreased kidney function in Cux1tm1Ejn/Cys1cpk mice. a: Cux1tm1Ejn mutation results in normal kidneys. Brightfield image of midagittal sections of 10-day-old age-matched kidneys from wild-type (WT) or Cux1tm1Ejn mice. Hematoxylin and eosin staining shows no phenotypic differences in between kidneys from Cux1tm1Ejn and WT mice. Bar = 1 mm. b: Gross appearance of Cys1cpk and Cux1tm1Ejn/Cys1cpk cystic 10-day-old kidneys. c and d: Kidney weight (KW) standardized as a percentage of body weight (BW) represented as mean values ± SE. *Kidneys from Cux1tm1Ejn/Cys1cpk 10-day-old mice were significantly larger (P ≤ 0.0001) than kidneys from Cux1tm1Ejn/Cys1cpk or Cys1cpk 10-day-old mice in c and that kidneys from Cux1tm1Ejn/Cys1cpk 3-day-old mice were significantly larger (P = 0.05) than kidneys from Cys1cpk 3-day-old mice in d. *Kidney weight (%BW) and BUN (mg/dl) were significantly greater (P ≤ 0.001) than BUN in 10-day-old Cux1tm1Ejn/Cys1cpk mice.
4% paraformaldehyde and blocked in paraffin. Sections were washed in PBS containing 1% Tween 20 (PBST) and blocked in 10% normal goat serum (NGS) at room temperature for 1 h. Rabbit Cux1 (Santa Cruz Biotechnology), mouse PCNA (Sigma), or rabbit Cathepsin-L (Calbiochem) primary antibody was applied to sections incubated at room temperature for 1 h at a concentration of 1:100, 1:3,000, and 1:50, respectively. Biotinylated goat anti-rabbit (1:400) was used to detect Cux1 and Cathepsin-L antibody. A horse anti-mouse Texas red conjugated secondary antibody (Vector) was utilized to detect PCNA antibody (1:400). Sections were then washed in PBST and incubated with either FITC-conjugated avidin (Vector) or incubated with avidin-biotin-peroxidase complex (ABC-Elite: Vector) and DAB. All sections were washed with PBST and then mounted with either Vectashield medium with Dapi (Vector) or dehydrated with graded ethanols, mounted with Permount (fisher), and covered with glass coverslips. All images were captured with an Optronics Magmasure digital camera.

Western blot analysis. Human ADPKD and normal human kidney (NHK) were harvested and nuclear extracts were prepared as previously described (8, 18). Nuclear extracts (45 μg) were loaded onto 4–15% SDS-PAGE gels and transferred to PVDF membranes where they were blocked in 5% milk PBST. Membranes were probed with Cux1 (1:50), Cathepsin-L (1:2,000), p27 (1:100), or p21 (1:100; Santa Cruz Biotechnology) primary antibody followed by PBST washes and horseradish peroxidase (1:10,000) secondary antibody application.

TUNEL assay. Sections were processed for terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-nick-end labeling (TUNEL) with the ApopTag Red InSitu Apoptosis Detection kit (Intergen) according to the manufacturer’s instructions. Sections were counter-
stained with DAPI, coverslipped, and visualized on a fluorescence microscope. Images were captured with an Optronics Magnafire digital camera.

Statistics. In all studies, a one-way ANOVA was performed. If significance between the genotypes existed ($P \leq 0.05$), post hoc analysis by least significant difference (LSD) was performed to determine statistical significance ($P \leq 0.05$) between groups. All statistical analyses were performed using the Statview statistical program.

RESULTS

We crossed Cux1tm1Ejn mice, which carry a 246 amino acid deletion in Cux1 that includes the first cut repeat (Cux1ΔCR1) (63), with Cystpk mice to generate mice homozygous for both genes (Cux1tm1Ejn/Cystpk). Mice carrying the Cux1ΔCR1 mutation have curly whiskers, wavy hair, and lactation defects (63), but have normal kidneys (Fig. 1a). Surprisingly, when this mutation was combined with the Cystpk mutation, the double homozygous (Cux1tm1Ejn/Cystpk) mice developed cystic kidneys that were significantly larger than the cystic kidneys from mice carrying only the Cystpk mutation (Fig. 1). Bilateral kidneys were harvested at postnatal day 10 (P10) and total KW and BW measurements were collected to calculate KW standardized as a percentage of BW, standard measurements to quantify renal cystic disease severity (24, 41). By gross appearance, kidneys from Cux1tm1Ejn/Cystpk mice were significantly larger than kidneys from Cystpk mice (Fig. 1b). Cux1tm1Ejn/Cystpk kidneys were significantly larger compared with Cystpk in regard to KW/BW, both at postnatal day 3 and 10 (Fig. 1, c and d). While Cystpk mice heterozygote for the Cux1tm1Ejn mutation showed a slight increase in KW/BW, it was not significant. Large cystic kidneys are directly correlated with reduced renal function and high BUN levels (13, 62). Accordingly, Cux1tm1Ejn/Cystpk animals had higher BUN levels than Cystpk mice (Fig. 1e), indicating impaired renal function.

