Regulation of cell proliferation and differentiation in the kidney

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1. ABSTRACT

The mammalian cut proteins are a broadly expressed family of nuclear transcription factors related to the Drosophila protein cut. One member of the cut family, Cux1, has been shown to function as a cell cycle dependent transcription factor, regulating the expression of a number of cell cycle regulatory proteins. Cux1 expression is developmentally regulated in multiple tissues suggesting an important regulatory function. Cux1 exists as multiple isoforms that arise from proteolytic processing of a 200 kD protein or use of an alternate promoter. Several mouse models of Cux1 have been generated that suggest important roles for this gene in cell cycle regulation during hair growth, lung development and maturation, and genitourinary tract development. Moreover, the aberrant expression of Cux1 may contribute to diseases such as polycystic kidney disease and cancer. In this review, we will focus on the phenotypes observed in the five existing transgenic mouse models of Cux1, and discuss the role of Cux1 in kidney development and disease.

2. INTRODUCTION

The "Cut wing" phenotype was first identified in Drosophila in the 1920s (1). Since then, numerous mutations associated with the partial or complete loss of cut expression have been identified in Drosophila (2-13). Our laboratory has been particularly interested in the cut mutation associated with the loss of the Malphigian tubules, the insect organ that functionally resembles a kidney (13-14). Using this information we, and others, have been able to identify a role for one of the mammalian cut proteins, Cux1, in the regulation of kidney development (15-22). For an excellent review on the role of cut in Drosophila and the evolutionary conservation of cut protein structure and function see Nepveu (23).

In recent years the cut family has grown and is now recognized as the cut superclass (24-25). The cut superclass consists of at least four classes of cut genes. The ONECUT family is characterized by having a single cut repeat domain and a homeodomain (24-26). The SATB (Special AT rich Binding domain) contains 1 COMPASS
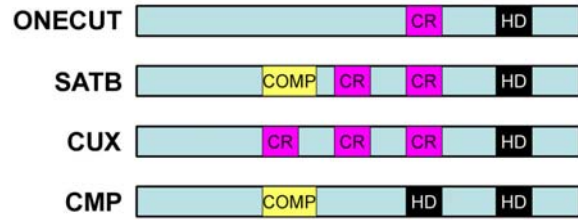
Figure 1. The Cut superclass family of homeodomain proteins. The Cut superclass homeodomain transcription factor family tree consists of the ONECUT family members, the SATB family members, the CUX family members, and the CMP family members. Motifs include cut repeats (CR), homeodomain (HD) and Compass domain (COMP).
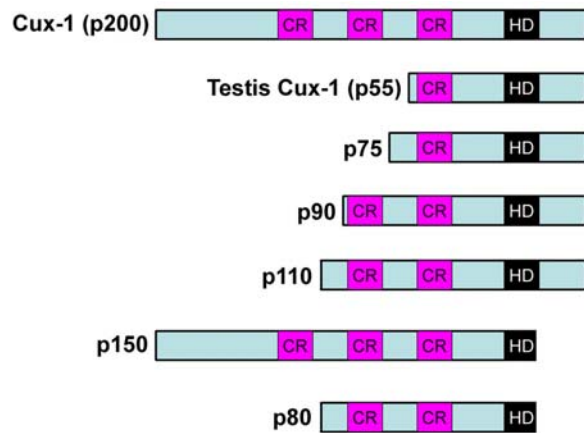
Figure 2. Structure of the Cux1 homeodomain proteins. The full-length Cux1 protein (p200) contains three cut repeat domains and the cut homeodomain. The testis specific Cux1 (p55) contains one cut repeat and the cut homeodomain (37). p75, contains one cut repeat and the cut homeodomain and is produced from an alternate promoter (38). p90, and p110 contain two cut repeats and the cut homeodomain and are products of proteolytic processing of p200 by a nuclear isoform of Cathepsin-L (41, 44). p150 is a product of C-terminal proteolytic processing within the homeodomain during mammary gland differentiation (42). p80 is a product of C-terminal proteolytic processing of p110 by a caspase (43).

domain, 2 cut repeat domains, and a homeodomain (25, 27). Cux proteins contain up to three cut repeats and a cut homeodomain, characterized by a histidine at position 50 (25, 28-33). And finally, the COMPASS family is included in the cut superclass, based on its similarity to the SATB family genes (25, 34-36). A figure showing the relationship between these family members is shown in Figure 1. This review will focus on the Cux class of cut related proteins.

