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PRINCIPAL INVESTIGATOR: Keri Drake

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Keri Drake

E-Mail: Keri.Drake@UTSouthwestern.edu

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14. ABSTRACT

Wilms tumor (WT) resembles the developing kidney, consisting of blastema/nephron progenitor cells (NPCs), epithelium, and stroma – indicating a link between the dysregulation of normal development and tumorigenesis. While activation of beta-catenin has been shown in a significant proportion of WTs, much remains to be understood about its role in tumor development. Previously, it has been assumed that NPCs act as a cancer stem cell; however, mouse models with activation of beta-catenin within the NPC lineage paradoxically show premature loss of blastema, a phenotype opposite to WT. Given that we and others have shown that disrupting signals from developing renal stroma results in abnormally maintained NPCs (reminiscent of WT nephrogenic rests), we hypothesized that signaling from the stroma, specifically activation of beta-catenin, contributes to Wilms tumorigenesis. Indeed, we have shown that activation of beta-catenin in stromal progenitors inhibits NPC differentiation. Additionally, comparisons of the transcriptomes from human WT to mutant mouse kidneys with beta-catenin activation in the stromal vs NPC lineages revealed that human WT more closely resembled stromal mutants, suggesting that stromal beta-catenin activation results in histological and molecular features of WT. Future studies will assess mechanisms of stromal-NPC crosstalk, both in normal development and human Wilms tumor samples.

15. SUBJECT TERMS
Wilms tumor, beta-catenin, CTNNB1, kidney development, stroma, nephron progenitor cells, blastema, single nuclei RNA seq, organoids

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1. INTRODUCTION:

Wilms tumor (WT), or nephroblastoma, is an embryonal tumor classically consisting of triphasic histology, with blastemal/nephron progenitor, epithelial, and stromal components resembling the fetal kidney. Although all WT appear to derive from aberrant renal development, no unifying mechanism has been identified. While activating mutations in CTNNB1, the gene encoding beta-catenin, are found in approximately 15% of WTs, tumors without mutations in CTNNB1 frequently show signs of upregulated beta-catenin activity, suggesting this pathway may drive tumorigenesis. However, beta-catenin signaling is highly cell-type and context-dependent with multiple functions in different aspects of renal development, with little understood about its role in WT. While it has previously been assumed that blastema/nephron progenitor cells (NPCs) act as a cancer stem cell, mouse models with activation of beta-catenin within the NPC lineage paradoxically show premature loss of the blastema, a phenotype opposite to WT. Using mutant mouse models, we and others have recently shown that signals from developing renal stroma somewhat surprising regulate NPCs, and loss of this regulation results in abnormally maintained nephron progenitors reminiscent of nephrogenic rests in WTs. Specifically, we have shown that activation of beta-catenin in the stromal progenitor population inhibits NPC differentiation and non-autonomously alters the molecular state of these cells. These findings demonstrate that disruption of the normal stromal microenvironment affects the balance between maintenance and differentiation of the neighboring NPC population. Additionally, comparisons of the transcriptomes of mutant mouse kidneys expressing an activated allele of beta-catenin in the stromal or nephron progenitor cells revealed that human WT more closely resembles stromal lineage mutants vs wild type kidneys and nephron progenitor lineage mutants. Overall, these results suggest that stromal beta-catenin activation results in histological and molecular features of human WT, supporting the hypothesis that aberrant signaling from the stroma, as mediated by activating mutations in beta-catenin, plays a role Wilms tumorigenesis. Future studies will assess underlying mechanisms of stromal-to-nephron progenitor cross-talk in normal development, specifically in the balance of NPC maintenance/differentiation, and further examine the roles of these signaling pathways in human Wilms tumor samples.

2. KEYWORDS:

Wilms tumor (WT), beta-catenin, CTNNB1, kidney development, stroma, nephron progenitor cells, blastema, single nuclei RNA seq, and organoids

3. ACCOMPLISHMENTS:

What were the major goals of the project?

The following table outlines the goals of the project from the approved statement of work, with the first colum including the proposed timeline and the second column showing the date and percentage completed.

Specific Aim 1: Determine the mechanism in which stromal beta-catenin inhibits nephron progenitor cell (NPC) differentiation
Major Task 1: Perform RNA-seq on isolated stroma from BcatEx3$^{flox/+}$ Foxd1Cre embryonic kidneys to identify stromal-specific candidate genes that function in stromal-NPC crosstalk

Subtask 1: Regulatory review and approval by the USAMRMC Animal Care and Use Review Office (ACURO) for animal work included in the institutionally approved PI's protocol (APN 2019-102701)	Proposed for months 1-3	100% complete in 9/2019
Subtask 2: Collect control and mutant kidneys (3 cre-negative controls and 3 BcatEx3$^{flox/+}$ Foxd1Cre mutant kidneys; will require a total of 3 timed pregnant female mice) and isolate stroma using FACs and submit to sequencing core	Proposed for months 3-4	Not complete
Subtask 3: Analyze RNA-seq data using institutional software pipelines to identify gene expression changes in mutant vs wild type kidneys	Proposed for month 5	Not complete
Subtask 4: Validate gene expression changes using qPCR and/or in situ hybridization on control and mutant kidneys (to be performed in triplicate, requiring 3 cre-negative controls and 3 BcatEx3$^{flox/+}$ Foxd1Cre mutant kidneys from 3 timed pregnant female mice to ensure reproducibility)	Proposed for months 6-9	50% complete in 8/2020
Milestone(s) Achieved: Identification of specific gene targets of beta-catenin activation in the renal stroma	Proposed for month 9	50% complete in 8/2020
Major Task 2: Test the functional ability of genes identified in aim 1a to regulate nephron progenitor maintenance/differentiation in vitro		
Subtask 1: Over-express genes in lentiviral vectors from aim1a in explant kidney culture to assess for effects on nephron differentiation (to be performed in triplicate using wild type kidneys, requiring 3 timed-pregnant female mice to assess ~10 genes)	Proposed for months 5-9	Not complete
Subtask 2: Knockdown of genes from aim1a in explant kidney culture to assess for effects on nephron differentiation (to be performed in triplicate using wild type kidneys, requiring 3 timed-pregnant female mice to assess ~10 genes)	Proposed for months 5-9	Not complete
Milestone(s) Achieved: Identification of stroma-specific genes that functionally inhibit nephron differentiation	Proposed for month 9	Not complete

Specific Aim 2: Evaluate human Wilms tumor for malignant potential of the stroma		
Major Task 1: Examine human samples for cell-lineage specific beta-catenin mutations to determine if stromal beta-catenin mutation can occur in isolation from blastema/epithelial cell populations		
Subtask 1: Regulatory review and approval by the USAMRMC Human Research Protection Office (HRPO) to obtained de-identified samples of humans Wilms tumor from our institutional biorepository per PI's IRB (STU-2019-1047)	Proposed for months 1-3	100% complete in 9/2019
Subtask 2: Obtain sections from de-identified human Wilms tumor samples from our institutional biorepository (8 samples, including 4 with beta-catenin activating mutations and 4 without will be obtained and used throughout the studies proposed in aim 2)	Proposed for month 3	50% complete in 8/2020
Subtask 3: Perform in-situ PCR to localize specific CTNNB1 mutations to stroma, blastema, or epithelial cells and determine if the same mutation is present in multiple cell lineages	Proposed for months 4-7	Not complete
Subtask 4: If necessary, perform laser microcapture of stromal and blastemal regions of human Wilms tumor and sequence for CTNNB1 mutations	Proposed for months 6-9	Not complete

Milestone(s) Achieved: Determine the cell types (ie: blastema vs stroma) that carry beta-catenin activating mutations in human Wilms tumor samples	Proposed for month 9	Not complete
Major Task 2: Evaluate the expression of stromal beta-catenin target genes in human Wilms tumor samples, using a targeted approach from the identified gene/pathways in our mouse model with stromal activation of beta-catenin (BcatEx3$^{flox/+}$ Foxd1cre) to determine if our mouse model recapitulates expression patterns of human Wilms tumor		
Subtask 1: Generate in-situ probes from gene list in aim 1	Proposed for months 8-9	Not complete
Subtask 2: Perform section in-situ hybridization on human Wilms tumor samples (obtained as above)	Proposed for months 9-11	Not complete
Milestone(s) Achieved: Localize the expression of beta-catenin target genes in human Wilms tumor samples, allowing molecular comparisons between human Wilms tumor and mouse models with Wilms tumor-like phenotypes	Proposed for month 12	Not complete
Major Task 3: Evaluate the expression of stromal beta-catenin target genes in human samples using unbiased, global gene expression profiling of human Wilms tumor samples.		
Subtask 1: Isolate nuclei from 3 human Wilms tumor samples and 1 control kidney (first run to be done at 6 months, allowing time for data to be analyzed, with a second run to be performed at 12 months; we will utilize the same samples obtained as described above)	Proposed for months 6, 12	Not complete
Subtask 2: Submit isolated nuclei to the sequencing core to generate single cell RNA seq data	Proposed for months 7-14	Not complete
Subtask 3: Analyze single cell RNA sequencing data using bioinformatic algorithms developed by Dr. Chaney to identify cell types (stroma vs blastema vs epithelial derivates) and evaluate their expression of beta-catenin target genes; perform regulon analysis to identify novel signaling pathways that may be regulated by beta-catenin in specific cell types	Proposed for months 15-18	Not complete
Milestone(s) Achieved: Generate molecular profiles of 15,000 – 20,000 single cells from human Wilms tumor samples and determine the differential gene expression of beta-catenin target genes in different cell types to further characterize the molecular profiles of Wilm tumor at the single cell level	Proposed for month 18	Not complete

Specific Aim 3: Evaluate cell-lineage effects of beta-catenin activating mutations to determine if beta-catenin activation in specific cell lineages during kidney development recapitulates human Wilms tumor		
Major Task 1: Examine mutant mouse lines for Wilms tumor-like phenotypes, including the following lines: 1) BcatEx3$^{flox/+}$ Six2cre, 2) BcatEx3$^{flox/+}$ Foxd1cre, 3) BcatEx3$^{flox/+}$ Six2cre+Foxd1cre, and 4) BcatEx3$^{flox/+}$ TcreERT2		
Subtask 1: Generate timed matings, isolate embryonic kidneys, and perform H&Es/in situs/immunoassays on mutants with activation of beta-catenin (BcatEx3$^{flox/+}$) in different compartments of the developing kidney (nephron progenitor lineage using Six2cre, stromal lineage using Foxd1cre, both nephron progenitor and stromal lineages using Six2cre+Foxd1cre double mutants, and a common progenitor using TCreERT2 with tamoxifen given at E8.0; analyses will be performed in triplicate for each genotype requiring approximately 20 timed pregnant female mice based on breeding schemes)	Proposed for months 3-10	100% complete in 4/2020
Subtask 2: Implant embryonic kidneys of the above genotypes under the kidney	Proposed for	100% complete in

capsule of immunocompromised mice to assess tumorigenic potential (to be performed in duplicate, with 2 kidneys implanted under one adult kidney capsule for each genotype, requiring 8 timed pregnant female mice and 15 NOD SCID mice)	months 4-12	2/2020
Milestone(s) Achieved: Determine if activation of beta-catenin in isolated stroma vs blastema vs both components of the developing kidney results in a Wilms tumor like phenotype	Proposed for month 12	100% complete in 4/2020
Major Task 2: Generate organoids using the above beta-catenin activation mutant lines, since this method preserves some wild type cell signaling that may be necessary in the development of Wilms tumor		
Subtask 1: Isolate embryonic kidneys from the above matings and generate organoids in vitro (to be performed in triplicate, requiring 16 timed pregnant female mice)	Proposed for months 13-15	Not complete
Subtask 2: Implant organoids under the kidney capsule of immunocompromised mice to assess for tumorigenic potential (to be performed in duplicate, requiring 15 NOD SCID mice)	Proposed for months 16-24	Not complete
Milestone(s) Achieved: Compare phenotypes of organoids vs embryonic kidneys with cell type specific beta-catenin activating mutations for proof of principle data (supporting the use of an organoid model to introduce multiple combinations of cell type specific mutations of Wilms candidate genes)	Proposed for month 24	Not complete

What was accomplished under these goals?

During this reporting period, major activities have included: 1) publication of a manuscript examining mutant mouse lines with beta-catenin activating mutations for Wilms tumor-like phenotypes, 2) analyses of embryonic kidneys implanted under the kidney capsule to assess to tumorigenesis, and 3) the development of protocols/additional methods to examine gene expression in human Wilms tumor to implement during the next funding period.

As described in the above statement of work, I proposed the following specific goals/objectives, including: 1) to determine the mechanism in which stromal beta-catenin inhibits nephron progenitor cell (NPC) differentiation, 2) to evaluate human Wilms tumor for malignant potential of the stroma, and 3) to evaluate cell-lineage effects of beta-catenin activating mutations to determine if beta-catenin activation in specific cell lineages during kidney development recapitulates human Wilms tumor. The significant results/key outcomes for each goal is outlined as follows:

For the first research goal, I had proposed to perform additional RNA-seq on BcatEx3$^{flox/+}$ Foxd1Cre (also referred to as Foxd1cre/Catnb$^{ex3/+}$) embryonic kidneys to identify candidate genes specifically expressed in the developing to stroma and test their functionality using lentiviral vectors in explant cultures. Since these studies required collecting fresh embryonic tissue for FACs/RNA-seq and explant assays, the experiments were interrupted due to an institutional wide laboratory shut down in April 2020 limiting our mouse work to only essential activities to maintain the colony. Since these studies could not be performed, we subsequently utilized the data that we had already generated from whole kidney RNA-sequencing to identify and validate potential candidate genes for this aim. Additionally, we observed that BcatEx3$^{flox/+}$ Foxd1Cre mutants lacked NPC differentiation earlier in development at E15.5, with abnormal expression of several markers of

induced NPCs, including C1qdc2 and Wnt4 (Fig. 1, panels W and X respectively). However, by E18.5 differentiating nephron structures marked by Lhx1 (Fig. 1, panel BB) were clearly observed. We could therefore take advantage of this observation and examine the two different time points for changes in stromal signaling that may be functioning to non-autonomously block NPC differentiation at E15.5, which is later lost or downregulated at E18.5, thus allowing NPC differentiation to proceed.

Through this approach, we confirmed that BcatEx3$^{flox/+}$ Foxd1Cre mutant kidneys show decreased expression of genes in the nephrogenic stroma, including Foxd1, Netrin, and Fat4 at E15.5 (Fig. 1, panels H, I, and K, respectively). Previous studies have shown that ablation of either Foxd1 or Fat4 resulted in abnormal NPC maintenance (Das, et. al, Nat Cell Bio, 2013). Since Foxd1 expression is specific to the stromal progenitor population, it was not surprising that is remained lost in mutant kidneys at E18.5 (Fig. 1, panel L). However, stromal expression of Fat4 interestingly appeared increased in mutant kidneys at E18.5 in comparison to E15.5 (Fig. 1, panel N), correlating with its proposed role in promoting NPC differentiation, as suggested in the previous literature.

