SHROOM3 IN NEPHRON FORMATION

CHARACTERIZATION OF THE ROLE OF SHROOM3 IN NEPHRON FORMATION

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TITLE: Characterization of the Role of Shroom3 in Nephron Formation

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ABSTRACT

Proper development of the nephron, the functional unit of the kidney, is essential for kidney function. The nephron develops from a pool of cap mesenchymal cells, as defined by a cluster of cells adjacent to the ureteric bud tips of branching ureteric epithelium, giving rise to two subset populations: the self renewing cells and the nephron progenitors. These nephron progenitors undergo mesenchymal-epithelial transition (MET) to develop into polarized renal vesicles (RV), and eventually fuse with the epithelial tubule to develop into a mature nephron. Although these processes are essential for the formation of functional kidneys, little is known about the molecular mechanisms that regulate them. In this study, we characterize several steps during cap mesenchyme and renal vesicle formation using our *Shroom3* knockout mouse kidney as our model. Previous researchers have associated Shroom3 with chronic kidney disease. Detecting and analyzing the genetic components of CKD is needed to improve our understanding of its pathogenesis. Shroom3 encodes an actin-binding protein that regulates cell shape changes through induction of apical constriction. However, there is a lack of evidence about Shroom3's expression pattern and functional role upstream of developed nephrons. Here, I defined the spatial and temporal expression of Shroom3 within the cap mesenchyme region. I investigated the nephron progenitors between Shroom3 wildtypes and mutants. Lastly, I analyzed the renal vesicle polarity in mutants, by analyzing apical membrane markers on RVs to characterize any abnormalities in their orientation and establishment of polarity.