There are two mammalian homologues of cut that have been identified in human (29), mouse (30, 32), dog (28), and rat (31). There is a confusing list of names that have been used to describe the cut genes, including
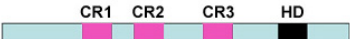
Cutl1 (<u>Cut </u>like homeobox gene <u>1</u>) and Cutl2, CDP (<u>C</u>CAAT <u>D</u>isplacement <u>P</u>rotein) and CDP2, Cux1 (<u>Cut</u> like homeob<u>ox</u> gene <u>1</u>) and Cux-2, and Clox (<u>C</u>ut <u>l</u>ike homeob<u>ox</u> gene). Recently, the Human Genome Organisation (HUGO) Gene Nomenclature Committee (HGNC) proposed changing the gene root for cut like homeobox genes to CUX1 and CUX2 for the human genes and Cux1 and Cux2 for the mouse orthologs. Since this review is focused on the murine mouse models of the cut homologues, we will use the Cux1 and Cux2 designations throughout this review. In addition to Cux proteins containing three cut repeat domains and the cut homeodomain, a number of truncated Cux proteins have been identified. These include a testis specific Cux1, which contains one cut repeat and the cut homeodomain (37), p75, a protein similar in structure to testis Cux1, that is produced from an alternate promoter (38), and CASP (Cut Alternately Spliced Protein), which contains amino terminal sequences from the gene, but none of the cut repeats or the homeodomain (39). CASP is a golgi protein (40), while testis Cux1 and p75 are nuclear proteins. In addition, there are at least four Cux1 proteins that appear to be products of proteolytic processing: p80, p90, p110, and p150 (41-45). The Cux1 proteins are diagrammed in Figure 2.

3. GENETICALLY ENGINEERED MOUSE MODELS OF CUX1

There have been 5 mouse models of *Cux1* that have been generated to date. Three gene targeting models have been generated to inactivate the *Cux1* gene: *Cux1^{tm1Ejn}* (Cux1ΔCR1) (46), *Cux1^{tm2Ejn}* (cux/CDP/ΔHD (47), CDP/Cux ΔC (48)), and *Cux1^{lacZ}* (49). In addition, two transgenic models were generated to overexpress the *Cux1* gene: *CMV/Cux1* Transgenic (17) and Cux1/p75 transgenic (50). The five models are summarized in Figure 5. Several common themes have emerged pointing to a role(s) for Cux1 in normal development and disease from the study of these mice and they will be presented in this review. In general, all of the Cux1 mutant mice displayed defects in growth regulation, with over expression of Cux1 resulting in organomegaly, and deletion of Cux1 resulting in growth retardation. Additional phenotypic changes that were described included defects in fertility, urogenital development, and hair growth and differentiation. We will begin by discussing the gene targeting models to knock out *Cux1*.

3.1. Cux1^{tm1Ejn} (Cux1ΔCR1) mice

Tufarelli *et al* designed a targeting construct to replace the first cut repeat (CR1) with a neomycin resistance cassette (46). Their goal was to introduce a nonsense mutation after position 1319 of the 4.5-kb reading frame of *Cux1* to generate a truncated product of ~60 kDa with this construct. However, the resulting mice expressed a mutant form of the protein with an internal deletion of 246 amino acids encompassing the first cut repeat domain, while the CR2, CR3, HD and C-tail of Cux1 remained intact. (46). The mRNA produced from the mutant allele was missing two exons, the exon encoding the first cut repeat and the subsequent exon. While at least two alternately spliced Cux1 transcripts have been identified

Model	Mutant Protein Structure	Reported Phenotype

Figure 3. Murine models for Cux1. There have been five genetically engineered mouse models for Cux1. The structure of the resulting Cux1 protein, as it exists within each mouse model, is represented next to the observed phenotype.

that are missing the second of these two exons, no endogenous transcript has been identified that is missing the exon encoding cut repeat 1 (51). This deletion, termed Cux1ΔCR1, resulted in mice displaying a relatively mild phenotype characterized by curly vibrissae and wavy hair. The authors observed that offspring of homozygote Cux1ΔCR1 females had a high degree of mortality. However, there was no correlation between the genotype of the pups and their survival. Moreover, the lethality was rescued by fostering litters of mutant females to lactating CD1 females. While there were decreased levels of ε-casein in the milk of homozygous mutant females at day 3 of lactation, by day 8 the levels were the same as in the wild-type milk. The decrease in one subpopulation of caseins could not explain the high degree of lethality, and histological analysis of mammary glands revealed no observable differences between Cux1ΔCR1 and wild-type lactating females. Thus, the cause of the lactation defect in Cux1ΔCR1 mutant mice remains unknown.

The levels and distribution of Cux1ΔCR1 mRNA appeared to be similar to that of wild-type mice. Since the mutation was not expected to affect the promoter, this was not surprising. However, the Cux1ΔCR1 protein was present at higher levels than wild-type Cux1 protein in spleen and thymus. These results are intriguing and raise the possibility that the mutant mRNA is translated more efficiently than the wild type, or that absence of cut repeat 1, or other sequences within the deleted region, alter the stability of the protein. In addition, the Cux1ΔCR1 protein appeared to exhibit altered DNA binding characteristics compared to the wild type Cux1 protein. In electromobility shift assays, the Cux1ΔCR1 protein was less readily competed off gp91-phox DNA promoter sequences with unlabeled oligonucleotide then the wild type Cux1 protein.

The relatively restricted phenotype of the Cux1ΔCR1 mice was also intriguing. Expression studies of Cux1 have indicated high levels of expression during the development of most tissues that correspond to regions of cell proliferation (15). The relatively mild phenotype observed in the Cux1ΔCR1 mice suggested that only select tissues required the first cut repeat. This supported a hypothesis that although Cux1 is expressed ubiquitously, it may function in a tissue specific manner (52). In this model, the four DNA binding domains were proposed to interact in different combinations to regulate the expression of various target genes in different tissues, or in different

stages of development. This was based on *in vitro* studies demonstrating that different combinations of the cut repeats, and/or homeodomain, were capable of, or required to, bind to specific DNA target sequences (52-57). The Cux1ΔCR1 phenotype suggested that cut repeat 1 (CR1) was required for hair and vibrissae development, but not for other developmental processes where Cux1 was known to be expressed. Thus, the Cux1ΔCR1 mutation was described as a hypomorphic allele.