To further analyze the Cux1tm1Ejn/Cystpk phenotype, cystic kidneys isolated from Cux1tm1Ejn/Cystpk and Cystpk mice were examined morphologically (Fig. 2, a and b). Histological analysis showed that the overall number of renal cysts was not different between the Cystpk and Cux1tm1Ejn/Cystpk mice (Fig. 2c). Rather the size of the cysts appeared to be increased in the kidneys from Cux1tm1Ejn/Cystpk mice. Cyst numbers were quantified in regard to the developmental stage of each individual cyst. The developmental stage of the renal cysts in Cux1tm1Ejn/Cystpk and Cystpk mice was determined by counting the number of cells lining the cysts (see MATERIALS AND METHODS). The results showed that kidneys from Cux1tm1Ejn/Cystpk mice had an increased number of advanced stage cysts compared with age-matched kidneys from Cystpk mice (Fig. 2d). This was associated with a reduced number of early stage cysts in the kidneys from Cux1tm1Ejn/Cystpk mice compared with kidneys from age-matched Cystpk mice. Moreover, the pattern of cystogenesis characteristic of the Cystpk mutation was unchanged. Cystpk mice undergo two phases of cystic disease (3, 17). Initially, Cystpk mice develop proximal tubule cysts that resolve by postnatal day 7. This is followed by a second phase of cystogenesis, in which cysts develop primarily from collecting ducts, resulting in massive enlargement of the kidneys. In kidneys from both Cux1tm1Ejn/Cystpk and Cystpk mice, the cysts were primarily restricted to the collecting ducts, with few proximal tubule cysts, although the collecting duct cysts in the Cux1ΔCR1/cpk kidneys were larger than those in the cpk kidneys (Fig. 2, e–h). Our results suggest that the Cux1tm1Ejn gene accelerates cyst growth, but does not induce cyst formation.
Cell proliferation is a key feature of cyst growth (4, 8, 22, 68). Moreover, there is a direct correlation between levels of cell proliferation and progression of cystic disease (8, 57, 62). Thus, one possibility is that the increased cyst size observed in the kidneys from Cux1^tm1Ejn/Cys1^cpk mice resulted from increased proliferation of the cyst-lining epithelial cells. In the developing kidney, Cux1 is expressed in the nephrogenic zone where it colocalizes with markers for cell proliferation, but is downregulated in maturing glomeruli and tubules (64). However, in Pkd1 null kidneys, Cux1 is ectopically expressed in the cyst-lining cells, and in normal appearing mature tubule epithelium, where it is associated with cell proliferation (53). Wild-type Cux1 protein was not expressed in the cyst-lining cells of kidneys isolated from 10-day-old Cys1^pk mice (Fig. 3, a–c), as described previously (53). In contrast, Cux1ΔCR1 protein colocalized with PCNA in the cyst-lining cells of kidneys isolated from 10-day-old Cux1^tm1Ejn/Cys1^cpk mice (Fig. 3, d–f). While there is limited apoptosis in the cysts of 10-day-old Cys1^pk mice, apoptosis is increased at later stages of cyst growth, where it is thought to contribute to cyst progression (8, 53). Moreover, inhibition of apoptosis slows cyst progression in mouse and rat models of PKD (8, 57). Consistent with an acceleration of cyst progression in the kidneys of Cux1^tm1Ejn/Cys1^pk mice, we observed an increase in TUNEL labeling in the cyst-lining cells of Cux1^tm1Ejn/Cys1^pk mice compared with Cys1^cpk mice, and an increase in activated caspase 3 in kidneys from Cux1^tm1Ejn/Cys1^cpk mice compared with Cys1^pk mice (Fig. 3, g–i).