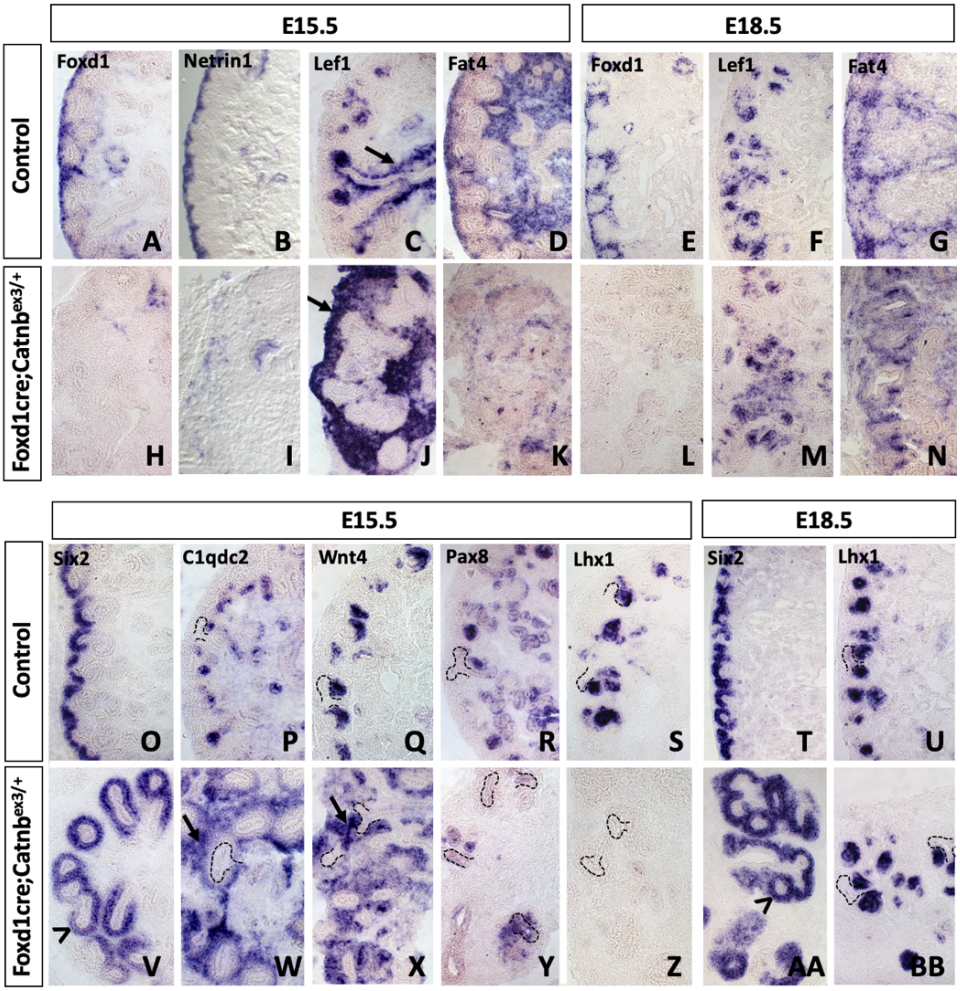

Figure 1. Activation of beta-catenin in the stromal progenitor population results in abnormal stromal patterning and with expanded NPCs that show delayed mesenchyme to epithelial transition. A-N) In comparison to control kidneys, BcatEx3$^{flox/+}$ Foxd1Cre mutants show disrupted stromal patterning, with loss

of Foxd1 (H), Ntn1 (I), and Fat4 (K) and ectopic expression of medullary markers, including Lef1 (J) at E15.5. At this same time point in development, the NPCs are expanded and do not undergo differentiation, as demonstrated by a lack of Lhx1 structures (Z). However, at E18.5, we observe changes in the stromal signaling, with increased Fat4 (N) and a loss of stromal Lef1 (M), which corresponds to the NPCs now undergoing differentiation into pre-tubular aggregate/renal vesicle structures (BB).

Additionally, further characterization of BcatEx3$^{flox/+}$ Foxd1Cre mutant kidneys showed ectopic expression of medullary stromal markers, including Lef1 (Fig. 1, panel J) and other markers included in our published manuscript (please see Fig. 5 in the attached manuscript Drake et. al, Development, 2020). These findings support that activation of beta-catenin in the stromal progenitor population leads to precocious and ectopic differentiation of a more medullary stromal cell type, and given that beta-catenin has previously been shown to be necessary for the development of the papillary stroma (Boivin and Bridgewater, Am J Physiol Renal Physiol, 2018 and Boivin et. al., J Pathol, 2016), our findings in this mutant model suggest it is also sufficient. Interestingly, at E18.5, mutant kidneys show a marked decrease in the strong stromal Lef1 expression that was observed at E15.5 (Fig. 1, panels N and J, respectively), making this another candidate gene, besides Fat4, that may function in non-autonomous signaling to regulate the balance of NPC differentiation/maintenance.

For major task 2 to test the functional ability of genes identified above to regulate nephron progenitor maintenance/differentiation in vitro, the subtasks of 1) over-expressing and 2) knocking down genes using lentiviral vectors in explant cultures were not performed, as described above. I am subsequently proposing an alternative method, to transfect isolated cell populations with lentiviral vectors and generate organoids, as outlined in the section below describing experiments planned in the next year.

For the second research goal to evaluate human Wilms tumor for malignant potential of the stroma, we were delayed in obtaining human WT samples from our institution's biorepository due to the COVID shutdown, which was closed for requests from April to July 2020. I have since been in close contact with the biorepository, who have identified samples and approved my request for tissue. I have received 3 samples of fresh/frozen WT from patients with known CTNNB1 mutations, and I am currently working on obtaining the additional stored samples for tissue sections. Thus, subtask 2 to obtain sections from de-identified human Wilms tumor samples (including 3 with beta-catenin activating mutations and 3 without) is approximately 50% complete, as the samples have been identified, our request has been approved, and I have received some tissue and am currently just waiting for the biorepository to provide us with the remaining requested samples. Following this, we will perform subtask 3 (in-situ PCR to localize specific CTNNB1 mutations to stroma, blastema, or epithelial cells and determine if the same mutation is present in multiple cell lineages), and the subtasks in major task 2 (to evaluate the expression of stromal beta-catenin target genes in human Wilms tumor samples, using a targeted approach from the identified gene/pathways in our mouse model with stromal activation of beta-catenin).

While we have been delayed in obtaining tissue, I have subsequently worked on implementing a new technique in the lab to validate human gene expression in Wilm tumor samples. Given that commericially available antibodies can be limited and have issues with non-specific binding and/or validating their specificity, as well as the drawbacks to generating conventional in-situ hybridization probes with some limitations in commercially available plasmids/genes, I have pursued utilizing

RNA scope to evaluate gene expression. This technique offers a reliable and robust method for the detection and quantifying gene expression and has been widely used in formalin fixed, paraffin embedded samples in other cancer research. I have received all the supplies and reagents in the lab and have completed my first control run to ensure the protocol is working (Fig. 2, panels A-F), with plans to subsequently examine selected genes in human tissue samples. For major task 3 (to evaluate human WT samples using unbiased, global gene expression profiling), I had proposed to submit one sample for sequencing during the first year of funding with the remainder of samples to be submitted during the second year; however, this was delayed due to an inability to obtain tissue. As described above, we have subsequently been able to work with the biorepository, and plan to obtain tissue shortly to submit for sequencing. In the interim, I have optimized our protocol for isolating nuclei by testing samples of embryonic kidney and human tissue, utilizing human tonsillar tissue provided by the biorepository to approximate the nuclei preparation for tumor tissue (Fig. 2, panels G-H).

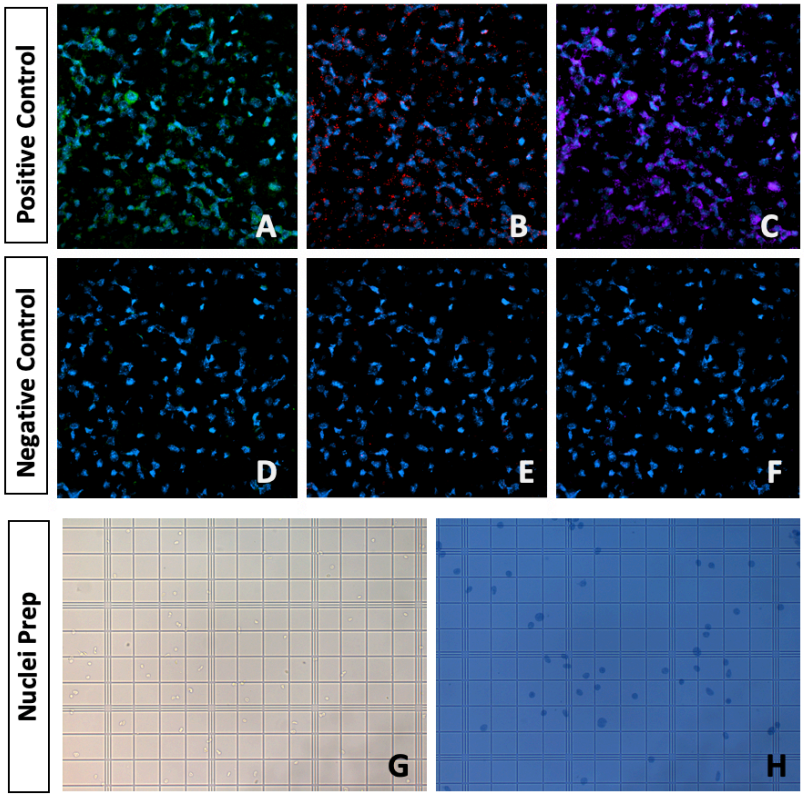

Figure 2. Strategies for examining gene expression human WT samples. A-C) Positive control probes along with a D-F) a negative control probe were tested on control slides to ensure our ability to perform RNA scope in the lab utilizing multiplexing, so multiple genes can be identified in a single sample. B) Representative images from single nuclei preparations in test samples of embryonic mouse kidneys and human tonsillar tissue (to approximate tumor tissue) were imaged in a hemacytometer with brightfield microscopy in unstained (G) and nuclei stained with trypan blue (H) to count/prepare nuclie in anticipation of submitting samples for single nuclei RNA seq.

For the third research goal to evaluate cell-lineage effects of beta-catenin activating mutations to determine if beta-catenin activation in specific cell lineages using mutant mouse models, subtask 1 (to generate timed matings, isolate embryonic kidneys, and characterize the effects of beta-catenin

activation in the nephron progenitor lineage using Six2cre, stromal lineage using Foxd1cre, both nephron progenitor and stromal lineages using Six2cre+Foxd1cre double mutants, and a common progenitor using TCreERT2) was completed. The manuscript detailing this work was accepted for publication (Drake et. al, Development, 2020) and is attached with this report. To briefly summarize our findings, we showed that mutant kidneys with beta-catenin activation mutations specifically targeting the stromal progenitor population form remnant epithelial structures surrounded by undifferentiated mesenchyme and spindle-shaped fibroblasts, similar to the histology of WT. Transcriptomes of mutant mouse kidneys with activating mutations in either the nephron progenitor lineage or the stromal lineage were compared to human WTs (using RNA seq obtained from the pubically available TARGET database), revealing that WT shares characteristic of the stromal-lineage mutants, more so than wild type developing kidney or NPC lineage mutants. We also showed that the expression of diagnostic markers of WT, including Six2, Cited1, and Ncam, is observed in the stromal lineage mutants, and not the NPC lineage mutants, since activation of beta-catenin in the nephrogenic lineage resulted in loss of nephron progenitor cell (NPC) renewal, a phenotype opposite to WT. Somewhat surprisingly, our examination of mice with mosaic activation of beta-catenin in early metanephric precursor lineages utilizing TcreERT2 revealed that mutant cells were either selected against or down-regulated the forced expression of beta-catenin, as these kidneys showed lineage-positive cells (suggesting of recombination) but lacked detectable beta-catenin activation and underwent grossly normal development. In contrast, simultaneous activation of beta-catenin in the nephron progenitor and stromal lineages showed severely perturbed development with the formation of bone-like tissue, with bone as well as other heterologous elements including cartilage and skeletal muscle being reported in some human WTs.

For subtask 2, we implanted 2-3 embryonic kidneys of the above genotypes under the kidney capsule of immunocompromised mice to assess tumorigenic potential. E15.5 kidneys were implanted in NOD SCID mice and examined at 2 month and 4 month time points. We utilized mutant mouse lines with the RosaTdtomato reporter (Fig. 3, B-E, inserts) to easily localize the implanted mutant kidney tissue. Results of these studies showed somewhat surprising results. First, control kidney showed some normally appearing kidney tissue with numerous glomeruli (Fig. 3, F); however, they also contained dilated cystic epithelial tubules positive for collecting duct markers (data not shown) that resembeled findings in some of the mutant kidney lines, including in the nephron progenitor lineage (BcatEx3$^{flox/+}$ Six2Cre; Fig. 3, G), dual stromal progenitor and nephron progenitor lineage (BcatEx3$^{flox/+}$ Six2Cre + Foxd1cre; Fig. 3, I), and early common progenitor (BcatEx3$^{flox/+}$ TCreERT2; Fig. 3, J) which we now know does not result in sustained activation of beta-catenin and resembles wild type kidneys. Furthermore, stromal lineage mutant kidneys (BcatEx3$^{flox/+}$ Foxd1Cre; Fig. 3, H), developed scar-like collagen, as evidenced by strong collagen staining with trichrome (data not shown). While beta-catenin activation has been previously shown to drive fibrogenesis and desmoid tumors through the proliferation of benign fibroblasts in skin (Lam and Gottardi, Curr Opin Rheumatol, 2011), this was a somewhat unexpected outcome to see from its activation in the developing renal stroma.

Based on these preliminary studies, further experiments to follow up the above findings include: 1) to exhaustively section BcatEx3$^{flox/+}$ Six2Cre + Foxd1cre implanted kidneys, since these kidneys develop a bone-like phenotype in vivo at E18.5, but when implanted under the capsule at E15.5, we did not observe the same phenotype in the sections obtained, and 2) to similarly further section BcatEx3$^{flox/+}$ Six2Cre implanted kidneys, since the phenotype appears remarkably different then

what is observed vivo. We also feel that the cystic collecting ducts may be proliferating/obscuring the NPCs/stroma in our models. One way to circumvent this would be to generate organoids from mutant embryonic kidneys, which could then be similarly implanted under the kidney capsule to assess for tumor potential. We have therefore moved forward to working on the proposed experiments in the second year of funding, generating organoids from the above beta-catenin mutants. We are currently optimizing protocols to isolate the nephrogenic zone from control and mutant kidneys, consisiting of NPCs, nephrogenic stroma, and some UB (but not fully formed collecting ducts as present in E15.5 kidneys).

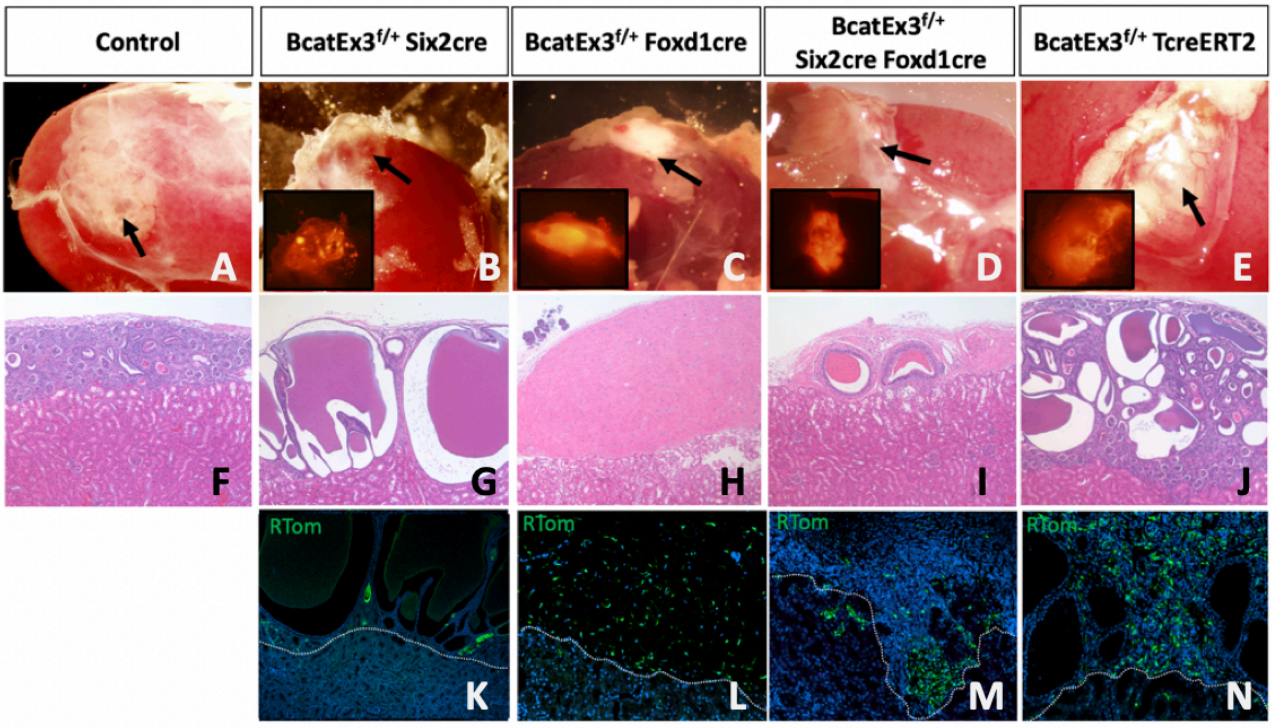

Figure 3. Characterization of embryonic kidneys with activation of beta-catenin in different cell lineages implanted under the kidney capsule of NOD SCID mice to assess tumorigenesis potential. A,F) Control (cre-negative kidneys) showed glomeruli (F) and some dilated tubules (data not shown) when examined 4 months after implantation. B, G, K) Mutant kidneys with activation of beta-catenin in the nephron progenitor lineage interestingly showed few reporter positive cells interspersed amongst the cystic tubules/collecting ducts. C, H, L) Mutant kidneys with activation of beta-catenin in the stromal progenitor lineage developed scar-like lesions. D, I, M) Mutant kidneys with activation of beta-catenin simultaneously in the nephron and stromal progenitor lineages did not show bone-like lesions in the preliminary analysis, though this needs to be further examined.