3.2. Cux1^{tm2Ejn} mice

Because the Cux1^{tm1Ejn} (Cux1ΔCR1) mouse was unexpected, and the strategy did not result in a Cux1 knockout mouse, another Cux1 knockout strategy was employed. A targeting construct was designed to delete a 4kb region that included portions of exons 20 and 21 encoding the homeodomain of Cux1. A stop codon was introduced within exon 20 to force premature translational termination at the beginning of the homeodomain, resulting in deletion of the C-terminus of Cux1 including the homeodomain while retaining CR1, CR2 and CR3 domains (47-48). This mutation, called Cux1^{tm2Ejn}, was generated in the laboratory of Dr. Ellis J. Neufeld and subsequently described by two different groups under two different names: cux/CDP/ΔHD (47) and CDP/Cux ΔC (48). Surprisingly, the two groups described slightly different phenotypes for this mutant mouse.

Immunofluorescent labeling revealed that a mutant protein was expressed from the Cux1^{tm2Ejn} targeted allele that was more abundant in the cytoplasm than in the nucleus (47). Moreover, Cux1 functions as the DNA binding subunit of the transcription complex HiNF-D to regulate the expression of histone genes (58-59). This DNA binding activity of HiNF-D was lost in nuclear extracts derived from homozygous mutant Cux1^{tm2Ejn} mice. Thus, the authors concluded that deletion of the C-terminus containing HD, results in a molecular phenotype reflected by the absence of Cux1 containing protein/DNA complexes.

The Cux1^{tm2Ejn} mutation resulted in mice with a more severe phenotype than Cux1^{tm1Ejn} mutant mice. Homozygous mutant Cux1^{tm2Ejn} pups appeared similar to their littermates at birth. The mutant pups nursed normally, and milk was observed within their stomachs during the first couple of days after birth, but they failed to thrive. By the end of the first post-natal week of life, many Cux1^{tm2Ejn} pups were smaller than their littermates. To determine whether the Cux1^{tm2Ejn} runted phenotype resulted from defective nutrient absorption within the gastrointestinal tract, morphological analysis was preformed. These studies revealed no obvious structural abnormalities in the small intestine of mutant mice. Moreover, no abnormalities were observed when other organs of the gastrointestinal (GI) tract were examined, such as the stomach and the colon. Therefore, the authors concluded that the Cux1^{tm2Ejn} mutation did not affect development of these organs. However, a repressor element within the sucrase isomaltase (SI) gene has been identified that is regulated by Cux1 (60). SI is expressed at relatively low levels early in development, but is significantly upregulated in the intestinal epithelium of the small intestine at the transition from suckling to weanling. Cux1 binds to and represses SI expression during the early stages of intestinal development, but upon down regulation of Cux1, SI is expressed. Consistent with this, the Cux1^{tm2Ejn} mutant mice showed increased SI expression in the intestine at early postnatal ages. The disruption of normal SI expression possibly reflects a disruption in intestinal development and/or function that could partially account for the growth defects in the Cux1^{tm2Ejn} mutant mice.

While Cux1^{tm2Ejn} mice were born at the expected mendelian frequency, most of the Cux1^{tm2Ejn} pups died within the first 10 days of postnatal life, and more than 70% of pups failed to survive to weaning age. The homozygous mutant mice that did survive to adulthood had a normal life span but were severely growth retarded weighing 30 to 50% less than their normal littermates, were very susceptible to bacterial infections, and had severe rhinitis.

Similar to the Cux1^{tm1Ejn} mice, Cux1^{tm2Ejn} mice had abnormal coat hair, although the phenotype was more severe. While the hair phenotype in the Cux1^{tm1Ejn} mice diminished with age, the surviving Cux1^{tm2Ejn} mice were completely bald by one month of age. Moreover, vibrissae were absent from the Cux1^{tm2Ejn} mice, while just curly or kinky shaped in the Cux1^{tm1Ejn} mice. Regrowth of the coat hair occurred over several months, but the hair was abnormal, with a gray appearance, and did not show the multiple hair types found in wild type mice. A striking finding was significantly reduced fertility in the male Cux1^{tm2Ejn} mice. It had previously been shown that the truncated testis specific Cux1 protein was absent from the testes of atrichotic (at/at) mice (37). These mice, in addition to having no hair, exhibited an absence of germ cells (61). Analysis of the testis specific transcript confirmed that this Cux1 mRNA species corresponded to spermatogenesis, and *in situ* studies localized the transcript to round spermatids. However, the infertility in the Cux1^{tm2Ejn} mice did not appear to result from testicular abnormalities, since the testis weight was comparable between wild type, heterozygote and homozygote mice. Moreover, there did not appear to be any morphological disruptions in spermatogenesis. Thus, spermatogenesis does not appear to be dependent on the presence of the testis Cux1 protein. Of interest, was a significant reduction in testosterone in the Cux1^{tm2Ejn} mice. However, this could not account for the reduced fertility, since heterozygous mice showed the same reduction in testosterone. Rather, the reduced infertility could result from disruptions in the pheromone sensory pathways, or from severe rhinitis that was observed in the mice.

