Tumorigenesis and Neoplastic Progression

β -Catenin and K-RAS Synergize to Form Primitive Renal Epithelial Tumors with Features of Epithelial Wilms' Tumors

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Wilms' tumor (WT) is the most common childhood renal cancer. Although mutations in known tumor-associated genes (*WT1*, *WTX*, and *CATNB*) occur only in a third of tumors, many tumors show evidence of activated β -catenin–dependent Wnt signaling, but the molecular mechanism by which this occurs is unknown. A key obstacle to understanding the pathogenesis of WT is the paucity of mouse models that recapitulate its features in humans. Herein, we describe a transgenic mouse model of primitive renal epithelial neoplasms that have high penetrance and mimic the epithelial component of human WT. Introduction of a stabilizing β -catenin mutation restricted to the kidney is sufficient to induce primitive renal epithelial tumors; however, when compounded with activation of K-RAS, the mice develop large, bilateral, metastatic, multifocal primitive renal epithelial tumors that have the histologic and staining characteristics of the epithelial component of human WT. These highly malignant tumors have increased activation of the phosphatidylinositol 3-kinase–AKT and extracellular signal–regulated kinase pathways, increased expression of total and nuclear β -catenin, and increased downstream targets of this pathway, such as c-Myc and survivin. Thus, we developed a novel mouse model in which activated K-RAS synergizes with canonical Wnt/

β -catenin signaling to form metastatic primitive renal epithelial tumors that mimic the epithelial component of human WT. (*Am J Pathol* 2011, 179:3045–3055; DOI: 10.1016/j.ajpath.2011.08.006)

Wilms' tumor (WT) is an embryonal tumor of the kidney that is the most common childhood renal cancer and the fourth most common childhood malignancy overall.^{1,2} Modern multimodal management can cure 95% of patients with WT with the most favorable risk profile^{3–7} but at the cost of significant short- and long-term morbidity.^{8–11} In addition, there remains a persistent cohort of children who ultimately fail therapy and die.³ Consequently, the main research priorities for WT are to lower the toxicity while maintaining efficacy of existing therapy for lower-risk patients and to develop novel therapeutics for higher-risk patients. Achieving these goals depends on understanding the mechanisms underlying WT oncogenesis and progression.

WTs are triphasic, embryonic tumors that classically have varying amounts of blastemal elements, primitive mesenchymal stroma, and primitive epithelia. They are generally thought to arise from nephrogenic rests (ie, embryonic tissue) derived from the metanephric mesenchyme during renal development. The genetic aberrations underlying this process are known to be heterogeneous¹² and can include inactivating mutations of Wilms' tumor 1 gene (*WT1*),^{13,14} Wilms' tumor gene found on chromosome X (*WTX*),^{15–17} and stabilizing/activating mutations of β -catenin (*CTNNB1*).^{18,19} The precise mecha-

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nism whereby these alterations lead to WT has not been defined, but all these mutations are associated with increased canonical Wnt/ β -catenin signaling.^{18,20–22} In fact, recent work demonstrated that Wnt/ β -catenin is activated in a chemically induced rat model of WT.²³ Indeed, there is evidence of activation of the canonical Wnt/ β -catenin pathway in up to 75% of WTs with *WT1* mutations, and some studies have suggested that more than half of all WTs show Wnt activation.^{24–26} However, when observed simultaneously in a large cohort of WTs, mutations of *WT1*, *CTNNB1*, and/or *WTX* could account for no more than a third of cases.¹⁴ Therefore, the mechanism underlying canonical Wnt/ β -catenin activation and genetic aberrations underlying WT genesis in the remaining cases is not known.

In addition to WT, aberrations of the canonical Wnt/ β -catenin pathway have been described in tumors of multiple tissues, including colon, breast, liver, and prostate. This pathway plays a central role in transcriptional regulation that impacts cell growth, development, and differentiation.^{27,28} β -Catenin exists in the cytosol as either cadherin associated or in a free form. The amount of free cytosolic β -catenin is regulated by a complex containing the adenomatous polyposis coli (APC) protein, axin, WTX, and glycogen synthase kinase-3 β . When excess free cytosolic β -catenin is present, the complex phosphorylates its serine/threonine residues on the N-terminus, leading to its degradation by the proteasomal machinery of the cell. However, on a Wnt-related signal, this process is inhibited, allowing β -catenin accumulation in the cytoplasm and translocation to the nucleus, where it interacts with TCF/LEF family transcription factors to promote specific gene expression. In cancers, the proteasomal degradation of cytosolic β -catenin is impaired due to different etiologies. The best example is in familial adenomatous polyposis, where there is a functional loss of APC. Similarly, in the sporadic forms of WT associated with Wnt/ β -catenin signaling abnormalities, there are mutations in serine/threonine residues at the N-terminal of β -catenin that are critical for its degradation.¹⁸ *WT1* and *WTX* have been shown to negatively regulate canonical Wnt/ β -catenin signaling; therefore, inactivating mutations of these genes, as is seen in a subset of WTs, would be expected to activate this pathway.^{20–22} However, the mechanism(s) underlying abnormal Wnt/ β -catenin activation in cases without mutations in *WT1*, *CTNNB1*, or *WTX* is not defined as yet. Neither is it clear whether activation of this pathway is sufficient for induction of these tumors.