Other achievements

Given that activating beta-catenin mutations have been shown to coincide with WT1 mutations in human WTs, we additionally generated mutant mouse lines with simulataneous knock of WT1 and activation of beta-catenin. Loss of WT1 in NPCs has been shown to block NPC differentiation (Berry et. al, Disease Models & Mechanisms, 2015). Since we and others have shown that beta-catenin activation in the NPC lineage resulted in a premature NPC differentiation, we hypothesized that loss of WT1 may affect this phenotype (ie: by maintaining NPCs and preventing their

differentiation driven by beta-catenin). However, □reliminary analyses of these mutant mice ($WT1^{c/c}$ $BcatEx3^{c/+}$ Six2cre) show a phenotype similar to the beta-catenin activating mutation, with no increased maintenance of NPCs as hypothesized, raising further questions about the interplay between how these two mutations contribute to WT.

What opportunities for training and professional development has the project provided?

Prior to the social distancing implementations due to COVID, I was participating in UTSW Kidney SPORE meetings held twice per month (subtask 1) and presented this project (subtask 2) at a meeting. Additionally, UTSW has formed a Wilms Tumor Group, which I presentated at and also participated in meetings (subtask 2). I have also been invited to present at the 2020 Kidney Cancer Research Summit that will be held virtually in October and hope to present this work at an additional national meeting in the second year of funding (subtask 4). Furthermore, I am currently working on developing additional aims to be included in an NIH Career Development grant, which I hope to submit in the next 3-6 months.

How were the results disseminated to communities of interest?

I have presented this project to groups at my institution (including the UTSW Kidney SPORE and Wilms Tumor Group, as described above). Additionally, work from this proposal was published the journal *Development* (see manuscript attached).

What do you plan to do during the next reporting period to accomplish the goals?

For research aim 1, to determine the mechanism in which stromal beta-catenin inhibits NPC differentiation, we have identified several candidate genes from the bulk RNA seq data was previously performed, making it less critical to isolate stroma and perform additional RNA seq studies. For the next step to test the functionality of these genes in the non-autonomous regulation of NPCs, I am proposing to use an alternative method to lentiviral transfection of explanted kidneys. As proposed in major task 2, over-expressing and knocking down genes using lentiviral vectors in kidney explants may not provide optimal transfection rates and could have off target effects on the NPCs, as the viral vectors are not cell type specific. Therefore, we expect to achieve more effective transfections with the ability to specifically target stromal cells if these studies were performed using organoid assays instead of explant cultures. To do this, the Carroll lab has developed techniques to isolate cells using magnetic beads (ie: stromal cells vs NPCs), which can then be transfected with lentiviral vectors when in a cell suspension, and used to generate organoids. This would have the added advantage of being able to target different mutations to different cell types/lineages, which would be a powerful tool to examine the roles of signaling pathways/genes identified in Wilms tumorigenesis.

For research aim 2, to examine human samples for cell-lineage specific beta-catenin mutations to determine if stromal beta-catenin mutation can occur in isolation from blastema/epithelial cell populations, I have been working closely with our institution's biorepository to identify and obtain samples, now that non-COVID studies are returning to operation. I have all ready obtained 3 samples of human WT with known CTNNB1 mutations, and I am planning to move forward with submitting a human tumor and control embryonic kidney for single nuclei sequencing in the next

month. Once additional tissue is obtained, I will move forward with performing in-situ PCR to localize specific CTNNB1 mutations to stroma, blastema, or epithelial cells and determine if the same mutation is present in multiple cell lineages, as well as the additional gene expression studies (using antibodies, in-situ hybridization, and/or RNA scope) on human WT samples.

For research aim 3, to evaluate cell-lineage effects of beta-catenin activating mutations to determine if beta-catenin activation in specific cell lineages during kidney development recapitulates human Wilms tumor, the remaining experiments to complete are outlined in major task 2, which is to generate organoids from these mutant lines and implant them under the kidney capsule of NOD SCID mice to assess for tumorigenic potential. These studies will provide an interesting comparison of whether or not maintaining residual "normal" cell signaling in the mileau of cells with beta-catenin activation affects the tumorigenic potential that was not observed in the in-vivo cre/lox models.

We expect to be able to carry out the above experiments now that research activity has resumed on campus, though at limited capacity and with social distancing. Additionally, the biorepository is now active, so we expect to be able to obtain the requested tissue in a timely manner.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

Our work as well, as previous studies, have shown that activating beta-catenin mutations are observed in both blastema and stromal components of WT. It has long been assumed that the causal mutation in WTs occurs in the blastemal component, with current models presuming that activation of beta-catenin in the blastemal lineage contributes to WT formation. However, we and others have shown that activation of beta-catenin in the nephron progenitor lineage paradoxically resulted in loss of NPC renewal in mice, a phenotype opposite toWT. Conversely, we showed that activation of beta-catenin in the stromal progenitor population resulted in mutant kidneys with remnant epithelial structures surrounded by undifferentiated mesenchyme and spindle-shaped fibroblasts, similar to the histology of WT. Furthermore, transcriptomes of mutant mouse kidneys with activating mutations in either the nephron progenitor lineage or the stromal lineage were compared with human WTs, revealing that WT shares characteristics of the stromal-lineage mutants more so than wild-type developing kidney or NPC lineage mutants. Taken together, these findings suggest that activation beta-catenin in the stroma contributes to WT pathogenesis, and further understanding of the lineage-specific effects of CTNNB1 mutations may lead to a better understanding of how the stromal microenvironment contributes to tumorigenesis.

What was the impact on other disciplines?

In normal kidney development, WNT/beta-catenin signaling regulates multiple aspects of nephrogenesis, including NPC maintenance, mesenchymal-to-epithelial transition, ureteric bud progenitor renewal, and differentiation of the interstitium. By examining the transcriptomic effects of beta-catenin activating mutations in multiple cell lineages of the developing kidney, we have further defined how abnormal activation of this signaling pathway exerts both cell-type specific and lineage-independent effects in renal development. While these findings are not only of

interest from the perspective of WT pathogenesis, they also aid in further understanding the roles of the renal stroma in regulating normal development. While distinctions between cortical and medullary interstitial cells have been previously recognized, recent work has revealed a surprising degree of heterogeneity in the embryonic renal interstitium (England et al., Development, 2020). These findings suggest that unique subpopulations or specific zones/regions of the developing renal interstitium may regulate the maturation or specification of adjacent cell types in the normally developing kidney. In support of this, inactivation of beta-catenin in the stromal progenitor population using Foxd1Cre has been shown to block development of the papillary interstitium as well as adjacent epithelial cells of the loop of Henle (England et al., Development, 2020 and Yu et al., Development, 2009). Conversely, in work performed under this study, we showed that activation of beta-catenin in the stromal progenitor population drives expression of papillary interstitial cells, with this abnormal interstitial patterning disturbing the normal stromal microenvironment, and leading to a lack of differentiation and altered gene expression in NPCs, with these findings contributing to the further understanding of nephrogenesis in the field of kidney development.

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

Nothing to report

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Overall, the work performed in the first year of funding was adjusted from the timeline previously proposed in the statement of work, primarily due to an institutional wide laboratory shut down in initiated in April 2020. This limited our mouse work to only essential activities to maintain the colony, with other experiments to generate tissue (ie: for RNA seq and explant studies) placed on hold as directed by our institition. Additionally, we have been delayed in obtaining human WT samples from our institution's biorepository, which was closed for requests from April to July 2020 due to the COVID shutdown. Given these obstacles, we worked with the data and tissue that we had already generated in an effort to continue to make progress toward each research aim as outlined in the accomplishments section of this report.

As described above in the planned experiments for the upcoming year, we are proposing a relatively minor change in approach to the first research goal. Since we were able to identify and validate several candidate genes from the bulk RNA seq data was previously performed, it is now less critical to perform additional RNA seq studies on isolated stroma from mutant kidneys. Instead, I am proposing to focus on the next step of testing the functionality of these genes in the non-autonomous regulation of NPCs. To do this, I plan to utilize lentiviral vectors to over-express and knock down genes in isolated cell-type specific suspensions that can then be used to generate organoids as described above. This will likely provide more effective transfection, with more robust gene expression changes, and allow us to target specific cell populations, potentially serving as a

novel technique to assess multiple genes/signaling pathways in generating Wilms tumor-like phenotypes in the developing kidney.

Actual or anticipated problems or delays and actions or plans to resolve them

As described above, we were delayed in obtaining biobank samples due to the institutional wide shutdown. To remedy this, I have been in close contact with members of biorepository, and while I have already obtained some frozen samples, we expect to be able to obtain all the requested tissue samples in the next 1-2 months.

Furthermore, despite resuming research with some social distancing/lab capacity restrictions, we have been able to resume work with our mouse colony to generate the needed tissue for the proposed experiments for the upcoming year.

Changes that had a significant impact on expenditures

Please see below for a description of the budget and justification, including any changes made to the budget for year 1 provided with the initial grant proposal:

PERSONNEL $82,919 (Year 1), $85,408 (Year 2)

No changes were made.

TRAVEL $1200 (Year 2)

No changes were made.

MATERIALS AND SUPPLIES $4,124 (Year 1), $436 (Year 2)

Due to the delayed in-vitro and cell culture work, expenses not utilized during year 1 are requested to be deferred to year 2.

OTHER EXPENSES (CORE SERVICES) $10,000 (Year 1), $10,000 (Year 2)

Due to the delay in obtaining human WT samples for sequencing and delay in utilizing the UTSW lentiviral vector core, expenses not utilized during year 1 are requested to be deferred to year 2.

ANIMAL COSTS $2,956 (Year 1), $2,956 (Year 2)

Due to the limited mouse work performed during our institutional COVID shutdown, expenses not utilized during year 1 are requested to be deferred to year 2.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

The regulatory review and approval by the USAMRMC Animal Care and Use Review Office (ACURO) for animal work included in the institutionally approved PI's protocol (APN 2019-102701) and the USAMRMC Human Research Protection Office (HRPO) obtain de-identified

samples of humans Wilms tumor from our institutional biorepository per PI's IRB (STU-2019-1047) were both completed in 9/2019, with no significant changes to report.

Significant changes in use or care of human subjects

Nothing to report

Significant changes in use or care of vertebrate animals

Nothing to report

Significant changes in use of biohazards and/or select agents

Nothing to report

6. PRODUCTS:

- **Publications, conference papers, and presentations** .

  Journal publications.

  Drake KA, Chaney CP, Das A, Roy P, Kwartler CS, Rakheja D, Carroll TJ. Stromal activation of beta-catenin in the developing kidney impacts nephron progenitor differentiation and may contribute to Wilms tumor. Development. 2020; 147(21). PMID: 32541007. Published in July 2020. Acknowledgement of federal support: Yes.

  Books or other non-periodical, one-time publications.

  Nothing to report

  Other publications, conference papers and presentations.

  Nothing to report

- **Website(s) or other Internet site(s)**

  Nothing to report

- **Technologies or techniques**

  Nothing to report

- **Inventions, patent applications, and/or licenses**

  Nothing to report

- **Other Products**

  Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: Keri Drake – No change
Name: Christopher Chaney – No change
Name: Mohita Patel – No change

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

The only change in support for personnel on the grant is for Keri Drake (PI), who's active funding from the CCRAC Early Career Research Award (9/2018 - 8/2020) has been completed.

What other organizations were involved as partners?

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

Nothing to report

9. APPENDICES:

Please see the attached published manuscript.

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RESEARCH ARTICLE

Stromal β-catenin activation impacts nephron progenitor differentiation in the developing kidney and may contribute to Wilms tumor

Keri A. Drake[1], Christopher P. Chaney[2], Amrita Das[3], Priti Roy[4], Callie S. Kwartler[5], Dinesh Rakheja[6] and Thomas J. Carroll[2,*]

ABSTRACT

Wilms' tumor (WT) morphologically resembles the embryonic kidney, consisting of blastema, epithelial and stromal components, suggesting tumors arise from the dysregulation of normal development. β-Catenin activation is observed in a significant proportion of WTs; however, much remains to be understood about how it contributes to tumorigenesis. Although activating β-catenin mutations are observed in both blastema and stromal components of WT, current models assume that activation in the blastemal lineage is causal. Paradoxically, studies performed in mice suggest that activation of β-catenin in the nephrogenic lineage results in loss of nephron progenitor cell (NPC) renewal, a phenotype opposite to WT. Here, we show that activation of β-catenin in the stromal lineage non-autonomously prevents the differentiation of NPCs. Comparisons of the transcriptomes of kidneys expressing an activated allele of β-catenin in the stromal or nephron progenitor cells reveals that human WT more closely resembles the stromal-lineage mutants. These findings suggest that stromal β-catenin activation results in histological and molecular features of human WT, providing insights into how alterations in the stromal microenvironment may play an active role in tumorigenesis.

KEY WORDS: β-Catenin, Wilms' tumor, Renal development, Stroma, Renal interstitium

INTRODUCTION

Wilms' tumor, or nephroblastoma, is an embryonal tumor classically consisting of triphasic histology, with blastemal/nephron progenitor, epithelial and stromal components thought to arise from disruptions in normal fetal nephrogenesis (Treger et al., 2019; Rivera and Haber; 2005; Hohenstein et al., 2015). During normal kidney development, nephron progenitor cells (NPCs) are maintained through renewing cell divisions. However, a subset of these cells simultaneously lose their progenitor cell identity and undergo mesenchymal-to-epithelial transition (MET) to form an immature tubule that will become a nephron, the functional unit of the kidney. A balance between self-renewal and differentiation is essential for generating kidneys with a sufficient number of nephrons necessary for function. This process is highly regulated and is known to rely on signals emanating from the epithelial cells of the ureteric bud as well as the surrounding stromal/interstitial signaling to the NPCs (Das et al., 2013; Fetting et al., 2014; Hum et al., 2014).

In a normal human kidney, the NPCs are exhausted prior to birth. However, in WT, blastemal cells/NPCs persist and continue proliferating well into the postnatal period. Still, much remains to be understood regarding the mechanism of this malignant transformation and resultant triphasic histology. Of particular relevance, the contribution of the stroma to Wilms' tumorigenesis remains largely unknown. While the stroma/interstitium plays multiple roles in supporting normal tissue development and homeostasis, it has also been shown to contribute to tumor formation, progression and metastasis in many cancers (Clark and Vignjevic; 2015; Li et al., 2007; Valkenburg et al., 2018). Tumor 'stroma' refers to all components of the interstitium, including fibroblasts, immune cells and vasculature (i.e. endothelium and endothelial-associated mural cells), as well as the basement membrane and extracellular matrix (Bremnes et al., 2011). In this study, we will focus on the non-immune, non-vascular, cellular components of the stroma, given that immature stroma/interstitial fibroblast cells abnormally persist and proliferate in WT.