Sinclair *et al* observed a lower than expected mendelian frequency of ~5% in the homozygous Cux1^{tm2Ejn} mice (47). However, when embryos were examined at 16 to 17 days post coitus, the normal mendelian frequency was observed, indicating that the homozygous mice were dying at birth. This is in contrast to the observations of Luong *et al* in which the Cux1^{tm2Ejn} mice survived to a week of age (48). Sinclair *et al* described a similar phenotype of wavy

hair and whiskers, balding between 2 and 3 weeks of age, and runting in the surviving mice. Interestingly, Sinclair *et al* also described a sexual dimorphism, as nearly all surviving *Cux1^{tm2Ejn}* mice were males. The *Cux1^{tm2Ejn}* mice also exhibited a muscle wasting phenotype, which was only observed in the homozygous animals and therefore unlikely to be related to the testosterone deficiency. The *Cux1^{tm2Ejn}* mice were generated in the laboratory of Dr. Ellis J. Neufeld and subsequently given to these two groups for analysis. However, Sinclair *et al* backcrossed the mutation to the C57BL/6 strain, suggesting that the differences observed in the *Cux1^{tm2Ejn}* mice between these two groups result from genetic background. Sinclair *et al* also generated the *Cux1^{tm2Ejn}* mutation on a RAG-2 deficient background to evaluate lymphoid differentiation.

The most striking phenotypic change was a decrease in B and T lymphocytes, with a dramatic reduction in the size of the thymus, with a particular loss of CD4(+)CD8(+) thymocytes. This was determined to result primarily from apoptosis. However, it was not clear whether this was induced by an overexpression of death inducing factors, or an absence of survival factors from the thymic stroma. One such factor might be TNF, which was increased in the thymus of *Cux1^{tm2Ejn}* mice. Moreover, TNF could also partially explain the wasting phenotype (62-63). Thus, TNF could be a candidate target of Cux1 repression. To directly test this concept, the authors crossed the *Cux1^{tm2Ejn}* mutation onto a TNF null background. The *Cux1^{tm2Ejn}* mutation on a TNF-/- background showed the same gross phenotype (curly hair, hair loss, curly whiskers, muscle wasting, smaller size). However, there was a partial rescue of thymocyte cell numbers (Jamie Lee, Angus Sinclair and Richard Scheuermann, written communication, May 2007). Thus, Cux1 may function to regulate normal hematopoiesis, possibly by modulating the expression levels of survival and/or apoptosis factors, such as TNF, within a given microenvironment.

3.3. Cux1^{lacZ} mice

The Cux1 gene was also targeted by Ellis *et al* (49). In contrast to the *Cux1^{tm2Ejn}* mice, this targeting construct was designed to disrupt the third cut repeat in addition to the homeodomain. This was expected to result in a deletion of domains essential for all Cux1 DNA binding activity (23), including that of the testis specific Cux1 protein (23, 37). *Cux1^{lacZ}* mutant mice were born at a normal mendelian frequency, but similar to *Cux1^{tm2Ejn}* mice, died shortly after birth because of delayed differentiation of lung epithelia. When the *Cux1^{lacZ}* mutation was placed on an outbred genetic background, there was a less pronounced delay in lung development, permitting the mice to survive beyond birth. Similar to the *Cux1^{tm2Ejn}* mutation, the *Cux1^{lacZ}* mutation resulted in the expression of a truncated Cux1 protein product that was localized in the cytoplasm. In addition, while the wild type Cux1 protein repressed an Sp1 containing promoter/luciferase construct, a known Cux1 target, the mutant protein failed to do so (64). Moreover, the mutant protein did not interfere with the repression by the wild type protein. Thus, similar to the *Cux1^{tm2Ejn}* mutation, the

*Cux1^{lacZ}* mutation appears to generate a reduced or non-functional protein.

An advantage of the targeting approach used to generate the *Cux1^{lacZ}* mutant mice, was the inclusion of the *lacZ* gene allowing the expression of Cux1 to be followed by β-galactosidase staining. This revealed broad expression of Cux1 in a number of different tissues, as had been described previously. However, the *Cux1^{lacZ}* mice did not show phenotypic changes in all of these tissues. In particular, in the testes there was distinct β-galactosidase activity in the round spermatids, with less in the elongating spermatids, but the mutant mice did not show a testis phenotype, regardless of the fact that the exons encoding the third cut repeat and homeodomain were deleted in this model. While there may have been some activity in the *Cux1^{tm2Ejn}* mice, since the third cut repeat remained, no testis Cux1 activity would be predicted in the *Cux1^{lacZ}* mice. In addition, male *Cux1^{lacZ}* mice on an outbred background did not show reduced fertility. This seems to further support the notion that the testis Cux1 protein is not required for spermatogenesis.

Similarly, β-galactosidase activity was observed in the developing kidney, although no renal phenotype was described.