Signaling pathways mediated by tyrosine kinase receptors in response to growth factors, such as insulin-like growth factor, epidermal growth factors, and vascular endothelial growth factor, have also been implicated in WT pathogenesis.^{29–35} The RAS family of membrane-bound G-coupled proteins plays a critical role in activating a series of signaling cascades downstream of these receptors, including the mitogen-activated protein kinase (MAPK) pathways and the phosphatidylinositol 3-kinase (PI3K)/AKT pathway. Activation of the MAPKs has been associated with a variety of malignancies and extracellular signal-regulated kinase (ERK), and PI3K/AKT have been

shown to modulate canonical Wnt/ β -catenin activation through various context-dependent mechanisms.^{36–40} In addition, ERK activation was recently demonstrated in a subset of human WT and in the only other mouse model of WT.³⁵ Constitutively active K-RAS has been implicated in numerous human cancers, including pancreas, lung, brain, and colon, owing to its ability to activate these downstream pathways.

Based on the strong evidence that the Wnt/ β -catenin pathway is important in the development of WT in humans, we sought to test whether constitutive activation of this pathway in mice would lead to tumors that would mimic features of human WT. We show that a conditional stabilizing mutation of the *CNNB1* gene (loss of exon 3 in the N-terminal half of the β -catenin protein) restricted to the kidney during renal development is sufficient to induce primitive renal epithelial neoplasms with histologic features seen in human WT. In addition, we show that simultaneous activation of K-RAS and β -catenin in the developing nephron increases the number and size of these tumors and induces distant metastases. Activated K-RAS mediates these effects by increasing the amount of total and nuclear β -catenin and downstream targets of the canonical Wnt/ β -catenin pathway, such as survivin and c-Myc. Thus, we developed a mouse model of spontaneously occurring primitive renal epithelial neoplasms that have high penetrance and closely mimic the features of the epithelial component of human WT.

Materials and Methods

Mice

Mice harboring γ GT-Cre recombinase were a gift from Dr. Eric Neilson (Vanderbilt University Medical Center, Nashville, TN).^{41–44} Mice with the tamoxifen-inducible Cited1-Cre recombinase (Cited1-CreER^{T2}) were developed as previously described.⁴⁵ Mice with a conditional activating mutation of *CNNB1* in which exon 3 is flanked by lox sites (Catnb^{lox(ex3)}) were a gift from Dr. Makoto M. Taketo (Kyoto University, Kyoto, Japan).⁴⁶ Mice with a conditional activating mutation of *Kras* (alias *Ki-ras*) (LSL-Kras^{G12D}) were obtained from Tyler Jacks (Massachusetts Institute of Technology, Cambridge, MA).⁴⁷ All the mice were bred and housed at Vanderbilt University Medical Center under an Institutional Animal Care and Use Committee-approved protocol. Mice were crossed to obtain mice with genotypes γ GT-Cre/Catnb^{+/lox(ex3)} (referred to as G-Catnb ^{Δ ex3}), γ GT-Cre/Kras^{+/G12D} (referred to as G-Kras^{G12D}), γ GT-Cre/Kras^{+/G12D}/Catnb^{+/lox(ex3)} (referred to as G-Kras^{G12D}/Catnb ^{Δ ex3}), and littermate controls. Similar breeding schemes were used to generate the same genotypes with Cited1-CreER^{T2} rather than γ GT-Cre (referred to as C-Catnb ^{Δ ex3}, C-Kras^{G12D}, and C-Kras^{G12D}/Catnb ^{Δ ex3}, respectively) and littermate controls. Tamoxifen (100 μ L of 15 mg/mL, dissolved by sonication in 10% ethanol/90% sunflower oil) was injected i.p. to the nursing mother starting on the day of birth and continued for 5 consecutive days as previously described.⁴⁵ For control litters, the equivalent volume of

diluent was injected. The number of generations of mixed breeding was capped at four; thus, all the data represent no more than the N4 generation of mixed breeding.