Activating mutations in the gene encoding β-catenin, *CTNNB1*, occur in about 15% of WTs (Li et al., 2004; Maiti et al., 2000). However, nuclear accumulation of β-catenin is observed in up to 50% of tumors (Koesters et al., 2003), suggesting that aberrant activation of this pathway is crucial in a significant proportion of WTs. β-Catenin is a component of the canonical Wnt signal transduction pathway. In the absence of a WNT ligand, β-catenin is phosphorylated, sequestered in the cytoplasm and tagged for degradation. However, in the presence of a WNT ligand, the cytoplasmic complex that phosphorylates β-catenin dissociates, freeing β-catenin to translocate into the nucleus and promote transcription. The mutations in the β-catenin gene observed in WT render the protein insensitive to degradation, thus resulting in a constitutively stabilized form lacking regulation of its transcriptional activity.

In normal kidney development, Wnt/β-catenin signaling regulates multiple aspects of nephrogenesis, including nephron progenitor renewal, mesenchymal-to-epithelial transition, ureteric bud progenitor renewal and differentiation of the interstitium (Boivin and Bridgewater; 2018; Boivin et al., 2016; Park et al., 2007; Sarin et al., 2014; Marose et al., 2008; Karner et al., 2011; Ramalingam et al., 2018). Which of these processes is perturbed in WT is still unclear.

[1]Division of Pediatric Nephrology, University of Texas Southwestern Medical Center, Dallas, TX 75390, USA. [2]Department of Molecular Biology and Internal Medicine, University of Texas Southwestern Medical Center, Dallas, TX 75390, USA. [3]Amgen, Inc., San Francisco, CA 94080, USA. [4]Department of Ophthalmology and Visual Sciences, Chicago, IL 60612, USA. [5]Division of Medical Genetics, Department of Internal Medicine, McGovern Medical School, University of Texas Health Science Center at Houston, Houston, TX 77030, USA. [6]Department of Pathology, University of Texas Southwestern Medical Center, Dallas, TX 75390, USA.

*Author for correspondence (thomas.carroll@utsouthwestern.edu)

T.J.C., 0000-0002-8322-4928

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Nuclear expression of β-catenin, as well as cells carrying *CTNNB1*-activating mutations, are observed among the different cell lineages of WT including blastemal, stromal, epithelial and even heterologous components such as skeletal muscle (Corbin et al., 2009; Duhme et al., 2019; Uschkereit et al., 2007). It has long been assumed that the causal mutation in WTs occurs in the blastemal component (Charles et al., 1998); however, recent studies performed in mice demonstrate that mutations in Wilms' candidate genes (including *Lin28* and *Wt1*/*Igf2*) in the blastemal/nephron progenitor component alone are not sufficient to cause WT (Urbach et al., 2014; Huang et al., 2016). It is becoming increasingly apparent that the balance between nephron progenitor maintenance and differentiation is regulated by signals from the renal stroma, and we and others have shown that perturbations in stromal differentiation result in abnormally maintained NPCs reminiscent of nephrogenic rests in WTs (Das et al., 2013; Hum et al., 2014). Given the established roles of the stroma in tumor progression and its developmental role in regulating nephron differentiation, and that WT stroma harbors β-catenin-activating mutations, we decided to interrogate the role of the stroma in WT.

Here, we characterize mice carrying an activating mutation of β-catenin specifically in the stromal progenitor population. We show that mutant kidneys form remnant epithelial structures surrounded by undifferentiated mesenchyme and spindle-shaped fibroblasts, similar to the histology of WT. Transcriptomes of mutant mouse kidneys with activating mutations in either the nephron progenitor lineage or the stromal lineage were compared with human WTs revealing that WT shares characteristics of the stromal-lineage mutants, more so than wild-type developing kidney or NPC lineage mutants. Indeed, expression of Six2, Cited1 and Ncam, diagnostic markers of WT, is observed in the stromal lineage mutants and not the NPC lineage mutants. Finally, we show that activation of β-catenin simultaneously in the nephron progenitor and stromal lineages (using dual Six2cre and Foxd1cre expression) results in severely disrupted kidney development with the formation of bone-like tissue, a phenotype that has been reported in human WT (Pritchard-Jones, 1997). These findings suggest that activation of β-catenin in the stroma contributes to WT pathogenesis. Further understanding of the lineage-specific effects of β-catenin-activating mutations, as well as a more-detailed analysis of the tumor stromal microenvironment, may aid in unraveling the molecular and cellular mechanisms underlying WT.

RESULTS

Activating mutations of β-catenin are found in both the stroma and NPCs of WT

Although it has long been known that WTs contain activating mutations in β-catenin, the role of these mutations (drivers versus passenger) and the cell type of origin has been somewhat controversial. Several groups have shown that activating mutations in β-catenin can be found in all cell types of WTs (Corbin et al., 2009; Duhme et al., 2019; Uschkereit et al., 2007). To confirm these observations, we isolated stromal and blastemal cells using laser capture microdissection from three different WT samples carrying activating mutations of β-catenin. In all three samples, the mutant allele was identified in both blastemal and stromal cellular components (representative data from one sample shown in Fig. 1). As the blastema and stroma arise from different cellular lineages in a normal kidney, and the two lineages appear to sort independently in early kidney development, the fact that the mutations are found in both cell populations within a tumor supports the claim that the mutagenic event occurred in a common precursor cell for both lineages (the intermediate mesoderm) and raises the possibility that activation in the stromal progenitor lineage contributes to the pathology of WT.

Activation of β-catenin within different lineages of the metanephros severely perturbs renal development, with stromal activation demonstrating histological characteristics of Wilms' tumor

β-Catenin is ubiquitously expressed in the developing kidney. Through the use of various lineage-restricted Cre lines and alleles of β-catenin that can be activated or inactivated by Cre, this factor has been shown to play a role in the balance of NPC maintenance and differentiation/mesenchymal-epithelial transition, as well as in the development of interstitial cells and the ureteric bud progenitors (Boivin and Bridgewater; 2018; Boivin et al., 2016; Park et al., 2007; Sarin et al., 2014; Marose et al., 2008; Karner et al., 2011; Ramalingam et al., 2018; Yu et al., 2009). Although previous studies have characterized kidneys carrying activated alleles of β-catenin ($Catnb^{ex3/+}$) within both the NPC and stromal lineages, we reanalyzed both mutants with a focus on their relationship to WT pathology. Six2Cre is active in the nephron progenitor cells, and Six2cre;$Catnb^{ex3/+}$ mutants lack developing nephrons and show premature loss of NPCs (Fig. 2C). Lineage-positive cells instead form aggregates of cells that persist throughout development and do not appear either mesenchymal or epithelial (Fig. 2D, Fig. S1).

Foxd1 is expressed in a population of mesenchymal cells in the cortex of the embryonic kidney, and lineage tracing studies have shown that Foxd1-derived cells give rise to non-endothelial non-immune stromal cells, including pericytes, fibroblasts, mesangium and vascular smooth muscle cells (Kobayashi et al., 2014). In comparison with activation with Six2Cre, activation of β-catenin with Foxd1Cre (Foxd1cre;$Catnb^{ex3/+}$, from here on referred to as stromal or interstitial activation) results in abnormal maintenance of the NPC population, with delayed nephrogenesis (Fig. 2E). Gross morphologic examination revealed that stromal mutant kidneys were fused to the body wall. E18.5 kidneys showed a complete absence of mature nephrons (Fig. 2F′) with expanded interstitial cells surrounding undifferentiated NPCs and immature epithelia resembling renal vesicles or s-shaped bodies, grossly resembling the morphology of WTs. Lineage tracing of mutant kidneys derived from the two different Cre driver strains confirmed recombination in the expected nephron progenitor and stromal lineages, with the majority of lineage-positive cells showing pathologically high levels of nuclear β-catenin (Fig. S1).

Activation of β-catenin within the stromal lineage results in expanded nephron progenitor populations with delayed mesenchymal-to-epithelial transition

WTs are characterized by the expression of a number of genes normally associated with undifferentiated NPCs, including *Six2*, *Pax8* and *Ncam*. We thus sought to assess the expression of these markers in kidneys with activating mutations of β-catenin in either lineage. Previous work suggests that activation of β-catenin in the NPC lineage promotes differentiation, with decreased numbers of NPCs, an increased number of pre-tubular aggregate structures expressing Wnt4, Pax8 and Lhx1, and a blockade in MET/differentiation (Park et al., 2007). Re-analysis of these mutants shows that this 'pre-tubular aggregate-like state' is transient, and by E15.5, NPCs lose Six2 expression as well as expression of Lhx1, Pax8 and Ncam (Fig. 3G,H). Thus, at a molecular level, activation of β-catenin within the NPC lineage does not lead to a WT-like phenotype nor does it lead to a maintenance of pre-tubular aggregate-like structures.

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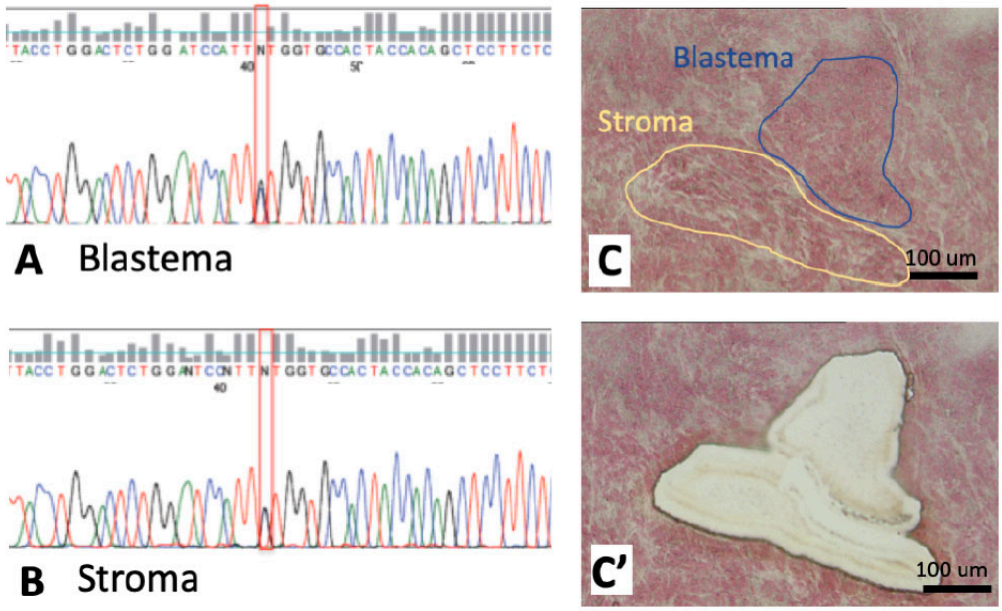

Fig. 1. Both blastemal and stromal components of human Wilms' tumor carry *CTNNB1*-activating mutations. (A-C′) Sequence reads of DNA extracted from blastema (A) and stroma/interstium (B) isolated using a laser microcapture dissection (C,C′) are shown from a representative tumor, demonstrating that both cell populations carry the same *CTNNB1* point mutation. Two additional tumors analyzed show the same frame shift in the second tumor and the same in/del in the third (data not shown). Scale bars: 100 µm.

Next, we further examined the abnormally maintained NPCs in stromal mutant kidneys. In contrast to activation within the NPC lineage, E15.5 Foxd1cre;Catnb$^{ex3/+}$ kidneys maintain expression of Six2 and Ncam (Fig. 3K,L and Fig. 4H). However, expression of pre-tubular aggregate/differentiating NPC markers is abnormal, with some markers, including *C1qdc2* and *Wnt4*, showing expanded expression surrounding the NPCs, while others, including Pax8 and Lhx1, are not expressed (Fig. 4I-L, respectively). At E18.5, a small number of structures expressing Lhx1 are present (Fig. 4N), corresponding to histologically identifiable renal vesicles and perhaps comma and S-shape bodies (Fig. 2F). These findings suggest that stromal activation of β-catenin not only non-autonomously blocks NPC differentiation, but also fundamentally alters the molecular state of these cells, demonstrating that disruption of the normal stromal microenvironment significantly affects the differentiation of the neighboring NPC population.

Foxd1Cre-mediated activation of β-catenin disrupts stromal patterning

Previously, it has been shown that ablation of the stromal progenitor population results in abnormally maintained NPCs that are reminiscent of nephrogenic rests (Das et al., 2013). Foxd1cre;Catnb$^{ex3/+}$ kidneys show some hallmarks of these stroma-less kidneys, including abnormally expanded Six2-expressing NPCs surrounding the UB (Fig. 3K). Given these findings, we previously hypothesized that nephrogenic stromal cells produce a signal that promotes differentiation or blocks renewal of the NPCs. With this in mind, we examined the molecular phenotype of stromal cells upon activation of β-catenin with Foxd1Cre. Foxd1cre;Catnb$^{ex3/+}$ kidneys show early loss of the stromal progenitor population, as indicated by decreased expression of Foxd1, *Ntn1* and *Smoc2* (Fig. 5G,H,S). Instead, several genes normally expressed in the papillary stroma were precociously and ectopically expressed in the cortex, including *Cpmx2*, *Sdc2*, *Dpp6* and *Wnt5a*, although *Wnt4* papillary stroma expression was lost (Fig. 5J-L,R,T) (Shan et al., 2010). Mutant kidneys showed decreased expression of the cortico-medullary stromal markers *Penk* and *Smoc2* (Fig. 5I,S). These findings suggest that activation of β-catenin in the stromal progenitor cells may be leading to precocious and ectopic differentiation of a more papillary stromal cell type. Given that β-catenin has previously been shown to be necessary for the development of the papillary stroma (Yu et al., 2009; Boivin and Bridgewater; 2018; Boivin et al., 2016), our findings suggest it is also sufficient.

Transcriptome profiling suggests human WT resembles mutant mouse kidneys with activation of β-catenin specific to the stromal lineage

Previous transcriptional analyses of human WT suggests the upregulation of numerous β-catenin targets genes (Gadd et al., 2017; Li et al., 2004; Zirn et al., 2006). However, as β-catenin is active in multiple lineages within the kidney and turns on unique targets in each lineage, we next sought to further characterize β-catenin target genes upregulated in human WT by investigating their expression in normal mouse kidneys, as well as our lineage-specific β-catenin mutants. This was accomplished by performing RNA-Seq on both Six2cre;Catnb$^{ex3/+}$ and Foxd1cre;Catnb$^{ex3/+}$ mutant kidneys (see Table S1). We next used BETA to integrate the differentially expressed genes from these mutant mouse models with beta-catenin CHIP-seq data (Table S2), thus generating a list of genes that are likely to be directly activated by β-catenin in each lineage.