An examination of hair follicle morphogenesis in the *Cux1^{lacZ}* mice revealed abnormal differentiation of the inner root sheath (IRS). This was associated with deregulated expression of Sonic hedgehog, suggesting that Cux1 regulates Shh expression in the IRS. Interestingly, β-galactosidase staining was expanded in the mutant hair follicles, suggesting that Cux1 can regulate its own expression. This is unlikely to be a direct regulation, however, and may be via the activation of a non-cell-autonomous signal. This may be similar to the regulation of cut in Drosophila by the Notch signaling pathway, where cut represses the ligands of Notch (11-12, 23, 65-79). Downregulation of Notch ligands results in reduced Notch signaling and the subsequent down regulation of cut. The expression of Notch1 in the developing hair follicle of the *Cux1^{lacZ}* mice was not different from wild type. This would be expected if Cux1 expression is regulated by Notch signaling, and is consistent with recent studies demonstrating that cut regulation by Notch is conserved across species (20, 80).

3.4. CMV/Cux1 Transgenic mice

An analysis of Cux1 expression during murine kidney development revealed that expression was highest in the nephrogenic zone, a region at the periphery of the kidney where new nephrons are being formed (15). This is a site characterized by high cell proliferation, as well as, extensive changes in cell morphology associated with the conversion of the metanephric mesenchyme to the epithelium that will compose the nephron (81). As nephrogenesis continues, cells exit the cell cycle, and terminal differentiation occurs. Strikingly, a sharp down regulation of Cux1 expression corresponded to the region where cells were exiting the cell cycle. While mature kidneys show very little cell proliferation, a number of
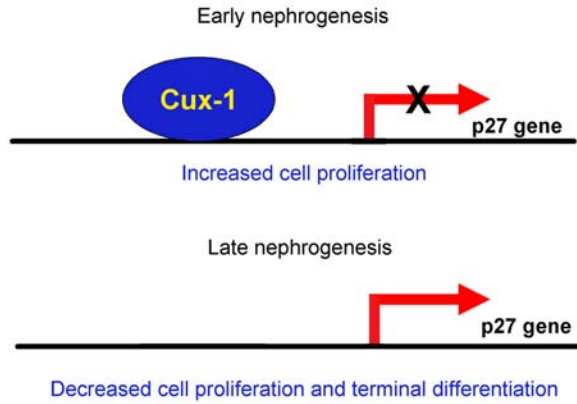
Figure 4. Cux1 regulation of p27 during kidney development. Cux1 is highly expressed in the early stages of nephrogenesis, where expression is associated with cell proliferation and repression of p27. At later stages of nephrogenesis, Cux1 is down regulated, which is permissive for p27 to be expressed resulting in cell cycle arrest and terminal differentiation.

disease states are characterized by cell cycle re-entry. One such disease is polycystic kidney disease, where cysts arising from the nephron are characterized by increased mitotic indices, and other phenotypic changes (82-83). When Cux1 expression was analyzed in the cpk mouse model of polycystic kidney disease, an increased and ectopic expression was observed (15). This suggested that the down regulation of Cux1 was necessary for normal kidney development. To test this hypothesis, transgenic mice expressing the full-length Cux1 cDNA under the control of the CMV immediate early gene promoter were generated (17). Based on the expression of Cux1 in the developing kidney, we anticipated that the ectopic expression of Cux1 would prevent maturation of the kidney, or perhaps induce cystogenesis. Surprisingly, the overexpression of Cux1 resulted in transgenic mice with multiorgan hyperplasia and organomegaly. Moreover, the enlarged organs retained a normal structure. The most extensive analysis was performed on the kidney. However, organomegaly was also observed in heart, testis, spleen, seminal vesicle and liver.

Kidneys from adult Cux1 transgenic mice exhibited a significant increase in the size of the renal cortex. However, there was not an overall increase in nephron number. Rather, the increased size of the kidneys resulted from an overall increase in the total number of cells in both the tubules and glomeruli, which correlated with an increase in cell proliferation. Important observations made by Coqueret *et al*, were the first to describe a mechanism by which mammalian cut proteins could regulate the cell cycle (84). They showed that Cux1 could repress the expression of the cyclin kinase inhibitor p21 in a cell cycle dependent manner (84). However, p21 knockout mice did not exhibit a hyperplastic phenotype, suggesting that repression of p21 by the ectopically expressed Cux1 was not resulting in the hyperplastic phenotype (85). Rather, the related cyclin kinase inhibitor

p27 was downregulated in CMV/Cux1 transgenic mice. Moreover, p27 knockout mice exhibited a multiorgan hyperplastic phenotype that was similar to the Cux1 transgenic mice (86-88). In addition, in a reporter assay, Cux1 protein repressed p27 promoter activity in a concentration dependent manner, implicating p27 as a potential target of Cux1 repression (17).

Proliferating cells were observed in both the tubules and glomeruli of kidneys from adult transgenic mice. In addition, the normal squamous epithelium that lined Bowman's capsule of the glomerulus was replaced with proximal tubule epithelium. Initially, this was interpreted as a homeotic mutation, converting the squamous epithelium of a more proximal structure to the cuboidal epithelium of a more distal structure. However analysis of developing kidneys showed that this was not the case. Rather, proliferating proximal tubule epithelium replaced the epithelium of Bowman's capsule. The multiorgan hyperplasia observed in the CMV/Cux1 transgenic mice partially phenocopied p27 knockout mice, and together with the important observations by Coqueret *et al*, showing that Cux1 repressed the cyclin kinase inhibitor, p21, led to the observation that Cux1 also represses p27. A model for the role of Cux1 in regulating cell proliferation in nephrogenesis is shown in Figure 4.