Antibodies

Antibodies used for immunohistochemical (IHC) analysis and/or Western blot analysis were as follows: Ki-67 and S-100 (Dako, Carpinteria, CA), Pax-2 (Covance, Princeton, NJ), Pax-8 (Proteintech Group Inc., Chicago, IL), c-Myc (Epitomics Inc., Burlingame, CA), actin (Sigma-Aldrich Corp., St. Louis, MO), WT-1 (Leica Microsystems GmbH, Wetzlar, Germany), CD56/neural cell adhesion molecule (Invitrogen, Carlsbad, CA), SALL4 (Abnova, Taipei, Taiwan), and p-ERK, p-AKT, p-P38, β -catenin, survivin, and cyclin D1 (Cell Signaling Technology Inc., Beverly, MA).

Histologic and IHC Analyses

The kidneys and other organs were harvested, fixed in 10% buffered formalin, processed, and paraffin embedded. Sections of the kidneys were either stained with H&E or underwent IHC analysis. H&E slides were reviewed by three independent pediatric pathologists (H.C., C.C., and E.J.P.). For IHC analysis, the slides were incubated with the primary antibodies described previously herein, after which they were exposed to a biotinylated secondary antibody that was incubated with an avidin-biotin complex-horseradish peroxidase complex (Vector Laboratories, Burlingame, CA) and then with liquid 3,3'-diaminobenzidine tetrahydrochloride (Dako liquid 3,3'-diaminobenzidine tetrahydrochloride + substrate chromogen system, catalog #2012-02). Stained tissue sections were photographed and processed using a Zeiss AX10 Imager.M1 microscope and AxioVision release 4.6 software (Carl Zeiss MicroImaging GmbH, Jena, Germany).

Immunoblotting

Snap-frozen kidneys were crushed rapidly on ice using a cold mortar and pestle, dissolved in lysis buffer (made fresh from 6 $\times$ stock solution of 2 mol/L Tris-HCl [pH 6.8], 20% SDS, glycerol, and protease inhibitors), and sonicated. The lysis was cleared by centrifugation. To obtain nuclear and cytoplasmic extracts, the NE-PER nuclear and cytoplasmic extraction reagent kit (Thermo Fisher Scientific Inc., Rockford, IL) was used. Protein concentration was determined using protein assay (Bio-Rad Laboratories, Hercules, CA), after which proteins were subjected to SDS-PAGE, transferred to Immobilon-P transfer membranes (Millipore, Billerica, MA), and subjected to immunoblotting using standard techniques. All the experiments were performed in duplicate with three to four animals of each genotype, and representative results are shown.

Statistics

Survival estimates are represented graphically using the Kaplan-Meier method, and comparisons across groups were performed using the log-rank statistic. Analysis was performed using GraphPad Prism v5.02 (GraphPad Software Inc., La Jolla, CA).

Results

Deleting Exon 3 of CNNTB1 in the Proximal Tubule Induces Primitive Epithelial Tumors with Histologic Features that Are Observed in Human WT

Mice harboring a Cited1-CreER^{T245} were bred with Catnb^{lox(ex3)} mice in which exon 3 of *CNNB1* is flanked by lox sites,⁴⁶ to investigate whether activation of β -catenin in the metanephric mesenchyme would result in renal tumors. Cited1 is expressed during metanephric mesenchyme (specifically the cap mesenchyme) development, and it becomes down-regulated during epithelial conversion and is only found at low levels in the first week of life in the mouse, after which it is no longer expressed.⁴⁸ Mice with the genotype Cited1-CreER^{T2}/Catnb^{+/lox(ex3)} (C-Catnb ^{Δ ex3}) were treated with 1.5 mg of tamoxifen or vehicle from postnatal days 1 to 5. C-Catnb ^{Δ ex3} were born at the expected frequency but demonstrated a modest but significant decrement in survival (median, 53 weeks) compared with control animals ($P < 0.0001$) (Figure 1A). In mice younger than 15 weeks ($n = 8$), there were no grossly visible renal abnormalities, but on microscopy, there were scattered tumor foci involving <1% of the renal parenchyma in 9 of 16 kidneys. In animals aged 15 to 25 weeks ($n = 8$), there were scattered tumor foci involving no more than 1% to 5% of the total renal parenchyma in 10 of 16 kidneys and gross tumors in 1 of 16 kidneys. In mice older than 25 weeks (up to age 62 weeks, $n = 10$), grossly visible tumors encompassing 5% to 10% of the renal parenchyma were present in 7 of 20 kidneys and small to moderate scattered foci in 18 of 20 kidneys (Figure 1B). On microscopic examination, these small, sometimes cystic, epithelial neoplasms are located primarily in the middle to deep renal cortex. They are predominantly composed of primitive epithelial clusters, adjacent to spindled mesenchymal stroma and rare undifferentiated cell nests. The epithelial cells in the smaller nodules are most commonly arranged in tubular structures, whereas in the larger lesions they form tubular, cribriform, cystic pseudopapillary, and glandular structures. The tubular epithelial cells tend to be columnar, with a moderate amount of pale eosinophilic cytoplasm toward the lumen. The cytologic profile is poorly differentiated, with an embryonal appearance. The nuclear chromatin is clumped with small nucleoli (Figure 1, C and D). The histologic appearance is most consistent with a primitive renal epithelial neoplasm. These lesions do not seem to be consistent with other epithelial neoplasms of the kidney, such as renal cell carcinoma.