To identify genes that are likely to be mis-regulated in WT directly due to activation of β-catenin, we then analyzed RNA-seq from WT samples in the TARGET database, comparing gene

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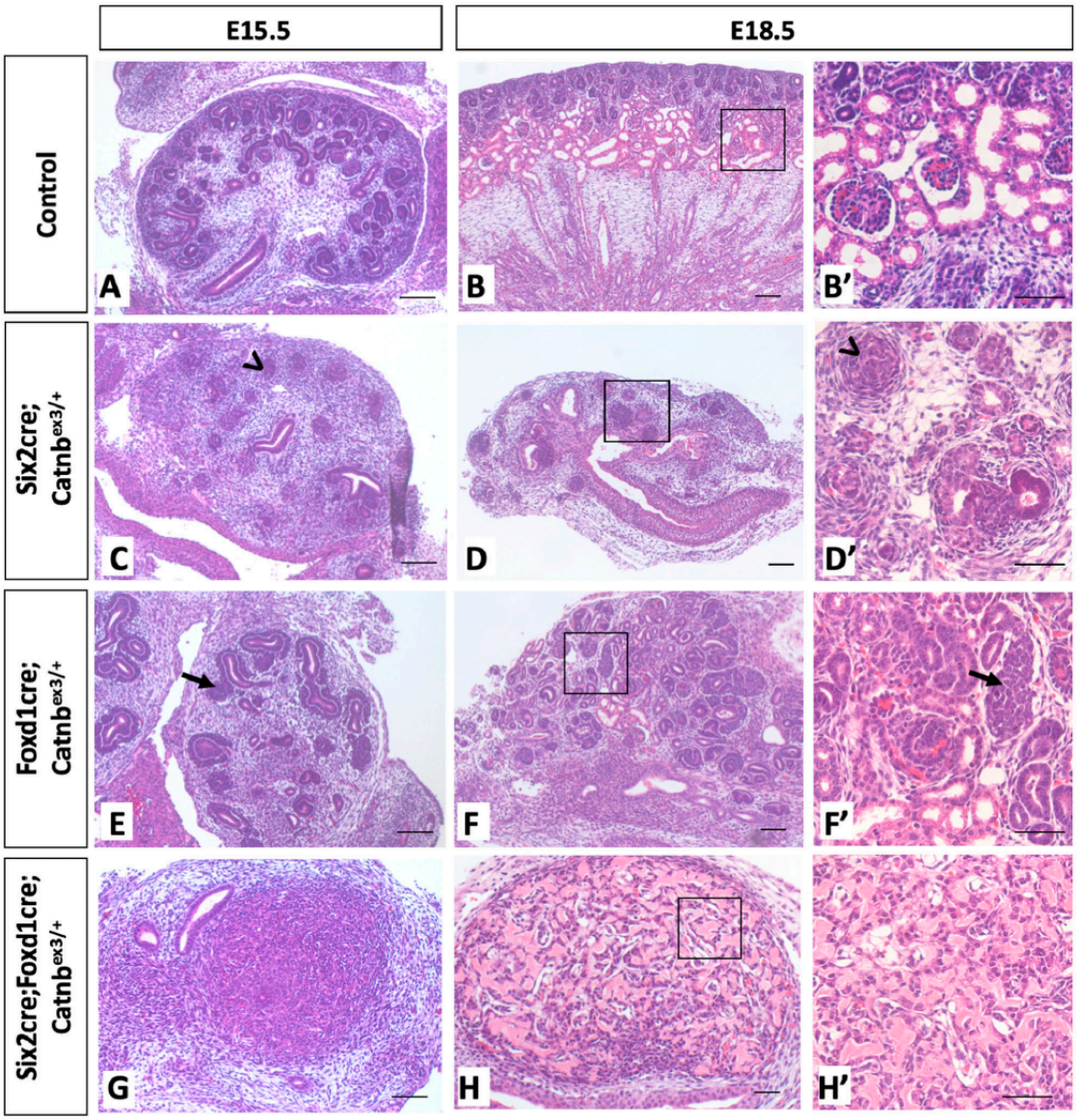

Fig. 2. Activation of β-catenin in different lineages of the developing kidney severely perturbs nephrogenesis, with stromal activation resulting in abnormal maintenance of NPCs and disrupted MET. (A-H′) In comparison with wild-type kidneys (A-B′), Six2cre;Catnb$^{ex3/+}$ kidneys (C-D′) show early loss of NPCs and lack of MET, whereas Foxd1cre;Catnb$^{ex3/+}$ kidneys (E-F') show abnormally maintained NPCs, lacking differentiating structures at E15.5 (E, arrow). By E18.5, some NPCs are induced and undergo nephrogenesis, but regions of abnormally maintained NPCs remain in the developing kidney (F′, arrow). (G-H′) Six2cre;Foxd1cre; Catnb$^{ex3/+}$ kidneys show little resemblance to the developing metanephros and form bone-like tissue later in development. Scale bars: 100 µm. *n*=3 for each timepoint/genotype.

expression profiles of human WTs with and without *CTNNB1*-activating mutations and cross-referenced these data with the list of likely direct β-catenin target genes from BETA (Table S3) and previously published targets. We next qualitatively compared this list with the lineage-specific mutant mouse models to determine whether one model more closely matched the expression pattern of WT with *CTNNB1*-activating mutations. There was no discernible alignment using this method or gene-set enrichment analyses. In fact, somewhat unexpectedly, both mouse mutants showed upregulation of several of the same WT-enriched target genes. This is surprising, given that many of these target genes, including *Nkd1*, *Gap43*, *Axin2*, *Notum*, *Mmp11* and *Apcdd1* are normally expressed specifically in the papillary stroma of wild-type kidneys (Fig. S2 and Fig. 6). Characterization of the expression of several of these genes in Six2cre;Catnb$^{ex3/+}$ and Foxd1cre;Catnb$^{ex3/+}$ kidneys showed that all β-catenin target genes assessed were precociously and ectopically expressed in both mutants (Fig. 6). However, in the Foxd1cre mutants, target expression was observed in all Foxd1cre

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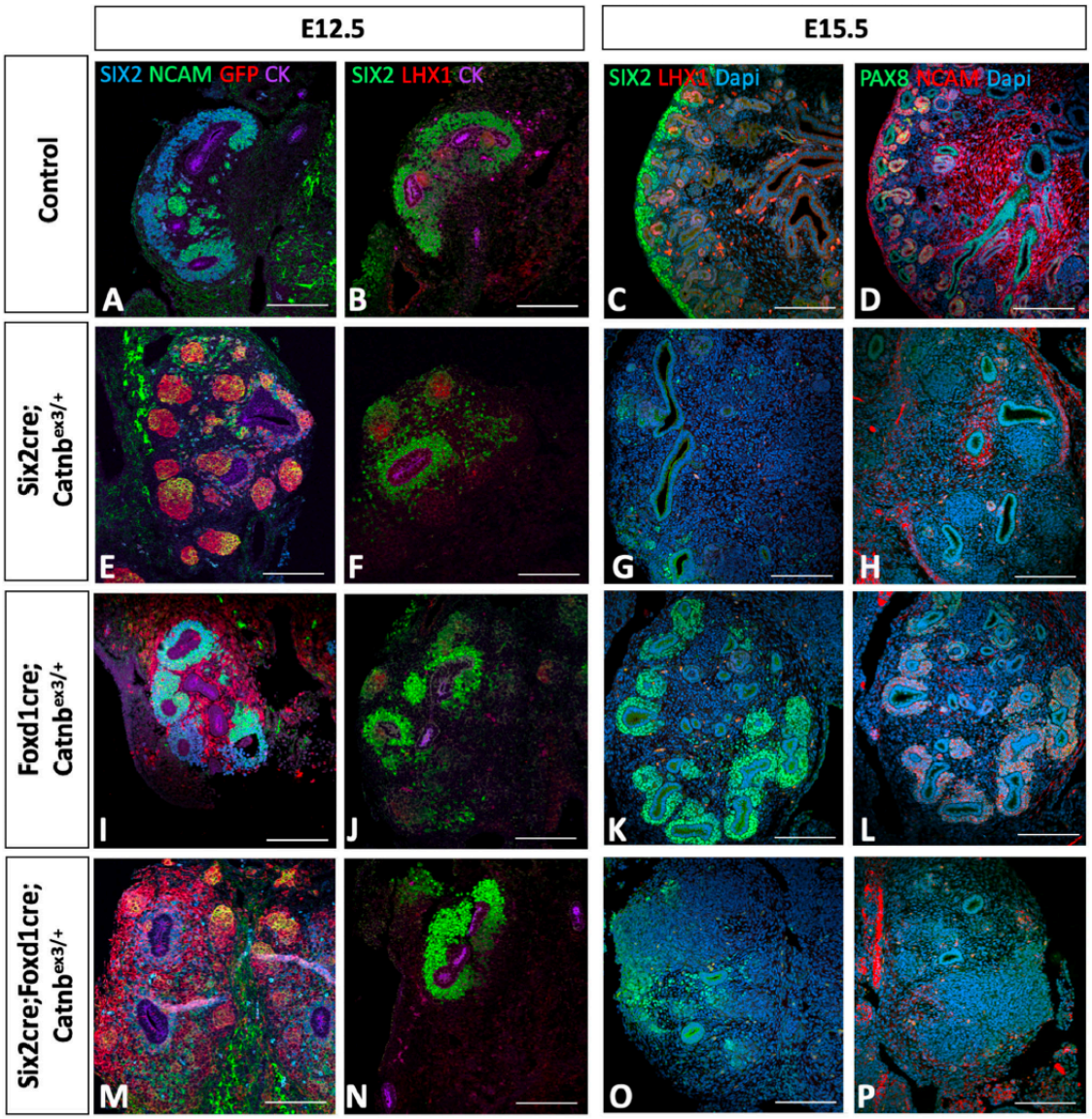

Fig. 3. β-Catenin activation within the nephron progenitor lineage results in premature loss of the nephron progenitor/blastemal cells, while the opposite phenotype is observed in response to β-catenin activation within the stroma. (A-P) In comparison with control kidneys (A-D), Six2cre;Catnb$^{ex3/+}$ mutant kidneys (E,F) show early loss of Six2-positive NPCs with transient expression of Ncam (E) and Lhx1 (F), consistent with a 'pre-tubular aggregate (PTA)-like state', as previously published. (G,H) However, by E15.5, these cells no longer express PTA or renal vesicle markers, including Lhx1, Pax8 and Ncam. (I-L) Foxd1cre;Catnb$^{ex3/+}$ show abnormally maintainted NPCs expressing Six2 and Ncam lacking Lhx1 (K) and Pax8 (L). (M-P) Six2cre;Foxd1cre;Catnb$^{ex3/+}$ mutants initially resemble Six2cre;Catnb$^{ex3/+}$ mutants at E12.5 (M-N), then lose expression of Six2 and Ncam-positive NPCs (O-P). Scale bars: 100 µm. *n*=3 for each timepoint/genotype.

lineage positive stroma (rather than being restricted to the medullary interstitium); in Six2cre mutants, expression was relatively normal in the papillary interstitium but was also observed ectopically in Six2cre-derived cells.

We next developed a more comprehensive method to compare β-catenin targets in human WT with our mutant mouse models. Given the considerable complexity and size of the TARGET dataset, we used a deep learning classification technique. Using a trained neural network classifier, the expression profiles for 124 Wilms' tumor samples were mapped to the expression data generated from kidneys of each of the three mouse genotypes (wild type, Six2cre;Catnb$^{ex3/+}$ and Foxd1cre;Catnb$^{ex3/+}$), resulting in a score ranging from 0 (no match) to 1.0 (representing a perfect match). As shown in Fig. 7, nearly all the human WT samples, including six tumors with known *CTNNB1*-activating mutations, showed almost exclusive mapping to the Foxd1cre;Catnb$^{ex3/+}$ transcriptome (scores ranging from 0.55 to 0.99), with only two samples showing an appreciable degree of similarity to wild-type kidneys (scores of 0.28 and 0.44),

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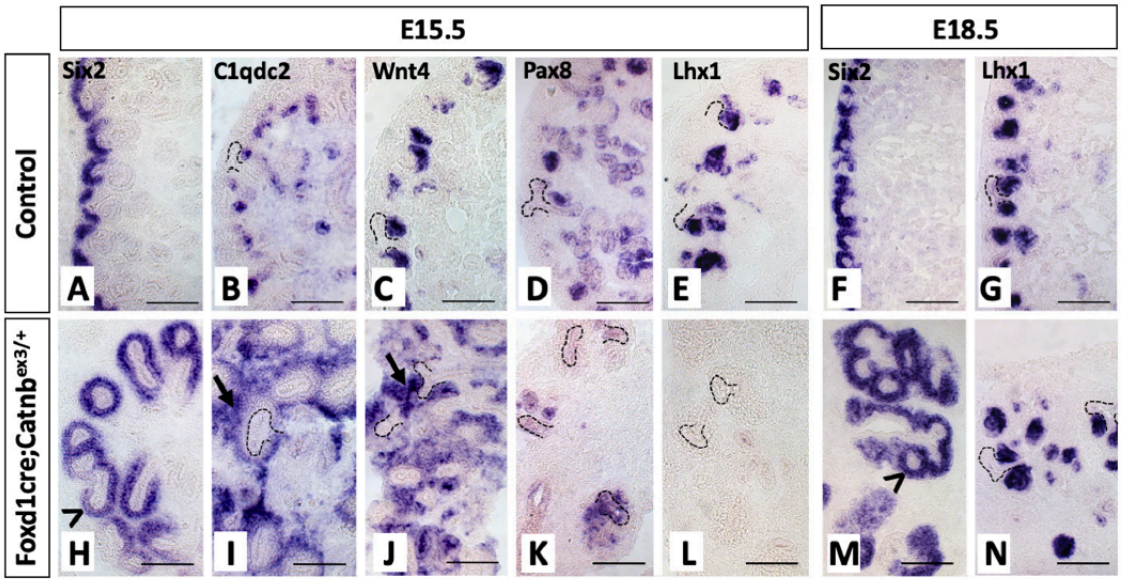

Fig. 4. β-Catenin activation in stromal lineage results in expanded nephron progenitor cells with delayed MET. (A-L) In comparison with control kidneys (A-G), NPCs of Foxd1cre;Catnb$^{ex3/+}$ mutant kidneys (H-L) show abnormal expansion at E15.5, expressing both markers of both self-renewal (H; Six2, arrowhead) and early commitment to differentiation/MET (I, C1qdc2; J, Wnt4; arrows) but lack expression of other MET markers (K, Pax8; L, Lhx1). (M,N) However, by E18.5, Six2-positive NPCs remained expanded (M, arrowhead) and Lhx1-positive structures are present (N), corresponding to histologically identifiable comma and S-shape-like bodies visualized using Hematoxylin and Eosin staining. Scale bars: 100 µm. *n*=3 for each timepoint/genotype.

and none of the samples mapping to the Six2cre;Catnb$^{ex3/+}$ expression profile (Table S4). This unbiased, quantitative approach, along with our histological studies, suggests that activation of β-catenin in the NPC lineage does not appear to transcriptionally recapitulate WT. Interestingly, despite human WT histologically resembling normal development, this analysis suggests that it is transcriptionally more similar to the mutant kidneys with stromal activation of β-catenin than to normal, wild-type embryonic kidney.

Activation of β-catenin simultaneously within both the NPC and stromal lineages results in ectopic bone formation

Although our data suggest WTs with activating mutations in β-catenin are most similar at the molecular level to mouse kidneys with an activating mutation in the stromal lineage, neither Foxd1cre;Catnb$^{ex3/+}$ or Six2cre;Catnb$^{ex3/+}$ mutants precisely mimic WT. As it is likely that WTs acquire mutations in β-catenin early in their history, we next sought to determine whether activation of β-catenin in both lineages was sufficient to lead to WT. We first sought to activate β-catenin in cells that represented a common progenitor to both the stroma and NPCs using T (brachyury)-creERT2. Using a Rosa-LSL-YFP lineage tracer, we observe recombination within the kidney when tamoxifen was administered to the TcreERT2;Catnb$^{ex3/+}$ embryos early in gestation at E7.5, E8.5 or E9.5; however, unexpectedly the kidneys appeared completely normal at E18.5 (Fig. 8) and at 4 months of age even though phenotypes outside the kidneys (such as curly tails) were visible (data not shown). Further analysis showed that lineage-positive cells within the kidney lacked detectable nuclear β-catenin (Fig. 8B). This was quite unexpected given the strong detectable nuclear β-catenin staining observed in the Six2cre;Catnb$^{ex3/+}$ and Foxd1cre;Catnb$^{ex3/+}$ mutants (Fig. 8C,D). This lack of nuclear β-catenin in the TcreERT2 mutants suggests that early renal precursor cells expressing increased levels β-catenin are preferentially selected against or subsequently downregulate β-catenin to allow continued development. Similar results were observed with another line driving Cre recombination in the intermediate mesoderm (Osr1CreERT2, data not shown).

To circumvent possible negative selection/cell competition, we simultaneously and uniformly activated β-catenin in both the NPC and stromal lineages by creating Six2cre;Foxd1cre;Catnb$^{ex3/+}$ mutant kidneys. As shown in Fig. 2, Six2cre;Foxd1cre;Catnb$^{ex3/+}$ mutant kidneys show no distinguishable features of normal kidney development at E15.5, with progenitor cells appearing to surround a primitive UB/nephric duct. Interestingly, by E18.5, these cells have a 'bone-like' appearance, with histology showing a non-calcified, bone-like matrix and strong alkaline phosphatase staining within the lineage-positive cells (Fig. 9A-C).