We found that liver hepatomegaly within CMV/Cux1 transgenic mice correlated with an increase in cell proliferation. In addition, the increase in Cux1 expression in transgenic livers was associated with a decrease in p21, but not p27, expression. Within transgenic livers, Cux1 was ectopically expressed in a population of small cells, but not in mature hepatocytes, and many of these small cells expressed markers of proliferation. Transgenic livers showed an increase in alpha-smooth muscle actin, indicating activation of hepatic stellate cells, and an increase in cells expressing chromogranin-A, a marker for hepatocyte precursor cells (89-91). Morphological analysis of transgenic livers revealed inflammation, hepatocyte swelling, mixed cell foci, and biliary cell hyperplasia. These results suggest that increased expression of Cux1 may play a role in the activation of hepatic stem cells, possibly through the repression of the cyclin kinase inhibitor p21.

3.5. p75 Transgenic Mice

A recent finding in the field of Cux1 biology was the identification of a nuclear isoform of Cathepsin-L that processes Cux1 in S-phase of the cell cycle (41). This exciting finding describes an internal start codon with the cathepsin-L mRNA that generates a truncated cathepsin-L protein. This isoform does not include the signal peptide, and its absence reveals a cryptic nuclear localization signal that directs it to the nucleus where it processes the full-length Cux1, a 200 kD protein, into a 110 kD protein that includes the second and third cut repeats and homeodomain, but lacks the amino-terminal domains. An additional Cux1 isoform of 90 kD, was recently described as also arising from cathepsin-L cleavage of the full length Cux1 protein (44). While the full length Cux1 protein engages in a rapid and transient repression of target genes,

the p110 protein is thought to stably bind DNA and both activate and repress gene expression, and can activate a number of genes upregulated in S-phase (45, 54, 92-93). While the absence of Cux1 delayed progression into S-phase of the cell cycle, increased expression of the p110 Cux1 isoform accelerated progression into S-phase (94). Moreover, the expression of cyclins A2 and E2 was dramatically increased by p110 and decreased in its absence. In addition, the expression of p27 was slightly decreased and the expression of DNA polymerase a slightly increased (94). These studies, together with the knockout and transgenic phenotypes, have established the role of Cux1 as a cell cycle regulator.

In addition to the p200, p110, and p90 Cux1 isoforms, a fourth Cux1 isoform, termed p75, was found to result from an alternative mRNA. The p75 isoform, which contains the CR3 and HD domains, is expressed primarily in the thymus and placenta (38). Interestingly, p75 expression is highest in CD4 (+)CD8 (+) T cells (38), which may explain the loss of these cells by apoptosis, observed in the $Cux1^{tm2Ejn}$ mutant mice. While the expression of p75 is tissue restricted normally, it is ectopically expressed in human breast cancers (38). Moreover, there was a statistically significant association between invasive tumors and p75 expression. To test the hypothesis that p75 expression may cause breast cancer, transgenic mice were generated ectopically expressing p75 under the direction of the mouse mammary tumor virus long terminal repeat (50). This construct was inserted into the hprt locus in ES cells, which were injected into blastocysts. Surprisingly, a third of these mice developed splenomegaly, hepatomegaly, and the infiltration of white blood cells into the kidneys and lungs. However, there was a low incidence of tumors in the mammary glands that may be explained by the weak expression of the p75 transgene in the mammary gland, despite the use of the MMTVLTR enhancer/promoter. The leukemia observed in the affected p75 transgenic mice was primarily neutrophilic and nonlymphoid. In the same study, the MMTVLTR enhancer/promoter was used to drive the expression of the p110 protein in transgenic mice. In contrast to p75, no hematopoeitic disorders were observed in these mice. In addition, the leukemia could not be transferred by transplanted splenocytes into irradiated mice. Thus, while the expression of p75 appeared to play a causative role in the leukemia, the activation of the transgene may require another event. This was supported by the fact that only 20 of 60 mice developed the myeloid proliferative phenotype. It is also interesting that splenomegaly and hepatomegaly were observed in both the CMV/Cux1 and Cux1 p75 transgenic mice. However, there are some important differences. The hepatomegaly observed in the CMV/Cux1 transgenic mice was 100% penetrant (89). Moreover, the hepatocellular phenotype resulted from in increase in intrinsic liver cells, and not from an infiltration of leukocytes (89).