Figure 1. An activating mutation of β -catenin restricted to the kidney proximal tubule is sufficient to induce primitive renal epithelial tumors in mice. **A:** Kaplan-Meier graph showing a modest decrease in overall survival between tamoxifen-treated *Cited1-CreER^{T2}/Catnb^{+/-lox(ex3)}* (C-Catnb^{Δex3}) and control mice ($P < 0.0001$). **B–D:** H&E examination shows small tumors in C-Catnb^{Δex3} mice with histologic findings consistent with a renal epithelial neoplasm with embryonal histologic features. Shown are a representative 2× power image of a kidney from a 62-week-old C-Catnb^{Δex3} mouse (**B**) and 20× (**C**) and 40× (**D**) magnification images. **E:** Kaplan-Meier graph showing minimal decrease in overall survival between *γGT-Cre/Catnb^{+/-lox(ex3)}* (G-Catnb^{Δex3}) and Catnb^{+/-lox(ex3)} control mice ($P = 0.043$). **F–H:** H&E examination shows small tumors in G-Catnb^{Δex3} mice with histologic findings consistent with a renal epithelial neoplasm with embryonal histologic features. Shown are a representative 2× power image of a kidney from a 28-week-old G-Catnb^{Δex3} mouse (**F**) and 20× (**G**) and 40× (**H**) magnification images. Scale bars: 50 μm (**C** and **G**); 25 μm (**D** and **H**).

To define whether activating mutations of β -catenin caused tumors only in the developing mesenchyme or whether this was a more generalized occurrence in the tumor, an activating mutation of β -catenin was knocked into the proximal tubular epithelium using a γ GT renal-specific cre recombinase to give rise to mice with the genotype γ GT-Cre/Catnb^{+/lox(ex3)} (G-Catnb ^{Δ ex3}).^{41–44} γ GT expression in the kidney is restricted to the proximal tubule and begins approximately on postnatal day 10, which is toward the end of nephron development.^{41,44} The G-Catnb ^{Δ ex3} mice were born at the expected frequency but had a minimal, but significant, decrement in survival compared with control animals ($P = 0.04$) (Figure 1E). In mice younger than 25 weeks ($n = 7$), there were no discernable gross or histologic abnormalities of the kidneys (data not shown). However, when the kidneys of animals aged 28 to 72 weeks ($n = 14$) were examined, minute to moderate-sized renal cortical tumors were found in 11 of 28 kidneys involving no more than 5% of the renal parenchyma (Figure 1F). These lesions histologically are virtually identical to those seen in the C-Catnb ^{Δ ex3} mice, except tumors were more frequently found in the midcortex (Figure 1, G and H). Thus, irrespective of the cre mice used, knocking in an activating mutant of β -catenin resulted in a renal epithelial neoplasm with embryonal histologic features.

An Activating K-RAS Mutant Synergizes with Abnormal β -Catenin Signaling to Induce Primitive Renal Epithelial Neoplasms Similarly Found in Human WT