Although β-catenin targets including Lef-1, a transcription factor involved in canonical Wnt/β-catenin signaling that has also been shown to be a direct target of β-catenin, are upregulated in lineage-positive cells in all three mutant lines (Fig. 9G-K), it is notable that the 'bone-like' phenotype, which is observed in some WTs, is observed only in the Six2cre;Foxd1cre;Catnb$^{ex3/+}$ mutants. This observation clearly supports the hypothesis that activation of β-catenin in the stromal lineage of WTs is not inert. We propose that activation of β-catenin in the stroma contributes in multiple ways to WT pathology.

DISCUSSION

WT, the most common type of kidney cancer in children, is thought to arise from transformed cells originating within the developing kidney. While WT1 and β-catenin mutations were some of the first known pathways proposed in WT pathogenesis, advances in sequencing technology have revealed WT to be genetically heterogenous, with a number of different genetic perturbations commonly resulting in a preserved nephron progenitor state and/or interrupted normal development (Gadd et al., 2017; Treger et al., 2019).

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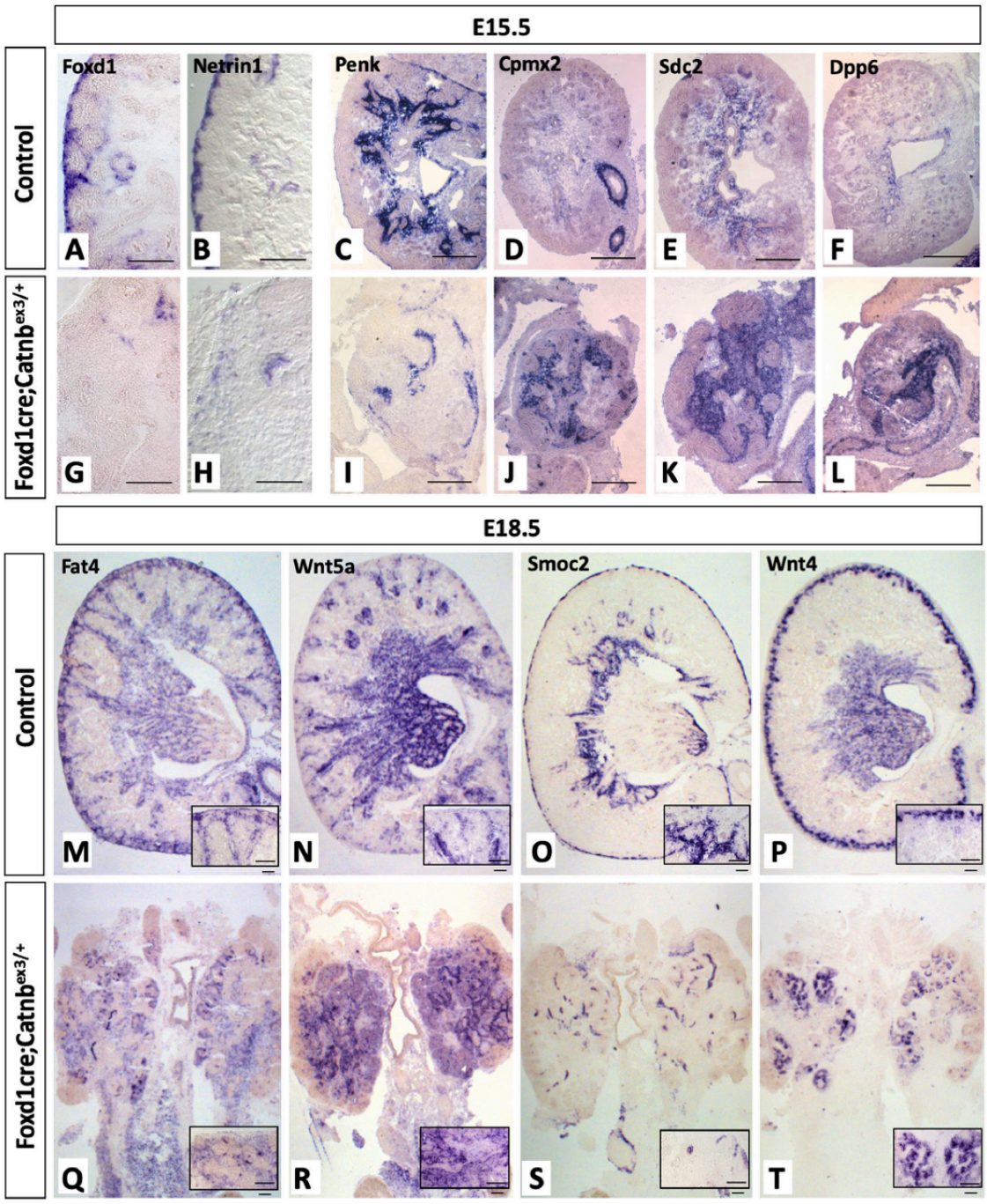

Fig. 5. β-Catenin activation in stromal lineage disrupts normal interstitial patterning. (A-T) Stromal markers in control kidneys (A-F,M-P) were compared with Foxd1cre;Catnb$^{ex3/+}$ mutants (G-L,Q-T), which show early loss of the Foxd1+ stromal progenitor population (G) and nephrogenic interstitial markers netrin 1 (H) and Smoc2 (S). Additionally, medullary stromal markers appear ectopically expressed in the cortex, including Cpmx2 (J), Sdc2 (K), Dpp6 (L) and Wnt5a (R), with a loss of expression of the corticomedullary markers Penk (I) and Smoc2 (S). Scale bars: 100 µm. *n*=3 for each timepoint/genotype.

Wnt/β-catenin signaling plays a crucial role in multiple aspects of normal kidney development and is upregulated in a significant proportion of human WTs. However, how β-catenin drives tumorigenesis remains unclear. By examining the transcriptomic effects of β-catenin-activating mutations in multiple cell lineages of the developing kidney, we have further defined how abnormal

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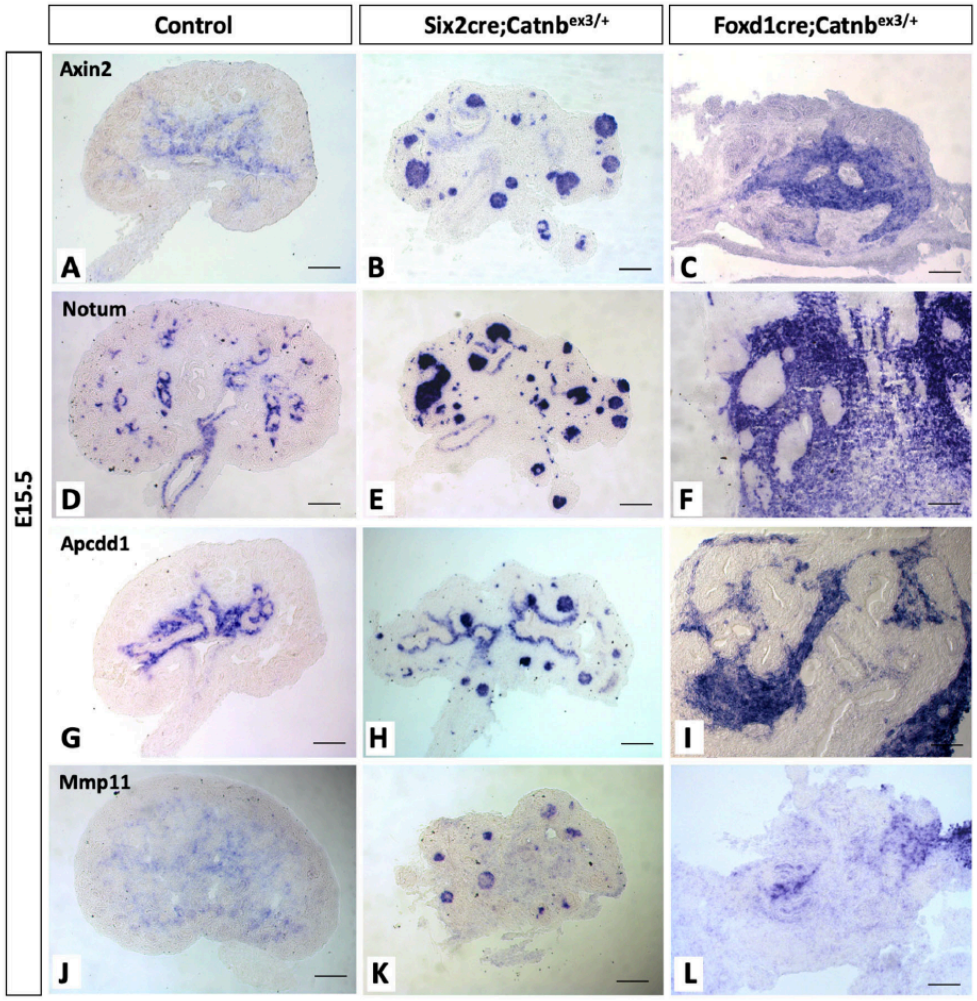

Fig. 6. β-Catenin activation in either the NPC or stromal lineages drives the expression of genes normally localized to the developing renal interstitium. (C,F,I,L) Foxd1cre;Catnb$^{ex3/+}$ mutants show upregulation of multiple stromal markers compared with controls (A,D,G,J), as expected given the known role of β-catenin in the development of the medullary interstitium. (B,E,H,K) However, these same target genes are strongly upregulated in Six2cre; Catnb$^{ex3/+}$ cells, somewhat unexpectedly given that these cells originate from a separate lineage where this transcriptional program is not active during normal development. Scale bars: 100 µm; *n*=3 for each timepoint/genotype.

activation of this signaling pathway exerts both cell-type specific and lineage-independent effects in renal development. Although it has been proposed that activation of β-catenin within the nephron progenitors or renal vesicles leads to tumor formation through inhibition of MET or activation of EMT, respectively, our findings suggest that activation of this gene within the mouse nephron progenitors and their derivatives does not lead to a WT-like phenotype at the molecular level. However, activation of β-catenin in the stromal progenitors results in impaired nephrogenesis, leading to nephrogenic rest-like structures that closely resemble human WTs at the transcriptional level. We show that Foxd1cre;Catnb$^{ex3/+}$ mutants maintain expression of Six2 and Ncam in the developing NPCs, similar to human WT blastema, whereas Six2cre;Catnb$^{ex3/+}$ mutants do not recapitulate this phenotype. Additionally, we show that the patterning of the renal interstitium is severely disrupted in Foxd1cre;Catnb$^{ex3/+}$ kidneys, with an early loss of Foxd1 progenitor cells and cortico-medullary stroma and a pronounced expansion of papillary stromal markers. Although distinctions between cortical and medullary interstitial cells have been previously recognized, recent work has revealed a surprising degree of heterogeneity in the embryonic renal interstitium (England et al., 2020), suggesting that unique subpopulations of interstitial cells regulate adjacent cell types in normal kidney development. In support of this, inactivation of β-catenin in the stromal progenitor population (using Foxd1Cre) blocks development of the papillary interstitium as well as adjacent epithelial cells of the loop of Henle (England et al., 2020; Yu et al., 2009). Conversely, we show here that activation of β-catenin in the stromal progenitor population drives expression of papillary interstitial cells. We hypothesize that the abnormal interstitial patterning in the Foxd1cre;Catnb$^{ex3/+}$ kidneys disturbs the normal stromal microenvironment present in developing kidneys, leading to the altered gene expression and lack of differentiation in the NPC

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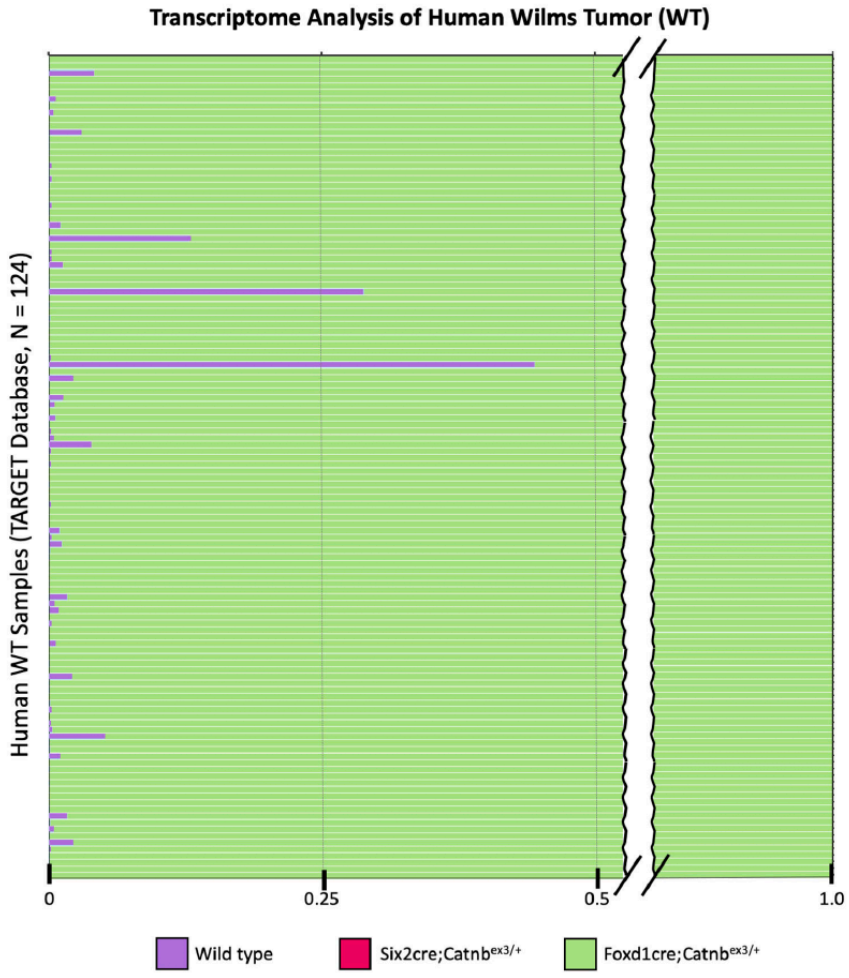

Fig. 7. Human WT shows molecular characteristics similar to mutant mouse kidneys with activation of β-catenin in stromal lineage. RNA-seq on E12.5 wild-type, Six2cre;Catnb$^{ex3/+}$ and Foxd1cre; Catnb$^{ex3/+}$ mutant kidneys (n=3 for each genotype) were compared with human WT RNA-seq data obtained from the publicly available TARGET database (n=124 samples). Using neural network classification, mapping scores ranging from 0 to 1.0 were generated for each human WT sample measuring similarity of expression of the 2806 identified likely direct targets of β-catenin with that of each of the mouse genotypes, with these results showing expression of these genes in the tumor samples was most similar to the Foxd1cre; Catnb$^{ex3/+}$ mouse model (green bars), with a few tumors showing a small degree of similarity to wild-type kidneys (purple bars), and none of the samples showing any significant degree of similarity to the Six2cre; Catnb$^{ex3/+}$ expression profile.

population. We hypothesize that a similar disruption to the stromal microenvironment contributes to Wilms' tumorigenesis, as has been suggested in numerous other tumors (Clark and Vignjevic; 2015; Mao et al., 2013; Bremnes et al., 2011; Valkenburg et al., 2018; Li et al., 2007).

It has long been assumed that driving mutations in WT develop in the NPC/blastemal component. However, more recently, studies using laser microcapture techniques to analyze different components of human WT, including data from three individuals in our study, demonstrate identical mutations in blastemal, stromal and epithelial components consistent with β-catenin mutations occurring in an early common precursor cell (Duhme et al., 2019; Uschkereit et al., 2007). Interestingly, our examination of mice with mosaic activation of β-catenin in early metanephric precursor lineages using TcreERT2 reveals that mutant cells were either selected against or downregulated the forced expression of β-catenin and underwent grossly normal development. In contrast, simultaneous activation of β-catenin in the nephron progenitor and stromal lineages showed severely perturbed development, with the formation of bone-like tissue. Interestingly, bone as well as other heterologous elements, including cartilage and skeletal muscle have been reported in human WTs. Although these observations are consistent with a model in which activating mutations must occur in both NPC and stromal lineages (or a common progenitor for both), we cannot rule out the possibility that the triphasic morphology of WTs is due to aberrant tumor cell differentiation (through either multi-lineage potential or an ability to transdifferentiate) or that activating mutations in epithelial structures lead to EMT. Although we feel that the molecular phenotype of tumor stroma along with the histology of mouse mutants makes these possibilities unlikely (specifically, lineage-traced NPC cells carrying an activated allele of β-catenin do not take on a stromal appearance), it is clear that activation of β-catenin alone is not sufficient to drive WT formation in mice and we cannot rule out contributions by other mutant genes.