The expanded expression of the Cux1ΔCR1 protein in the cystic kidneys was surprising since this deletion would not be expected to affect the regulatory elements in the Cux1 gene. Moreover, the levels of Cux1^tm1Ejn mRNA are not different from that of wild-type Cux1 (p200) mRNA (63). However, the levels of Cux1ΔCR1 protein appear to be elevated in some tissues compared with Cux1 (p200). To determine whether the Cux1ΔCR1 protein was elevated in the kidney, we performed Western blot analysis on kidneys isolated from postnatal day 10 wild-type, Cys1^pk, and Cux1^tm1Ejn mice, and Cys1^pk mice carrying one or two mutant Cux1 alleles (Cux1^tm1Ejn+/−/Cys1^pk or Cux1^tm1Ejn/Cys1^pk). Figure 4 shows the expression of Cux1 (p200) in wild-type mice, which is completely processed to the p110 form in Cys1^pk mice. This corresponded to an increase in nuclear cathepsin-L in Cys1^pk mice, compared with wild-type mice. In contrast, the Cux1ΔCR1 protein is not processed in Cys1^pk mice. Moreover, while p21 and p27 are both highly expressed in Cys1^pk mice, they are downregulated in Cys1^pk mice carrying one or two mutant Cux1 alleles (Fig. 4). In addition, Cux1 is not expressed in the cyst-lining cells of Cys1^pk mice (Fig. 5, a and j), which is associated with the ectopic expression of p21 and p27 in the cyst-lining cells (Fig. 5, d, g, m, p). In contrast, the Cux1ΔCR1 protein is ectopically expressed in the cyst-lining cells of Cux1^tm1Ejn/Cys1^pk mice (Fig. 5, c and l), and this is associated with reduced expression of p21 and p27 in the cyst-lining cells (Fig. 5, f, i, o, r). Interestingly, Cys1^pk mice heterozygote for the Cux1^tm1Ejn mutation (Cux1^tm1Ejn+/−/Cys1^pk) showed expression of the Cux1/Cux1ΔCR1 protein in cyst-lining cells (Fig. 5, b and k) that was associated with a reduction in p21 expression, but not p27 expression in the cyst-lining cells (Fig. 5, e, h, n, q).

The Cux1^tm1Ejn phenotype is similar to previously described mouse mutants in the EGFR pathway, including wa-1 (35, 38) and wa-2 (16, 36). The role of EGFR in PKD has been well-described (18, 29, 32, 49, 54). Moreover, blocking EGFR activity, either genetically using the wa-2 mutation, or pharmacologically, results in decreased cyst formation and improved kidney function (49, 55, 56). To determine whether changes in EGFR signaling were disrupted in Cux1^tm1Ejn/Cys1^pk mice, we examined the expression of EGFR in Cys1^pk and Cux1^tm1Ejn/Cys1^pk mice. Figure 5 shows that no differences in expressions were found between Cys1^pk and Cux1^tm1Ejn/Cys1^pk mice (Fig. 5, s and t). This is consistent with previous reports suggesting that the Cux1^tm1Ejn phenotype does not result from disruption of the EGFR pathway (63).
Fig. 5. Ectopic expression of Cux1ΔCR1 protein in cystic kidneys results in the downregulation of p21 and p27 in Cux1tm1Ejn/Cys1cpk mice. Localization of Cux1 (a, j), Cux1/Cux1ΔCR1 (b, k), Cux1ΔCR1 (c, l), p21 (d-f, m-o), p27 (g-i, p-r), and EGFR (s, t) in kidneys isolated from 10-day-old Cys1cpk (a, d, g, j, m, p, s), Cux1tm1Ejn/Cys1cpk (b, e, h, k, n, q), and Cux1tm1Ejn/Cys1cpk (c, f, i, l, o, r, t) mice. a and j: Cux1 protein is restricted to interstitial cells in cystic kidneys from Cys1cpk mice (arrowheads in j) and is not expressed in cyst-lining cells (arrows in j). b and k: In Cux1tm1Ejn/Cys1cpk mice, Cux1/Cux1ΔCR1 protein is expressed in interstitial cells (arrowheads in k), with few cysts exhibiting ectopic expression in cyst-lining cells (arrows in k). c and l: Cux1ΔCR1 protein is ectopically expressed in cyst-lining cells in cystic kidneys from Cux1tm1Ejn/Cys1cpk mice (arrows in l), as well as in interstitial cells (arrowheads in l). d-f, m-o: p21 protein is ectopically expressed in cyst-lining cells in Cys1cpk mice (d, and arrows in m), but is downregulated in cyst-lining cells in both Cux1tm1Ejn/Cys1cpk and Cux1tm1Ejn/Cys1cpk mice (e, f, and arrows in n and o). g-i, p-r: p27 protein is ectopically expressed in cyst-lining cells in both Cys1cpk and Cux1tm1Ejn/Cys1cpk mice (g, h, and arrows in p and q) but is downregulated in cyst-lining cells in Cux1tm1Ejn/Cys1cpk mice (i, and arrows in r). s and t: EGFR protein is apically expressed in some cyst-lining cells in Cys1cpk mice (arrow in s) and Cux1tm1Ejn/Cys1cpk mice (arrow in t). Bar = 50 μm (a-i). Bar = 50 μm (j-t).
We previously reported differences in Cux1, p21, and p27 expression in mice with ARPKD (Cys1<sup>fpk</sup>) and ADPKD (Pkd1 null) (53). To determine whether these expression differences might result from differences in the proteolytic processing of Cux1, we evaluated Cux1 expression in epithelial cells isolated from cysts of human ADPKD kidneys or tubules of NHKs. We were unable to detect full-length Cux1 (p200) protein in nuclear extracts from NHK (Fig. 6a). However, there was an accumulation of full-length Cux1 (p200) protein in the nuclear extracts of cells isolated from the cysts of ADPKD kidneys (Fig. 6a). To determine whether this increase in full-length Cux1 (p200) protein was from decreased proteolytic processing, we compared nuclear cathepsin-L levels in NHK and ADPKD cells. Figure 6b shows that nuclear cathepsin-L was significantly reduced in ADPKD cells. When we examined cathepsin-L expression in kidneys isolated from embryonic wild-type and Pkd1 null mice, we found that nuclear cathepsin-L was similarly reduced in Pkd1 null mice (Fig. 6c and d).