To define whether an activating mutant of K-RAS, which is sufficient to induce tumors in other organs, such as the lung,⁴⁷ would accelerate Catnb ^{Δ ex3}-induced tumor formation, we simultaneously instituted an activating mutant of *Kras* (LSL-Kras^{G12D}). Expressing the *Kras*^{G12D} mutant alone by crossing either the Cited1-CreER^{T2} (with tamoxifen given to the nursing mother for 5 days starting at birth as previously) or the γ GT-Cre with the LSL-Kras^{G12D} mice did not induce renal tumors (data not shown). However, when we crossed the Cited1-CreER^{T2} or γ GT-Cre mice with Catnb ^{Δ ex3} and LSL-Kras^{G12D} (C-Kras^{G12D}/Catnb ^{Δ ex3} and G-Kras^{G12D}/Catnb ^{Δ ex3}, respectively), aggressive tumors that presented at an early age were induced. Median overall survival was significantly shorter than that of Catnb ^{Δ ex3} and control animals, with a median life expectancy of 15.3 weeks for C-Kras^{G12D}/Catnb ^{Δ ex3} and 18.8 weeks for G-Kras^{G12D}/Catnb ^{Δ ex3} ($P < 0.001$) (Figure 2, A and B). Note a decrease in survival in C-Kras^{G12D} and G-Kras^{G12D} mice, but this was not due to renal tumor formation or any apparent histologic changes in the kidneys. Other potential sites of recombination, including the liver, thyroid, mammary epithelium, and cardiac trabeculae, were also free of gross or histologic changes. The cause of death in these animals is currently unknown. All animals sacrificed at 10 to 20 weeks old ($n = 11$ for C-Kras^{G12D}/Catnb ^{Δ ex3} and $n = 18$ for G-Kras^{G12D}/Catnb ^{Δ ex3}) had large-volume, bilateral, solid, and cystic masses that, in many cases, replaced most of the normal renal parenchyma (Figure 2, C and D). Most lesions were in the renal cortex, but they were also found in the corticomedullary junction, the collecting ducts, and the

Figure 2. An activating mutation of *Kras* and β -catenin restricted to the kidney proximal tubule induces numerous large metastatic primitive renal neoplasms. Kaplan-Meier graphs showing significantly lower overall survival for C-Kras^{G12D}/Catnb ^{Δ ex3} mice compared with Catnb ^{Δ ex3} and control animals (A) and for G-Kras^{G12D}/Catnb ^{Δ ex3} mice compared with Kras^{G12D}, Catnb ^{Δ ex3}, and control animals (B) ($P < 0.0001$ for both). C: A gross picture of the kidneys from a 15-week-old G-Kras^{G12D}/Catnb ^{Δ ex3} mouse. D–H: H&E examination shows high-volume, multifocal tumors in Kras^{G12D}/Catnb ^{Δ ex3} mice consistent with epithelial-dominant WT with metastases to the lung. Shown are a 2 $\times$ power image of a kidney from a different 15-week-old G-Kras^{G12D}/Catnb ^{Δ ex3} mouse (D); $\times 10$ (E), $\times 20$ (F), and $\times 40$ (G) magnification images of the kidney from a 13-week-old G-Kras^{G12D}/Catnb ^{Δ ex3} mouse; and a $\times 20$ magnification image of the lung from the same 13-week-old G-Kras^{G12D}/Catnb ^{Δ ex3} mouse (H). Scale bars: 100 μ m (E); 50 μ m (F and H); 25 μ m (G).

papilla. Although the histologic appearance is similar to that of the lesions described previously herein in Catnb ^{Δ ex3} mice, these lesions are larger (Figure 2E), and on microscopy the cells had a higher nuclear/cytoplasmic ratio, large

nuclei with open chromatin, and small to prominent nucleoli. There was also significant variability in size and shape. Although mitoses were frequently encountered, no atypical mitoses were present. Some lesions contain small clusters of blastemal cells with hyperchromatic nuclei and a high nuclear/cytoplasmic ratio, and other lesions contain spindle mesenchymal cells intermixed between the tubular epithelial elements (Figure 2, F and G). Central necrosis was occasionally present, especially in larger lesions. The tumors in the C-Kras^{G12D}/Catnb^{Δex3} and G-Kras^{G12D}/Catnb^{Δex3} mice are not consistent with the recognized histologic subtypes of renal cell carcinoma. Rather, the histologic profile is most consistent with primitive renal epithelial neoplasms with histologic features seen in some human WT. Importantly, three G-Kras^{G12D}/Catnb^{Δex3} animals (17% of those >10 weeks of age) had metastases to the lung (Figure 2H), the most common site of metastases in pediatric WT. Given the presence of metastases in the G-Kras^{G12D}/Catnb^{Δex3} mice, we focused our attention on these animals for the remaining studies.

Given the similarities to the epithelial components of human WTs, we then stained for markers that have been used to differentiate WT from other renal neoplasms. The tumors from G-Kras^{G12D}/Catnb^{Δex3} mice were positive for Pax-2 and Pax-8, both of which are consistent with a renal epithelial origin for the tumors (Figure 3, A and B), although not specific to WTs per se.^{49,50} As is the case for human WT specimens, the tumors were negative for S-100 and epithelial membrane antigen (Figure 3, C and D). The tumors were negative for WT-1 and CD56/neural cell adhesion molecule (Figure 3, E and F). WT-1 is often positive in the blastemal elements of human WT but is often negative in the differentiating epithelial and stromal components.^{50–52} CD56/neural cell adhesion molecule expression is present in virtually all WTs that have been tested, regardless of the histologic subtype, but is not specific for WT. To our knowledge, it has not been tested in the rare subtype of WTs that are epithelial predominant. SALL4 is a recently described marker for the epithelial component of human WTs, and these tumors stained weakly positive for this by IHC staining (Figure 3G).⁵³ Taken together, the expression pattern of these tumors reflects their epithelial dominance and suggests features that have been identified in some human WTs.