4. CUX1 AS CELL CYCLE REGULATOR

Collectively, the examination of the different Cux1 mutant mouse models provides insight into its function during development and disease. A common phenotype observed in all of the Cux1 models was the regulation of growth. In the case of the $Cux1^{tm2Ejn}$ and the $Cux1^{lacZ}$ mice, this was evident in the smaller size of the mice resulting from growth retardation (47-49). Conversely, the two transgenic models exhibited organomegaly of some organs, resulting from hyperplasia of the endogenous cells of the kidney and liver (17), or from the infiltration of proliferating myeloid cells into the kidney, liver, and spleen (50). Together with *in vitro* studies, the transgenic mouse models indicate that increased Cux1 expression promotes progression through the cell cycle. While the full-length Cux1 protein, p200, is capable of repressing p21 and p27, expression of the truncated p110 Cux1 protein in cells is capable of accelerating entry into S-phase by the activation of cyclin A2 and, possibly, cyclin E2 (94). Renal mesangial cells isolated from the CMV/Cux1 transgenic mice similarly exhibit accelerated progression into S-phase, compared to mesangial cell isolated from wild type mice (95). In contrast, initial examination of cell proliferation in fibroblasts isolated from $Cux1^{tm2Ejn}$ or $Cux1^{lacZ}$ mutant mice revealed no differences in cell cycle progression in Cux1 mutant mice (47, 49). Rather, an increase in apoptosis in the thymic and bone marrow lymphoid populations of $Cux1^{tm2Ejn}$ mice was observed, although this was thought to result from changes in the tissue stroma, since transplantation of mutant bone marrow into wild type mice had normal thymus size (48). The runted phenotype in the $Cux1^{tm2Ejn}$ and $Cux1^{lacZ}$ mutant mice, nevertheless, suggested that cell growth was disrupted. This was confirmed by Sansregret *et al*, who performed a comprehensive analysis of cell cycle progression in fibroblasts isolated from embryonic $Cux1^{lacZ}$ mice, which revealed that the absence of Cux1 delayed the progression into S-phase, compared to wild type cells (94). Thus, a disruption of cell cycle progression occurred in Cux1 mutant cells, and this was associated with a decrease in Cyclin E expression.

5. CUX1 IN KIDNEY DEVELOPMENT

In addition to the tubular hyperplasia in the kidneys of the CMV/Cux1 mice, we found that increased expression of Cux1 in mesangial cells resulted in cell proliferation and mesangial expansion (18). This was consistent with studies demonstrating that decreased expression of p27 results in increased mesangial cell proliferation in culture, and reduced renal function in glomerulonephritis (96-97). We also observed a disruption in podocyte architecture that may have contributed to the albuminuria observed in the Cux1 transgenic mice (18). Whether the podocyte foot process effacement resulted from aberrant signaling between the mesangial cells and podocytes, or form mechanical stress, is not known. However, it is clear that increased expression of Cux1 is sufficient to induce the early events of mesangioproliferative glomerulonephritis. Given the expression pattern of Cux1 in the developing kidney (15), and the dramatic changes in cell proliferation in the kidneys of the CMV/Cux1 transgenic mice (17), it was surprising that no kidney phenotypes were reported for any of the

Cux1 targeted mice. During kidney development, maturing nephrons proceed through an orderly sequence of developmental stages that can be discerned morphologically. These stages are renal vesicle (stage I), comma- and S-shaped body (stage II), developing capillary loop (stage III), and maturing glomerulus (stage IV) (98-101). Cux1 is most highly expressed in stages I-III, and is down regulated after the developing capillary loop stage. Recently, an examination of kidney development in the *Cux1^{tm2Ejn}* mice revealed disruptions in nephrogenesis (102). While nephrogenic stages I and II were normal, developing capillary loop staged nephrons had significantly fewer cells than wild type. Moreover, the missing cells were the presumptive endocapillary cells that will develop into mesangial cells. Together with the increase in mesangial cell number observed in the CMV/Cux1 transgenic mice, a role for Cux1 in mesangial cell differentiation, proliferation, and/or survival can be postulated. Although Cux1 is expressed at earlier stages in the forming nephron, no abnormalities were observed. The most likely explanation for this is compensation from other cut related genes, such as Cux2 (103). Cux2 expression has been observed in the early developing genitourinary tract supporting this notion. Alternatively, the *Cux1^{tm2Ejn}* mutation may generate a partially functional protein permitting the early events in kidney development to occur. Thus, the role of Cux1 in early nephrogenesis remains to be defined.

A rather striking finding was the ability of ectopically expressed Cux1 to induce cell proliferation in the kidneys of CMV/Cux1 transgenic mice, without disrupting differentiation (17). The expression pattern of Cux1 in the developing kidney suggested that Cux1 must be down regulated for normal renal tubule differentiation to take place. However, the kidneys of CMV/Cux1 transgenic mice were structurally normal, indicating this was not the case. Although cell cycle arrest and differentiation seem to be coupled in the developing kidney, the continued cell proliferation in the adult kidneys of CMV/Cux1 transgenic mice suggests that cell proliferation can be unlinked from differentiation. Moreover, this suggests that terminally differentiated renal tubule cells have the capacity to re-enter the cell cycle without first undergoing a de-differentiation event. This has important implications for kidney disease, including polycystic kidney disease.