β-Catenin plays multiple, cell type-specific roles during kidney development by activating different target genes (Pan et al., 2017). Surprisingly, stabilization of β-catenin in either the stroma or the NPCs results in activation of the same stromal target genes. Previous studies have shown that different targets of β-catenin are regulated in a dose-specific manner (Ramalingam et al., 2018). It is possible that the stromal targets represent genes activated by the highest levels of β-catenin, independent of cell type. Another possibility is that the expression of co-regulators determines target gene expression. It is interesting to note that Lef1 was over-expressed in all gain-of-function models (Six2cre, Foxd1cre and dual Six2cre;Foxd1cre) examined. The 'stromal targets' of β-catenin

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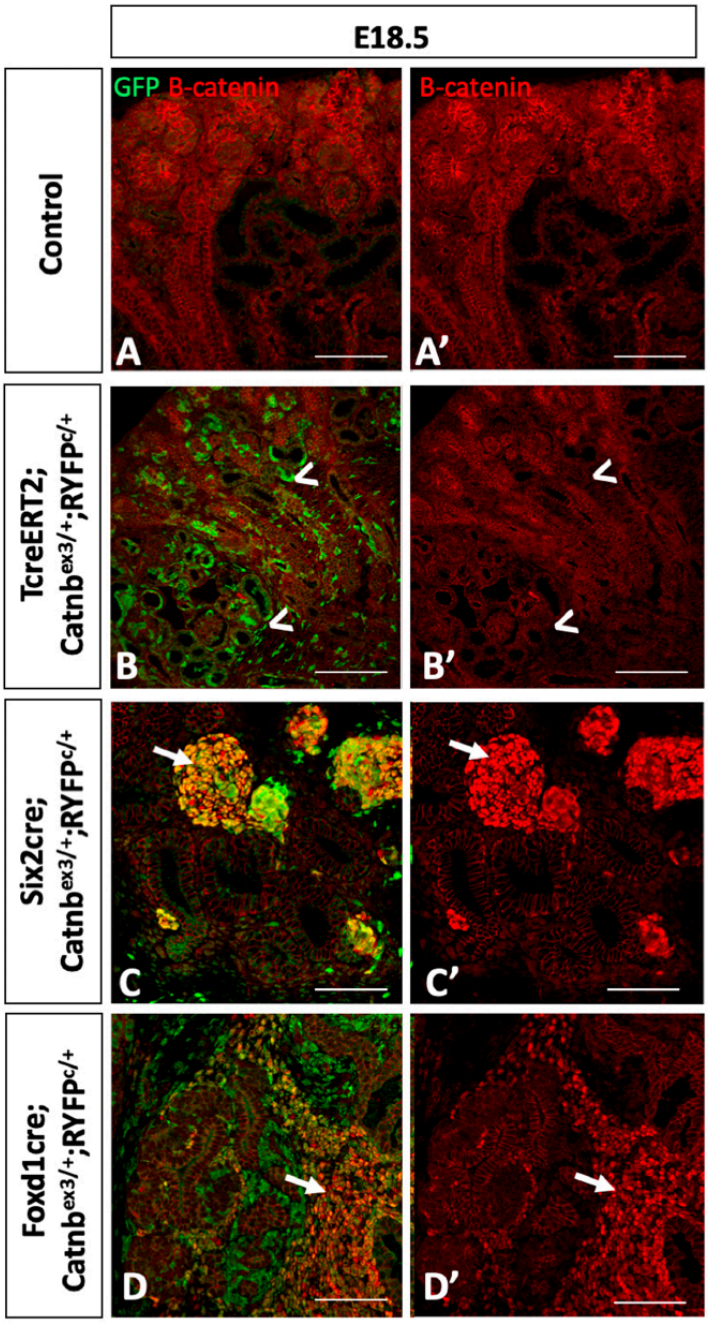

Fig. 8. β-Catenin activation in early metanephric kidney precursors does not result in nuclear β-catenin despite evidence of recombination. (A,B′) β-Catenin expression in control kidneys (A,A′) was compared with TcreERT2; Catnb$^{ex3/+}$;RosaYFP$^{c/+}$ mutants given 2 mg per 40 g body weight of tamoxifen at E9.5 (B,B′), which demonstrate recombination by the presence of lineage-traced cells (B, arrowheads); however, these cells unexpectedly lack detectable expression of β-catenin (B′, arrowheads). (C-D′) Conversely, strong nuclear expression is observed in Six2cre and Foxd1cre mutant kidneys (arrows). Scale bars: 100 µm. *n*=3 for each timepoint/genotype.

may actually be specific targets of a Lef1/β-catenin complex. These two hypotheses (which are not mutually exclusive) will need to be further explored.

Surprisingly, we observed no gross abnormalities in adult kidneys upon mosaic activation of β-catenin in the NPC, stromal or intermediate mesoderm lineages even though we did detect lineage positive cells. This is in contrast to a previous publication that found that activation of β-catenin with Six2cre but not Foxd1cre resulted in tumor like structures in adult mice (Huang et al., 2016). We cannot explain the discrepancy; however, it is important to note that Huang et. al performed limited molecular characterization of their dysplastic tissues. Whether these lesions truly represent models of WT is still unclear. As we are unable to detect cells with nuclear β-catenin in E18.5 or adult kidneys after mosaic activation of β-catenin using standard immunofluorescence techniques, we hypothesize that, under otherwise normal conditions, strong β-catenin activity either leads to precocious differentiation or is detrimental to cell survival (or both). In that case, additional mutations in genes affecting cell survival or

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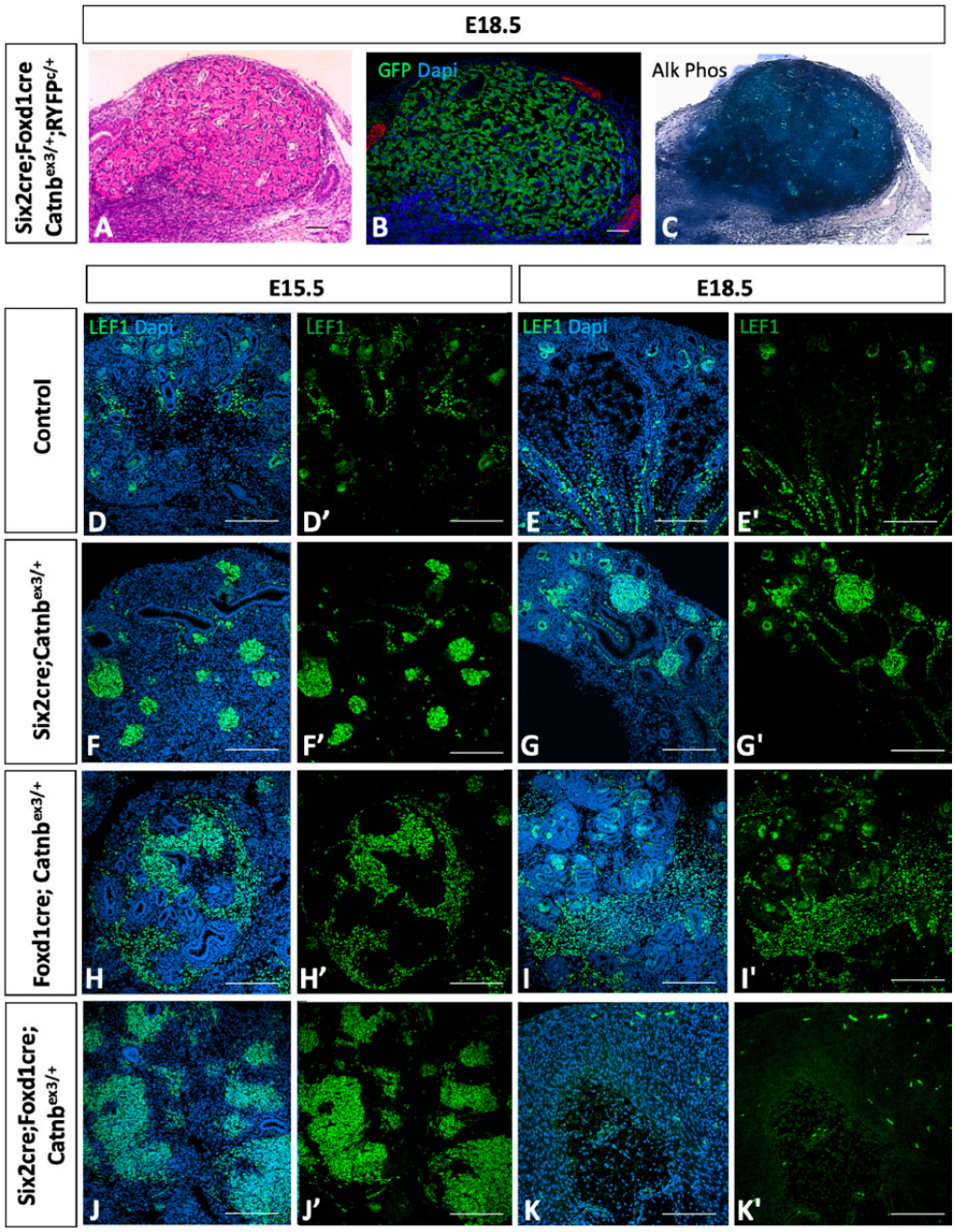

Fig. 9. β-Catenin activation in dual NPC and stromal lineages results in the development of bone. (A-C) Six2cre;Foxd1cre;Catnb$^{ex3/+}$ mutant kidneys resemble 'bone-like' tissue at E18.5 (Hematoxylin and Eosin staining, A), with reporter expression confirming these cells originated from the targeted cell populations (B), and demonstrate strong expression of the bone marker alkaline phosphatase (C). (D-K′) Although Lef-1, a transcription factor previously shown to interact with β-catenin to promote osteoblast activity (Hoeppner et al., 2011; Li et al., 2018), is upregulated in all mutant lines (F-I′), the 'bone-like' phenotype is observed only in the Six2cre;Foxd1cre;Catnb$^{ex3/+}$ mutants (J-K′). Scale bars: 100 µm; *n*=3 for each timepoint/genotype.

differentiation may help to maintain these cells postnatally. Indeed, mutations in the *WT1* gene, which affects NPC differentiation, as well as in *TP53*, which impacts cell survival, are frequently found in tumors with β-catenin mutation (Huff, 2011; Maiti et al., 2000). It will be interesting to see whether mutation of either of these interacts with β-catenin activation, especially in the TcreERT2 context.

Given the heterogeneous nature of WT, understanding the effects of β-catenin activation in different cell lineages of the developing kidney in comparison with the molecular changes of human WT will aid in unraveling the pathogenesis of this embryonal tumor. Although our data suggest that activation of β-catenin in the stromal lineage of a mouse kidney is sufficient to alter the microenvironment in which

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nephrogenesis occurs and can lead to WT-like phenotypes, they certainly do not prove that this occurs in WTs, as the lineage of the different cell types observed in WTs is still not known. Application of single cell transcriptomic techniques to WTs of known genetic background will certainly be enlightening. No matter what, our findings provide additional insights into the genetic programs driven by β-catenin in the developing kidney and suggest further studies are necessary to understand the role of stromal signaling in the development of WT.

MATERIALS AND METHODS

Mouse models

Catnb$^{ex3/+}$, Six2cre, Foxd1cre and TcreERT2 mouse lines in *Mus musculus* were used as previously described (Harada et al., 1999; Kobayashi et al., 2014; Kobayashi et al., 2008; Imuta et al., 2013). All mice were bred on a mixed genetic background. For experimental assays described below, Catnb$^{ex3/ex3}$ females were crossed with male Cre-line mice, with day of plug counted as E0.5. Pregnant females were sacrificed at various gestational time points. Lineage-tracing experiments were performed by crossing Rosa26YFP (JAX Stock #006148) or Rosa26Tomato (JAX Stock #007909) reporter mice with the above mouse lines. Mice with the desired genotype were randomly selected regardless of sex with Cre-negative littermates used as controls. Tamoxifen (Sigma, T5648) was administered by gavage at a dose of 2 mg per 40 g body weight. All animals were housed, maintained and used according to National Institutes of Health (NIH) and Institutional Animal Care and Use Committees (IACUC) approved protocols at the University of Texas Southwestern Medical Center (OLAW Assurance Number D16-00296).

Kidney sample preparation, immunostaining and *in situ* hybridization assays

Embryonic tissue was fixed in 4% paraformaldehyde, embedded in paraffin, sectioned into 5 µm slices, and subjected to Hematoxylin and Eosin staining or immunofluorescence (IF). Slides for IF were immersed and boiled with either 10 mM sodium citrate or TE antigen retrieval buffer, and blocked with a solution of 5% FBS/PBS for 1 h at room temperature followed by the application of primary antibodies diluted in blocking solution. The following antibodies were used: anti-GFP (Aves, GFP-1020, 1:200), anti-RFP (Rockland, 600-401-379, 1:200), anti-Ncam (Sigma, C9672, 1:200), anti-CK (DSHB, TROMA-I-s, 1:50), anti-Six2 (Proteintech, 11562-1-AP, 1:200; Abnova, H000110736-M01, 1:200), anti-β-catenin (Sigma, C7207, 1:200), anti-Lhx1 (DSHB, 4F2-c, 1:200), anti-Pax8 (Proteintech, 10336-1-AP, 1:200) and anti-Lef1 (Proteintech, 2230S, 1:200). For *in situ* hybridization assays, tissue was fixed with 4% paraformaldehyde, cryoprotected with 30% sucrose, embedded in OCT medium (TissueTek), sectioned into 10 µm slices, rehydrated with PBS before being treated with 15 µg/ml proteinase K for 10 min and fixed in 4% PFA followed by an acetylation step. Slides were then washed and incubated with pre-hybridization buffer for 1 h at room temperature before being hybridized with the specific probe overnight at 65°C. Slides were then washed in 0.2×SSC then transferred to NTT before blocking with 2% blocking solution (Roche) for at least 1 h at room temperature. Slides were then incubated with anti-Dig alkaline phosphatase-conjugated antibody (Roche, 1:4000) overnight at 4°C. The next day, slides were washed in 3×NTT and 3×NTTML before incubating with BM purple (Roche) for color reaction. After color reaction, slides were fixed with 4% PFA and mounted using Permount. E18.5 Six2cre; Foxd1cre;Catnb$^{ex3/+}$ bone-like tissue was fixed in 4% paraformaldehyde, decalcified with EDTA, sectioned into 5 µm slices, and subjected to Hematoxylin and Eosin staining, immunofluorescence and alkaline phosphatase staining performed by our institutional histology core.

RNA-sequencing and analyses of gene expression data from the TARGET database

RNA-Seq was performed on mouse kidneys (E12.5 whole kidneys; n=3 Cre-negative controls, 3 Foxd1cre;Catnb$^{ex3/+}$ mutants and 3 Six2cre; Catnb$^{ex3/+}$ mutants). RNA was isolated from dissected kidneys stored in RNA later solution (Invitrogen, AM7020). RNA-Seq was performed using single-end 75 bp with a minimum of 20 million reads per sample. Transcript abundance was estimated without aligning reads using Salmon (Patro et al., 2017) against an index of coding sequences from the Ensembl GRCm38 assembly. Transcript-level abundance was imported, and count and offset matrices generated using the tximport R/Bioconductor package (Soneson et al., 2015). Differential expression analysis was performed using the DESeq2 R/Bioconductor package (Love et al., 2014). WT expression data was downloaded from the TARGET database using the TCGAbiolinks R/Bioconductor package (Colaprico et al., 2016). A variance-stabilizing transformation implemented in the vst function of DESeq2 was applied prior to neural network processing.