Renal Tumors in Kras^{G12D}/Catnb^{Δex3} Mice Have Elevated ERK and PI3K/AKT Signaling

Because the MAPK and PI3K signaling pathways are known to be downstream of RAS, we hypothesized that the increased tumorigenicity in Kras^{G12D}/Catnb^{Δex3} relative to the Catnb^{Δex3} mice is associated with increased activation of these pathways. We, therefore, determined the level of phosphorylated AKT, ERK, and P38 MAPK in the tumors of G-Catnb^{Δex3} and G-Kras^{G12D}/Catnb^{Δex3} mice relative to renal parenchyma in wild-type and Kras^{G12D} mice. There was an equal increase in AKT phosphorylation in the tumors of G-Catnb^{Δex3} and G-Kras^{G12D}/Catnb^{Δex3} mice (Figure 4A). In contrast, phospho-ERK was increased only in tumors from G-Kras^{G12D}/Catnb^{Δex3} mice (Figure 4B). P38 MAPK

was not increased in tumors from either of the mice genotypes (Figure 4C). Thus, activation of the PI3K pathway seems to be associated with the induction of tumors in Catnb^{Δex3} and Kras^{G12D}/Catnb^{Δex3} mice, and ERK activation seems to be associated with the transformation of the relatively benign tumors found in Catnb^{Δex3} mice to the highly aggressive tumors found in Kras^{G12D}/Catnb^{Δex3} mice.

Renal Tumors in Kras^{G12D}/Catnb^{Δex3} Mice Have Increased β-Catenin Expression and Nuclear Translocation

AKT and ERK signaling have previously been shown to positively regulate β-catenin expression levels *in vitro*,

Figure 3. Kras^{G12D}/Catnb^{Δex3} tumors show staining consistent with the epithelial component of some human WTs. The tumors, marked by an asterisk, from Kras^{G12D}/Catnb^{Δex3} mice were stained for the following well-defined markers of WT: Pax-2 (A), Pax-8 (B), S-100 (C, arrowheads indicate examples of positively staining normal renal tubules), epithelial membrane antigen (EMA) (D, arrowhead indicates normal glomerulus), WT-1 (E, arrowhead indicates normal glomerulus with positively staining podocytes), CD56/neural cell adhesion molecule (NCAM) (F), and SALL4 (G). Scale bars: 50 μm.

Figure 4. Kras^{G12D}/Catnb^{Δex3} tumors exhibit AKT and ERK activation. Kidneys from Kras^{G12D}/Catnb^{Δex3}, Kras^{G12D}, Catnb^{Δex3}, and control mice were stained for phosphorylated (p) AKT (A), ERK (B), and P38 MAPK (C). Tumors are denoted by asterisks. Scale bars: 50 μm.

suggesting synergy between these two pathways in WT formation.^{36,37,54–56} We, therefore, performed IHC analysis for β -catenin expression in Catnb^{Δex3}, Kras^{G12D}, and Kras^{G12D}/Catnb^{Δex3} mice. β -catenin expression was not increased in the Catnb^{Δex3} and Kras^{G12D} kidney tissue relative to wild-type animals (data not shown) but was highly expressed in tumors found in Kras^{G12D}/Catnb^{Δex3} mice, where a nuclear preponderance was seen at high magnification (Figure 5A). To define the compartmentalization and expression of β -catenin in more detail, Western blot analyses of whole kidney lysates from all three mutant mice and wild-type controls were performed. Consistent with the IHC analysis results, high levels of β -catenin were present in whole cell lysates and in the nuclear fractions from Kras^{G12D}/Catnb^{Δex3} kidneys (Figure 5, B and C). There was also increased β -catenin expression in the whole kidney cell lysates and in nuclear fractions from Kras^{G12D} but not Catnb^{Δex3} mice. Taken together, these data demonstrate that activation of K-RAS increases total and nuclear β -catenin protein levels, and this is even more accentuated in Kras^{G12D}/Catnb^{Δex3} mice.