**DISCUSSION**

Cux1 is highly expressed in the nephrogenic zone during normal kidney development where it functions to repress p27 gene expression promoting cell proliferation (30, 64). Transgenic mice expressing the Cux1 (p200) cDNA using the CMV immediate early gene promoter develop multiorgan hyperplasia and exhibit continued cell proliferation in adult kidneys (30). However, these mice do not develop renal cysts, suggesting that cell proliferation alone is insufficient for cystogenesis. Cux1<sup>tm1Ejn</sup> mice carry an in-frame deletion of Cux1, encompassing exons 15 and 16 (60). The Cux1<sup>tm1Ejn</sup> phenotype is similar to previously described mouse mutants in the EGFR pathway (16, 35, 36, 38). The role of EGFR in PKD has been well-described (18, 29, 32, 49, 54). Moreover, blocking EGFR activity, either genetically or pharmacologically, results in decreased cyst formation and improved kidney function (49, 55, 56). To determine whether the Cux1<sup>tm1Ejn</sup> would similarly alter cyst progression, we crossed the Cux1<sup>tm1Ejn</sup> mice with Cys1<sup>fpk</sup> mice to generate Cux1<sup>tm1Ejn/Cys1<sup>fpsk</sup></sup> mice. Our results show that an in-frame deletion of Cux1, encompassing exons 15 and 16, modifies the progression of PKD in Cys1<sup>fpk</sup> mice. However, rather than slowing the progression of cystogenesis, the presence of the Cux1<sup>tm1Ejn</sup> mutation accelerated cyst growth. Given the similarity between phenotypes of Cux1<sup>tm1Ejn</sup> mice and mouse mutants in the EGFR pathway, one possibility is that the EGFR pathway is disrupted in the Cux1<sup>tm1Ejn/Cys1<sup>fpk</sup></sup> mice. However, the Cux1<sup>tm1Ejn</sup> phenotype does not appear to result from a disruption of the EGFR pathway, as no change in the expression of EGFR or TGF-α was found in Cux1<sup>tm1Ejn</sup> mice (63). Moreover, Cux1 expression was not changed in epidermoid carcinoma cells that overexpress the EGFR (A431 cells) treated with EGF, compared with untreated cells, suggesting that Cux1 is not regulated by EGFR. In addition, EGFR expression was not altered between Cys1<sup>fpk</sup> and Cux1<sup>tm1Ejn/Cys1<sup>fpk</sup></sup> mice.