6. CUX1 IN MOUSE MODELS OF POLYCYSTIC KIDNEY DISEASE

In addition to the "loss of function" and "gain of function" mutations of the Cux1 gene itself, the examination of Cux1 in other mutant mice has provided additional insight into Cux1 function. Initially, it was observed that Cux1 expression is expanded in the cpk mouse model of polycystic kidney disease (15). As the genes mutated in autosomal dominant polycystic kidney disease (ADPKD) and autosomal recessive polycystic kidney disease (ARPKD) have been identified (for reviews on PKD see 82-83), increasing efforts have been undertaken to identify the function of these gene products. One of these genes, Pkd1, which encodes polycystin 1, has

been found to regulate cell cycle progression. In a landmark paper, Bhunia *et al*, showed that polycystin-1 signals through the Jak-Stat pathway to upregulate the expression of the cyclin kinase inhibitor p21, in a Pkd2 dependent manner (104). Mutations in Pkd1, such as those observed in ADPKD patients, would then be expected to result in a decrease in p21 and increased cell proliferation. Since p21 is a target of repression by Cux1, the role of Cux1 in PKD was further investigated. In addition to the cpk model, it was observed that Cux1 expression is expanded in the Pkd1 null mouse model of PKD (21). However, the pattern and timing of expression in these two mouse models is very different. In the cpk model, Cux1 is not abnormally expressed until very late, when cysts are large and encompass most of the kidney. At this stage, the increased expression of Cux1 is associated with an increase in apoptosis. In contrast, Cux1 is highly and abnormally expressed at very early stages of cystogenesis in the Pkd1 null mouse model. In fact, increased expression of Cux1 may precede cyst development, with expression in normal differentiated appearing tubules in the cystic kidney (21). However, the increased expression of Cux1 does not cause cysts, as evidenced by the absence of cysts in the CMV/Cux1 transgenic mice. Rather, the upregulation of Cux1 may be necessary for cyst growth. The availability of Cux1 and Pkd1 mutant mice will allow this to be tested directly. In contrast, it was recently shown that transgenic mice ectopically expressing the p75 isoform of Cux1 using the CMV immediate early gene promoter develop renal abnormalities including renal tubule hyperplasia and cystic dilations with long latency (105).

7. CUX1 IN CALCINEURIN MUTANT MICE

The transgenic mouse models suggest that increased expression of Cux1 may contribute to the deregulated cell proliferation observed in diseases such as cancer and polycystic kidney disease. Thus, understanding the mechanisms of Cux1 regulation may lead to a better understanding of these diseases. In addition to the proteolytic processing of Cux1 by Cathepsin-L, Cux1 function also appears to be regulated by phosphorylation and dephosphorylation. A number of serine/threonine kinases have been identified that phosphorylate specific sites in the cut repeat domains of Cux1. Phosphorylation of Cux1 by Casein Kinase II, Protein Kinase C, cyclin A/Cdk1, and cAMP mediated Protein Kinase A has been demonstrated *in vitro* and *in vivo* (106-109). Phosphorylation of Cux1 reduces DNA binding, while dephosphorylation by cdc25A phosphatase increases Cux1 DNA binding ability (84).

The serine/threonine phosphatase calcineurin is composed of the catalytic subunit, Calcineurin A (CnA), and the regulatory subunit, Calcineurin B (CnB) (110). Calcineurin phosphatase activity is initiated only when CnB is bound to both calmodulin and calcium. Two isoforms of CnA, CnA-α and CnA-β are widely expressed, while expression of a third isoform, CnA-γ, is restricted to testis and brain (111-112). CnA-α knockout mice have a reduction in the nephrogenic zone resulting from reduced cell proliferation and increased apoptosis, and an absence

of mesangial cells in the glomeruli that develop postnatally (113). The underlying mechanism appears to be the deregulated expression of the cyclin kinase inhibitor p27 (113-114). While Cux1 was expressed normally in the developing kidneys of CnA-α knockout mice, it did not localize to proliferating cells, suggesting that Cux1 function was inhibited in the absence of calcineurin (115). Moreover, ectopic expression of Cux1 rescued the growth inhibition in kidney organ cultures treated with the calcineurin inhibitor cyclosporin by repressing p27 (115). Thus, examining Cux1 activity in the context of additional mouse models has revealed a potential role for the phosphatase calcineurin in regulating Cux1 activity. Interestingly, three-week-old CnA-α knockout mice and six-month-old CnA-α heterozygous mice exhibit extracellular matrix expansion and reduced kidney function, associated with increased TGF-β (116). It has recently been shown that Cux1 is a downstream effector of TGF-β signaling, and is involved in regulating genes important for cell motility, invasion, and extracellular matrix expression (117-118). The ability of ectopic Cux1 expression to rescue calcineurin inhibition, suggests that Cux1 is required for early growth in the developing kidney, however, the CMV/Cux1 transgenic mice also demonstrate that increased Cux1 expression in fully mature kidneys leads to glomerulosclerosis and fibrosis. Thus, Cux1 may be a key factor in cyclosporin induced nephrotoxicity.

8. CONCLUDING THOUGHTS

Cux1 is a complex protein with multiple DNA binding domains that is highly expressed in multiple tissues during development. In the kidney, the expression of Cux1 is associated with cellular proliferation in the nephrogenic zone. Moreover, the ectopic expression of Cux1 results in renal hyperplasia, and Cux1 expression is increased in polycystic kidney disease. However, the ectopic expression of Cux1 does not result in cyst formation. This suggests that the increased expression of Cux1 may be required for cyst formation, a hypothesis that can be tested using available animal models, and/or that altered expression or function of Cux1 may modify cyst progression. In either case, understanding the mechanisms of Cux1 regulation may lead to new therapeutic targets for the treatment of PKD.

9. ACKNOWLEDGMENTS

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