To verify that this increased expression of β -catenin observed in the kidney of Kras^{G12D}/Catnb^{Δex3} mice was functionally significant, we assessed expression levels of downstream targets of the canonical Wnt/ β -catenin pathway, such as c-Myc and survivin. Expression of c-Myc was significantly increased in Kras^{G12D}/Catnb^{Δex3} mice compared with Catnb^{Δex3} mice as determined by IHC analysis and immunoblotting (Figure 5, D–F). Similar results were observed when survivin was assessed by immunoblotting (Figure 5F). As would be anticipated in tumors harboring high levels of c-Myc, IHC studies for G-Kras^{G12D}/Catnb^{Δex3} mice demonstrated high rates of proliferation, as evidenced by high levels of Ki-67 (Figure 5G) and cyclin D1 (Figure 5H). Conversely, the tumors demonstrated low levels of apoptosis, as shown by IHC staining for cleaved caspase 3 (Figure 5I), consistent with high levels of survivin. Taken together, these findings demonstrate that simultaneous activation of Kras and β -catenin is associated with up-regulation of canonical Wnt/ β -catenin pathway downstream target genes that play a critical role in modulating cell proliferation and apoptosis.

Discussion

In this study, we present a transgenic mouse model of highly penetrant metastatic primitive renal epithelial tumors that share the histologic features of some human WT. This model was achieved by simultaneous expression of activated K-RAS and Δ exon 3 β -catenin in the developing metanephric mesenchyme or proximal tubules of the kidney. We further demonstrate that although a stabilizing mutation of β -catenin is sufficient to induce tumors in the kidney, they are not highly proliferative, and they lack the ability to metastasize. This kidney phenotype contrasts with that of the gastrointestinal tract, where the Δ exon 3 β -catenin mutation induces multiple neoplastic polyps in the colon.⁴⁶ We also demonstrate that

Figure 5. $Kras^{G12D}/Catnb^{\Delta ex3}$ tumors have increased levels of total and intranuclear β -catenin, increased levels of c-Myc and survivin, increased markers of cell proliferation, and decreased apoptosis. **A:** Low-power magnification images ($\times 5$) of a kidney from a $Kras^{G12D}/Catnb^{\Delta ex3}$ mouse stained for β -catenin. Tumors are denoted by **asterisks**. Scale bar = 200 μm . Evidence of nuclear β -catenin (**arrows**) is seen on a $\times 40$ magnification image (**inset**). Scale bar = 25 μm . **B:** Western blot analysis of whole kidney lysates for β -catenin and actin loading controls. Wild-type β -catenin (**top band**) and Δ exon 3 β -catenin (**bottom band**) are visualized. **C:** Western blot analysis of nuclear fractionated protein lysates of the kidney stained for β -catenin and histone (nuclear fraction) loading control. A representative example of individual mice is shown. Kidneys from $Catnb^{\Delta ex3}$ (**D**) and $Kras^{G12D}/Catnb^{\Delta ex3}$ (**E**) mice were stained for c-Myc. Tumors are denoted by **asterisks**. Scale bars: 100 μm . **F:** Western blot analysis of whole kidney protein for c-Myc and survivin and corresponding actin loading controls. A representative example of individual mice is shown. The tumors were stained for the proliferative markers Ki-67 (**G**) and cyclin D1 (**H**) and for a marker of apoptosis, cleaved caspase 3 (**I**). Tumors are denoted by **asterisks**. Scale bars: 100 μm .

activating mutations of *Kras* alone are not sufficient to induce malignancies in the kidney. This situation is similar to their role in the gastrointestinal tract, where they induce hyperproliferation but not tumors,⁵⁷ and unlike their role in the lungs, where K-RAS is sufficient to induce tumors.⁴⁷ Thus, the effects of these oncogenes differ depending on their tissue expression; and in the context of the kidney, co-expression of a Δ exon 3 β -catenin and a $Kras^{G12D}$ activation mutant induces aggressive, primitive renal epithelial neoplasms, which can metastasize to the lung and have features consistent with some human WTs.

The $Kras^{G12D}$ kidneys and tumors in the $Kras^{G12D}/Catnb^{\Delta ex3}$ mice do not show features of renal cell carcinoma or any other carcinoma found in either the pediatric or adult kidney. This is surprising considering the strong

association between activation of tyrosine kinase-dependent pathways and mutations in the von Hippel-Lindau gene that are found in familial and sporadic clear cell renal cell carcinomas.⁵⁸ Although not well studied, there is no clinical association between *RAS* mutations and sporadic WTs in humans.⁵⁹ This contrasts with microarray data, which suggest that members of the *RAS* family are target genes of the Wnt/ β -catenin pathway in patients with WT,⁶⁰ and with studies showing synergy of the Wnt/ β -catenin and *RAS* activation in other organs, such as the colon and breast.^{56,61–67} In addition, tyrosine kinase receptors such as insulin-like growth factor and vascular endothelial growth factor have been implicated in WT and often signal through *RAS*.^{29–35} The present data support the notion that even in the absence of identified *KRAS*

mutations, activation of downstream components of the K-RAS pathway that have been identified in WTs cooperate with β -catenin to promote renal tumorigenesis in humans.