We propose an alternate model to explain the acceleration of cyst progression in Cys1<sup>fpk</sup> mice expressing the Cux1<sup>tm1Ejn</sup> mutation in which the Cux1ACR1 protein is ectopically expressed resulting in the repression of p21 and p27, increased cell proliferation, and a more rapid progression of cystic disease. Several observations are consistent with this model. First, expression analysis suggests that the mutant Cux1ACR1 protein was more broadly expressed than the Cux1 (p200) protein in the cystic kidneys isolated from 10-day-old Cux1<sup>tm1Ejn/Cys1<sup>fpk</sup></sup> or Cys1<sup>fpk</sup> mice, respectively. Second, the ectopically expressed Cux1ACR1 protein is associated with increased cell proliferation in cystic kidneys from Cux1<sup>tm1Ejn/Cys1<sup>fpk</sup></sup> mice. Third, while p21 and p27 are upregulated in kidneys isolated from Cys1<sup>fpk</sup> mice (47), both are downregulated in cystic kidneys from Cux1<sup>tm1Ejn/Cys1<sup>fpk</sup></sup> mice.
Cux1 (p200) represses p21 and p27 promoter activity in a concentration-dependent manner (10, 25). The decreased expression of p21 and p27 in the cystic kidneys expressing the Cux1ΔCR1 protein suggests that this protein can function similar to the Cux1 (p200) protein. It is of interest that p27, but not p21, was ectopically expressed in the cyst-lining cells of Cux1tm1Ejn /Cys1cpk mice. Moreover, the kidneys from Cux1tm1Ejn /+ /Cys1cpk mice were not significantly enlarged compared with kidneys from Cys1cpk mice. The reduced expression of p21 in the Cux1tm1Ejn / + / Cys1cpk mice, with no significant change in kidney size, suggests that loss of p21 does not contribute significantly to the Cux1tm1Ejn /Cys1cpk phenotype. This is consistent with previous studies showing that p21 null mice do not exhibit a cell proliferation defect (14), while p27 knockout mice develop hyperplasia (15, 28, 42).

The mechanism underlying the elevated levels of the mutant protein observed in cystic kidneys from Cux1tm1Ejn /Cys1cpk mice, compared with the levels of Cux1 (p200) in Cys1cpk mice, is not clear at present. Importantly, the Cux1tm1Ejn mutation does not alter the promoter and, thus, does not change the pattern of expression. This suggests that the ectopic expression of the Cux1tm1Ejn gene results from the Cys1cpk phenotype, while the Cux1ΔCR1 protein accumulation results from some other mechanism.

During S-phase of the cell cycle, a nuclear isoform of cathepsin-L proteolytically processes the Cux1 (p200) at amino acids 643, 747, and 755 to generate the p110 isoform (21). The deletion in the Cux1ΔCR1 protein includes the first cut repeat and the cathepsin-L site at position 643, while the cathepsin-L sites a positions 747 and 755 remain in the mutant protein. While Cux1 (p200) is completely processed to the p110 isoform in kidneys from Cys1cpk mice, the Cux1ΔCR1 protein is not processed in kidneys from Cux1tm1Ejn /Cys1cpk mice, suggesting the mutant Cux1ΔCR1 protein is more stable than the wild-type protein.

In contrast to Cys1cpk mice, nuclear cathepsin-L is reduced in both human ADPKD cells and in Pkd1 null kidneys. This suggests a mechanism in which reduced processing results in the accumulation of Cux1 (p200) leading to downregulation of p21 and p27 and increased cell proliferation in ADPKD. In contrast, the levels of nuclear cathepsin-L were not reduced in kidneys from Cys1cpk mice compared with kidneys from wild-type mice. Thus, in Cys1cpk mice, Cux1 (p200) would not escape proteolytic processing, and therefore would not accumulate. However, deletion of the first cathepsin-L cleavage site in Cux1ΔCR1 reduces proteolytic processing resulting in Cux1ΔCR1 protein accumulation.

It is of interest that reduced activity of cathepsin-L has previously been reported in the Han:SPRD rat model of PKD (52) and in human ADPKD cells (25). In both, the reduced activity was not a result of decreased gene expression, but of abnormal targeting of the enzyme. It is similarly possible that reduction of the nuclear isoform of cathepsin-L in human ADPKD cells and in kidneys from Pkd1 null mice is the result of abnormal targeting.

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