To elucidate the relationship between activation of β-catenin in either Six2 or Foxd1 lineage cells and Wilms' tumor, we performed the following analysis. First, we used BETA (Wang et al., 2013) to integrate the results of RNA-Seq of whole kidneys from Foxd1cre;Catnb$^{ex3/+}$, Six2cre;Catnb$^{ex3/+}$ and wild-type comparator mice with the previously published β-catenin ChIP-seq data (Park et al., 2012). This resulted in a list of 2806 likely direct targets of β-catenin. A neural network was then trained to classify the expression profiles of Foxd1cre;Catnb$^{ex3/+}$, Six2cre;Catnb$^{ex3/+}$ and wild-type kidneys based on the expression of these 2806 genes. Next, the expression profiles for 124 WT samples curated in the TARGET database were mapped from human genes to mouse orthologues and then input to the neural network classifier. A sequential neural network with two hidden layers each containing 512 nodes was trained with the Adam optimizer using sparse categorical cross entropy loss using the TensorFlow platform ('TensorFlow White Papers|TensorFlow', n.d.). Five-fold cross validation was used during training. Classification scores ranging from 0 to 1.0 were assigned to each human WT sample for each mouse genotype (wild type, Foxd1cre;Catnb$^{ex3/+}$ or Six2cre;Catnb$^{ex3/+}$). The scores sum to 1 for each tumor sample and so can be interpreted as a probability of identity. None of the samples showed significant similarity to Six2cre;Catnb$^{ex3/+}$ mutants.

Statistical analysis

Data presented in figures are representative examples from one of at least three different experiments on at least three different embryos/organs. No significant variability was noted in tissues of the same genotype; all animals with correct genotypes were included in the analysis. Bioinformatic statistics were carried about as described above; algorithms and software availability are provided in Table S5.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: T.J.C.; Methodology: K.A.D., A.D., T.J.C.; Software: C.P.C.; Formal analysis: K.A.D., C.P.C., A.D., P.R., C.S.K., T.J.C.; Investigation: T.J.C.; Resources: D.R., T.J.C.; Writing - original draft: K.A.D., C.S.K., T.J.C.; Writing - review & editing: K.A.D., C.P.C., T.J.C.; Supervision: T.J.C.; Project administration: K.A.D., T.J.C.; Funding acquisition: K.A.D., T.J.C.

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Data availability

RNA-Seq data presented in this manuscript has been deposited in GEO under accession number GSE150074.

Supplementary information

Supplementary information available online at https://dev.biologists.org/lookup/doi/10.1242/dev.189597.supplemental

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References

Boivin, F. J. and Bridgewater, D. (2018). beta-Catenin in stromal progenitors controls medullary stromal development. *Am. J. Physiol. Renal. Physiol.* **314**, F1177-f1187. doi:10.1152/ajprenal.00282.2017

Boivin, F. J., Sarin, S., Dabas, P., Karolak, M., Oxburgh, L. and Bridgewater, D. (2016). Stromal beta-catenin overexpression contributes to the pathogenesis of renal dysplasia. *J. Pathol.* **239**, 174-185. doi:10.1002/path.4713

Bremnes, R. M., Dønnem, T., Al-Saad, S., Al-Shibli, K., Andersen, S., Sirera, R., Camps, C., Marinez, I. and Busund, L.-T. (2011). The role of tumor stroma in cancer progression and prognosis: emphasis on carcinoma-associated fibroblasts and non-small cell lung cancer. *J. Thorac. Oncol.* **6**, 209-217. doi:10.1097/JTO.0b013e3181f8a1bd

Charles, A. K., Brown, K. W. and Berry, P. J. (1998). Microdissecting the genetic events in nephrogenic rests and Wilms' tumor development. *Am. J. Pathol.* **153**, 991-1000. doi:10.1016/S0002-9440(10)65641-6

Clark, A. G. and Vignjevic, D. M. (2015). Modes of cancer cell invasion and the role of the microenvironment. *Curr. Opin. Cell Biol.* **36**, 13-22. doi:10.1016/j.ceb.2015.06.004

Colaprico, A., Silva, T. C., Olsen, C., Garofano, L., Cava, C., Garolini, D., Sabedot, T. S., Malta, T. M., Pagnotta, S. M., Castiglioni, I. et al. (2016). TCGAbiolinks: an R/Bioconductor package for integrative analysis of TCGA data. *Nucleic Acids Res.* **44**, e71. doi:10.1093/nar/gkv1507

Corbin, M., de Reyniès, A., Rickman, D. S., Berrebi, D., Boccon-Gibod, L., Cohen-Gogo, S., Fabre, M., Jaubert, F., Faussillon, M., Yilmaz, F. et al. (2009). WNT/beta-catenin pathway activation in Wilms tumors: a unifying mechanism with multiple entries? *Genes Chromosomes Cancer*, **48**, 816-827.

Das, A., Tanigawa, S., Karner, C. M., Xin, M., Lum, L., Chen, C., Olson, E. N., Perantoni, A. O. and Carroll, T. J. (2013). Stromal-epithelial crosstalk regulates kidney progenitor cell differentiation. *Nat. Cell Biol.* **15**, 1035-1044. doi:10.1038/ncb2828

Duhme, C., Busch, M., Heine, E., De Torres, C., Mora, J. and Royer-Pokora, B. (2019). WT1-Mutant Wilms Tumor Progression Is Associated With Diverting Clonal Mutations of CTNNB1. *J. Pediatr. Hematol. Oncol.* doi:10.1097/mph.0000000000001697.

England, A. R., Chaney, C. P., Das, A., Patel, M., Malewsak, A., Armendariz, D., Hon, G., Strand, D., Drake, K. A. and Carroll, T. J. (2020). Characterization of interstitial heterogeneity in the developing kidney. BioxRiv.

Fetting, J. L., Guay, J. A., Karolak, M. J., Iozzo, R. V., Adams, D. C., Maridas, D. E., Brown, A. C. and Oxburgh, L. (2014). FOXD1 promotes nephron progenitor differentiation by repressing decorin in the embryonic kidney. *Development* **141**, 17-27. doi:10.1242/dev.089078

Gadd, S., Huff, V., Walz, A. L., Ooms, A., Armstrong, A. E., Gerhard, D. S., Smith, M. A., Auvil, J. M. G., Meerzaman, D., Chen, Q.-R. et al. (2017). A Children's Oncology Group and Target initiative exploring the genetic landscape of Wilms tumor. *Nat. Genet.* **49**, 1487-1494. doi:10.1038/ng.3940

Harada, N., Tamai, Y., Ishikawa, T., Sauer, B., Takaku, K., Oshima, M. and Taketo, M. M. (1999). Intestinal polyposis in mice with a dominant stable mutation of the beta-catenin gene. *EMBO J.* **18**, 5931-5942. doi:10.1093/emboj/18.21.5931

Hoeppner, L. H., Secreto, F. J., Razidlo, D. F., Whitney, T. J. and Westendorf, J. J. (2011). Lef1DeltaN binds beta-catenin and increases osteoblast activity and trabecular bone mass. *J. Biol. Chem.* **286**, 10950-10959. doi:10.1074/jbc.M110.165100

Hohenstein, P., Pritchard-Jones, K. and Charlton, J. (2015). The yin and yang of kidney development and Wilms' tumors. *Genes Dev.* **29**, 467-482. doi:10.1101/gad.256396.114

Huang, L., Mokkapati, S., Hu, Q., Ruteshouser, E. C., Hicks, M. J. and Huff, V. (2016). Nephron Progenitor But Not Stromal Progenitor Cells Give Rise to Wilms Tumors in Mouse Models with beta-Catenin Activation or Wt1 Ablation and Igf2 Upregulation. *Neoplasia* **18**, 71-81. doi:10.1016/j.neo.2015.12.001

Huff, V. (2011). Wilms' tumours: about tumour suppressor genes, an oncogene and a chameleon gene. *Nat. Rev. Cancer* **11**, 111-121. doi:10.1038/nrc3002

Hum, S., Rymer, C., Schaefer, C., Bushnell, D. and Sims-Lucas, S. (2014). Ablation of the renal stroma defines its critical role in nephron progenitor and vasculature patterning. *PLoS One* **9**, e88400. doi:10.1371/journal.pone.0088400

Imuta, Y., Kiyonari, H., Jang, C.-W., Behringer, R. R. and Sasaki, H. (2013). Generation of knock-in mice that express nuclear enhanced green fluorescent protein and tamoxifen-inducible Cre recombinase in the notochord from Foxa2 and T loci. *Genesis* **51**, 210-218. doi:10.1002/dvg.22376

Karner, C. M., Das, A., Ma, Z., Self, M., Chen, C., Lum, L., Oliver, G. and Carroll, T. J. (2011). Canonical Wnt9b signaling balances progenitor cell expansion and differentiation during kidney development. *Development* **138**, 1247-1257. doi:10.1242/dev.057646

Kobayashi, A., Mugford, J. W., Krautzberger, A. M., Naiman, N., Liao, J. and Mcmahon, A. P. (2014). Identification of a multipotent self-renewing stromal progenitor population during mammalian kidney organogenesis. *Stem Cell Reports* **3**, 650-662. doi:10.1016/j.stemcr.2014.08.008

Kobayashi, A., Valerius, M. T., Mugford, J. W., Carroll, T. J., Self, M., Oliver, G. and Mcmahon, A. P. (2008). Six2 defines and regulates a multipotent self-renewing nephron progenitor population throughout mammalian kidney development. *Cell Stem Cell* **3**, 169-181. doi:10.1016/j.stem.2008.05.020

Koesters, R., Niggli, F., Von Knebel Doeberitz, M. and Stallmach, T. (2003). Nuclear accumulation of beta-catenin protein in Wilms' tumours. *J. Pathol.* **199**, 68-76. doi:10.1002/path.1248

Li, C. M., Kim, C. E., Margolin, A. A., Guo, M., Zhu, J., Mason, J. M., Hensle, T. W., Murty, V. V., Grundy, P. E., Fearon, E. R. et al. (2004). CTNNB1 mutations and overexpression of Wnt/beta-catenin target genes in WT1-mutant Wilms' tumors. *Am. J. Pathol.* **165**, 1943-1953. doi:10.1016/S0002-9440(10)63246-4

Li, H., Fan, X. and Houghton, J. (2007). Tumor microenvironment: the role of the tumor stroma in cancer. *J. Cell. Biochem.* **101**, 805-815. doi:10.1002/jcb.21159

Li, Z., Xu, Z., Duan, C., Liu, W., Sun, J. and Han, B. (2018). Role of TCF/LEF Transcription Factors in Bone Development and Osteogenesis. *Int. J. Med. Sci.* **15**, 1415-1422. doi:10.7150/ijms.26741

Love, M. I., Huber, W. and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* **15**, 550. doi:10.1186/s13059-014-0550-8

Maiti, S., Alam, R., Amos, C. I. and Huff, V. (2000). Frequent association of beta-catenin and WT1 mutations in Wilms tumors. *Cancer Res.* **60**, 6288-6292.

Mao, Y., Keller, E. T., Garfield, D. H., Shen, K. and Wang, J. (2013). Stromal cells in tumor microenvironment and breast cancer. *Cancer Metastasis Rev.* **32**, 303-315. doi:10.1007/s10555-012-9415-3

Marose, T. D., Merkel, C. E., Mcmahon, A. P. and Carroll, T. J. (2008). Beta-catenin is necessary to keep cells of ureteric bud/Wolffian duct epithelium in a precursor state. *Dev. Biol.* **314**, 112-126. doi:10.1016/j.ydbio.2007.11.016

Pan, X., Karner, C. M. and Carroll, T. J. (2017). Myc cooperates with β-catenin to drive gene expression in nephron progenitor cells. *Development* **144**, 4173-4182. doi:10.1242/dev.153700

Park, J. S., Valerius, M. T. and Mcmahon, A. P. (2007). Wnt/beta-catenin signaling regulates nephron induction during mouse kidney development. *Development* **134**, 2533-2539. doi:10.1242/dev.006155

Park, J. S., Ma, W., O'brien, L. L., Chung, E., Guo, J. J., Cheng, J. G., Valerius, M. T., Mcmahon, J. A., Wong, W. H. and Mcmahon, A. P. (2012). Six2 and Wnt regulate self-renewal and commitment of nephron progenitors through shared gene regulatory networks. *Dev. Cell* **23**, 637-651. doi:10.1016/j.devcel.2012.07.008

Patro, R., Duggal, G., Love, M. I., Irizarry, R. A. and Kingsford, C. (2017). Salmon provides fast and bias-aware quantification of transcript expression. *Nat. Methods* **14**, 417-419. doi:10.1038/nmeth.4197

Pritchard-Jones, K. (1997). Malignant origin of the stromal component of Wilms' tumor. *J. Natl. Cancer Inst.* **89**, 1089-1091. doi:10.1093/jnci/89.15.1089

Ramalingam, H., Fessler, A. R., Das, A., Valerius, M. T., Basta, J., Robbins, L., Brown, A. C., Oxburgh, L., Mcmahon, A. P., Rauchman, M. and et al. (2018). Disparate levels of beta-catenin activity determine nephron progenitor cell fate. *Dev. Biol.* **440**, 13-21. doi:10.1016/j.ydbio.2018.04.020

Rivera, M. N. and Haber, D. A. (2005). Wilms' tumour: connecting tumorigenesis and organ development in the kidney. *Nat. Rev. Cancer* **5**, 699-712. doi:10.1038/nrc1696

Sarin, S., Boivin, F., Li, A., Lim, J., Svajger, B., Rosenblum, N. D. and Bridgewater, D. (2014). beta-Catenin overexpression in the metanephric mesenchyme leads to renal dysplasia genesis via cell-autonomous and non-cell-autonomous mechanisms. *Am. J. Pathol.* **184**, 1395-1410. doi:10.1016/j.ajpath.2014.01.018

Shan, J., Jokela, T., Skovorodkin, I. and Vainio, S. (2010). Mapping of the fate of cell lineages generated from cells that express the Wnt4 gene by time-lapse during kidney development. *Differentiation* **79**, 57-64. doi:10.1016/j.diff.2009.08.006

Soneson, C., Love, M. I. and Robinson, M. D. (2015). Differential analyses for RNA-seq: transcript-level estimates improve gene-level inferences. *F1000Res* **4**, (1521) doi:10.12688/f1000research.7563.1

Treger, T. D., Chowdhury, T., Pritchard-Jones, K. and Behjati, S. (2019). The genetic changes of Wilms tumour. *Nat Rev Nephrol* **15**, 240-251. doi:10.1038/s41581-019-0112-0

Urbach, A., Yermalovich, A., Zhang, J., Spina, C. S., Zhu, H., Perez-Atayde, A. R., Shukrun, R., Charlton, J., Sebire, N., Mifsud, W. et al. (2014). Lin28 sustains early renal progenitors and induces Wilms tumor. *Genes Dev.* **28**, 971-982. doi:10.1101/gad.237149.113

Uschkereit, C., Perez, N., De Torres, C., Kuff, M., Mora, J. and Royer-Pokora, B. (2007). Different CTNNB1 mutations as molecular genetic proof for the independent origin of four Wilms tumours in a patient with a novel germ line WT1 mutation. *J. Med. Genet.* **44**, 393-396. doi:10.1136/jmg.2006.047530

Valkenburg, K. C., De Groot, A. E. and Pienta, K. J. (2018). Targeting the tumour stroma to improve cancer therapy. *Nat Rev Clin Oncol* **15**, 366-381. doi:10.1038/s41571-018-0007-1

Wang, S., Sun, H., Ma, J., Zang, C., Wang, C., Wang, J., Tang, Q., Meyer, C. A., Zhang, Y. and Liu, X. S. (2013). Target analysis by integration of transcriptome and ChIP-seq data with BETA. *Nat. Protoc.* **8**, 2502-2515. doi:10.1038/nprot.2013.150

Yu, J., Carroll, T. J., Rajagopal, J., Kobayashi, A., Ren, Q. and Mcmahon, A. P. (2009). A Wnt7b-dependent pathway regulates the orientation of epithelial cell division and establishes the cortico-medullary axis of the mammalian kidney. *Development* **136**, 161-171. doi:10.1242/dev.022087

Zirn, B., Samans, B., Wittmann, S., Pietsch, T., Leuschner, I., Graf, N. and Gessler, M. (2006). Target genes of the WNT/beta-catenin pathway in Wilms tumors. *Genes Chromosomes Cancer* **45**, 565-574. doi:10.1002/gcc.20319