The present data show that activation of K-RAS in the kidney increases total and nuclear mutant (exon 3 deleted) and wild-type β -catenin levels, suggesting that it increased canonical Wnt/ β -catenin transcriptional activation. This is further supported by the increased levels of canonical Wnt/ β -catenin targets, such as c-Myc, survivin, and cyclin D1. AKT activation is increased in $\text{Catnb}^{\Delta\text{ex}3}$ and $\text{Kras}^{\text{G12D}}/\text{Catnb}^{\Delta\text{ex}3}$ mice, suggesting that activation of PI3K might play a critical role in the formation of all the tumors. The fact that ERK is increased only in tumors from $\text{Kras}^{\text{G12D}}/\text{Catnb}^{\Delta\text{ex}3}$ mice suggests that ERK-dependent pathways are involved in the synergy between activated K-RAS and canonical Wnt activation in renal tumor development. This is in agreement with a recent study by Hu et al³⁵ that demonstrated ERK activation in a subset of human WT. The mechanisms whereby AKT and ERK might regulate β -catenin signaling to induce renal tumors are currently unclear; however, AKT activation can lead to phosphorylation of the c-terminus of β -catenin, increasing its stability and transcriptional regulation.³⁹ Furthermore, ERK activation can lead to increased transcription and mRNA levels of the *CNNB1* gene,³⁷ and activation of AKT and ERK can regulate the phosphorylation of glycogen synthase kinase-3 β , which is a critical component of the regulatory complex that marks β -catenin for degradation by the proteasome.^{36,68}

Tumors in $\text{Kras}^{\text{G12D}}/\text{Catnb}^{\Delta\text{ex}3}$ mice have dramatically increased levels of the canonical Wnt/ β -catenin targets c-Myc, survivin, and cyclin D1. c-Myc almost certainly plays a critical role in inducing the malignant transformation of these tumors owing to its multiple roles in regulating gene transcription.⁶⁹ c-Myc is likely regulated at many levels because ERK and AKT have been shown in other contexts to play a direct role in increasing c-Myc levels by increasing its transcription and stabilization.⁴⁰ It is possible that this could, in turn, act to stabilize and increase β -catenin transcriptional regulation, leading to further increases in c-Myc.^{38,70} Such a positive feedback loop could potentially be playing a role in promoting the aggressive phenotype seen in these mice. The primitive renal epithelial tumors seen in $\text{Kras}^{\text{G12D}}/\text{Catnb}^{\Delta\text{ex}3}$ mice also have elevated levels of survivin, a member of the inhibitor of apoptosis proteins that have a role in suppressing apoptosis across many tumor types.⁷¹ Similar to c-Myc, this protein is regulated by numerous signaling pathways, including β -catenin and ERK- and PI3K-dependent signaling pathways.

The present transgenic mouse model is one of only two that harbor tumors with features of human WT. The other model was developed through mosaic, somatic ablation of *Wt1* combined with constitutional up-regulation of *Igf2* (referred to as *Wt1-Igf2*).³⁵ The *Wt1-Igf2* tumors histologically showed areas with predominant epithelial cells, had up-regulation of Pax-2, and showed activation of ERK, as seen in $\text{Kras}^{\text{G12D}}/\text{Catnb}^{\Delta\text{ex}3}$ mice. However, there were frequently areas of predominant blastemal cells, which were rare in $\text{Kras}^{\text{G12D}}/\text{Catnb}^{\Delta\text{ex}3}$ mice. In addition, the

frequency of tumor formation was lower (64% versus 100% in $\text{Kras}^{\text{G12D}}/\text{Catnb}^{\Delta\text{ex}3}$), the burden of disease in individual mice was lower (bilateral or multicentric in 54% versus bilateral and multicentric in 100%), and there was no evidence of metastases (none versus 17%). Finally, canonical Wnt/ β -catenin activation in *Wt1-Igf2* mice was not reported but would be an interesting avenue to explore.

In summary, we developed a transgenic mouse model of primitive epithelial renal tumors with features that strongly resemble those seen in some human WTs with respect to their natural history, histologic findings, and expression of IHC markers. This model provides a powerful new tool for studying the biology of primitive renal epithelial neoplasms such as those seen as a part of WTs and suggests a novel important pathway(s) in renal tumor formation that could be therapeutically targeted in the future.

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