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LIST OF ABBREVIATIONS

AB - Antibody

BC - Bowman's Capsule

BMP7 - Bone Morphogenetic Protein 7

CD - Collecting Duct

CKD – Chronic Kidney Disease

CM - Cap Mesenchyme

DAPI - 4′,6-diamidino-2-phenylindole

EYA1 - Eyes Absent Homolog 1

FGF8 - Fibroblast growth factor 8

FHH - Fawn-Hooded Hypertensive

G - Glomerulus

GDNF - Glial cell line-derived neurotropic factor

GWAS – Genome Wide Association Studies

H&E - Hematoxylin and Eosin

IF - Immunofluorescence

IHC - Immunohistochemistry

LHX1 – Lim homeobox 1

MET - Mesenchymal to Epithelial Transition

MLC - Myosin Light Chain

MM - Metanephric Mesenchyme

NCAM – Neural cell adhesion molecule

NMII - Non Muscle Myosin II

NP - Nephron Progenitor

OSR1 - Odd-Skipped Related Transcription Factor 1

PAR3 – Partitioning Defective 3

PE - Parietal Epithelium

RET - Glial cell line-derived neurotropic factor receptor

RV - Renal Vesicle

SALL1 - Spalt Like Transcription Factor 1

SBD - Shroom3 Binding Domain

SIX2 - Sine oculis-related homeobox 2

SNP – Single Nucleotide Polymorphism

SSB - S-Shaped Body

UB - Ureteric Bud

UE - Ureteric Epithelium

UMOD – Uromodulin

WNT9B – Wnt family member 9

WNT4 – Wnt family member 4

WT - Wildtype

WT1 – Wilms Tumor Protein 1

DECLARATION OF ACADEMIC ACHIEVEMENT

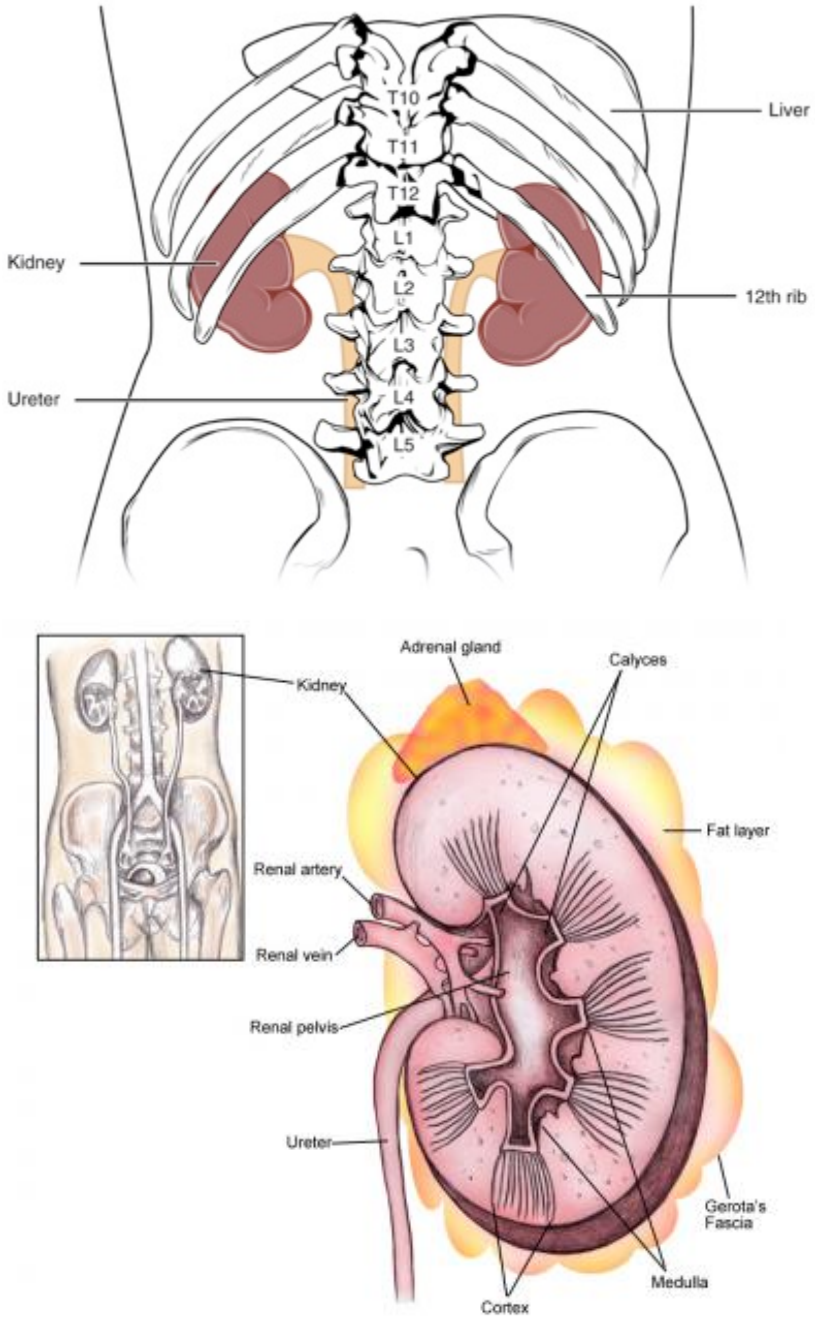
I personally accomplished all the research and necessary tasks for Figure 1-17.

1. BACKGROUND:

1.1 Kidney Anatomy and Function

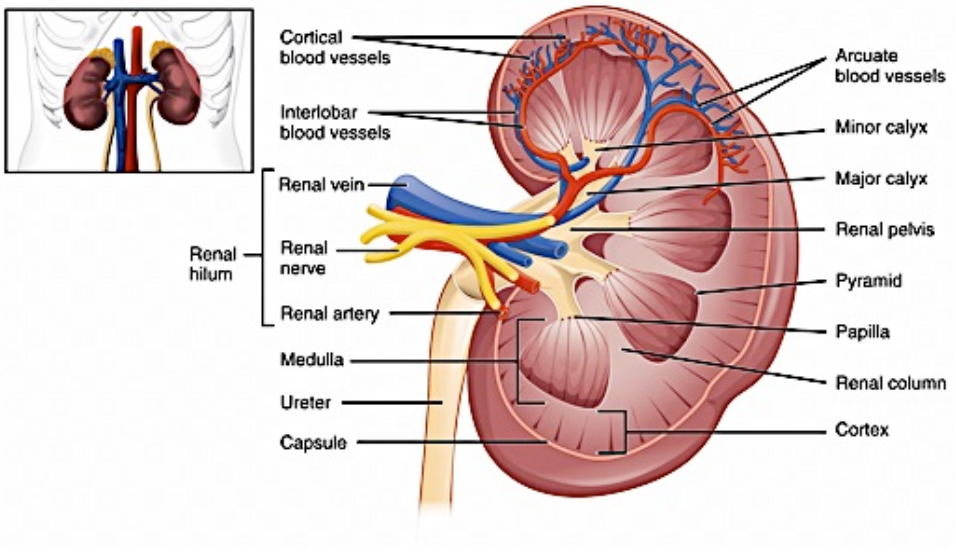
1.1.1 Gross Kidney Anatomy

The developed kidneys are bean-shaped organs approximately 4-5 inches in length residing on either side of the spine in the retroperitoneal space. The kidneys receive 20% of cardiac output when at rest through their vascularized system. The ribs protect the upper region of the kidneys. However, the right kidney sits lower than the left, due to the slight displacement by the liver (Supplementary Figure 1A). A connective tissue capsule allows the kidneys to hold their shape and allow protection, which is further covered by an adipose tissue layer for shock absorbance, called the renal fat pad. These excretory organs are further protected by strong fibrous connective tissue called the renal fascia. The renal fascia and the peritoneum serve to anchor the kidneys in the posterior region of the abdominal wall (Supplementary Figure 1B). On the superior region of the kidney resides the adrenal gland.



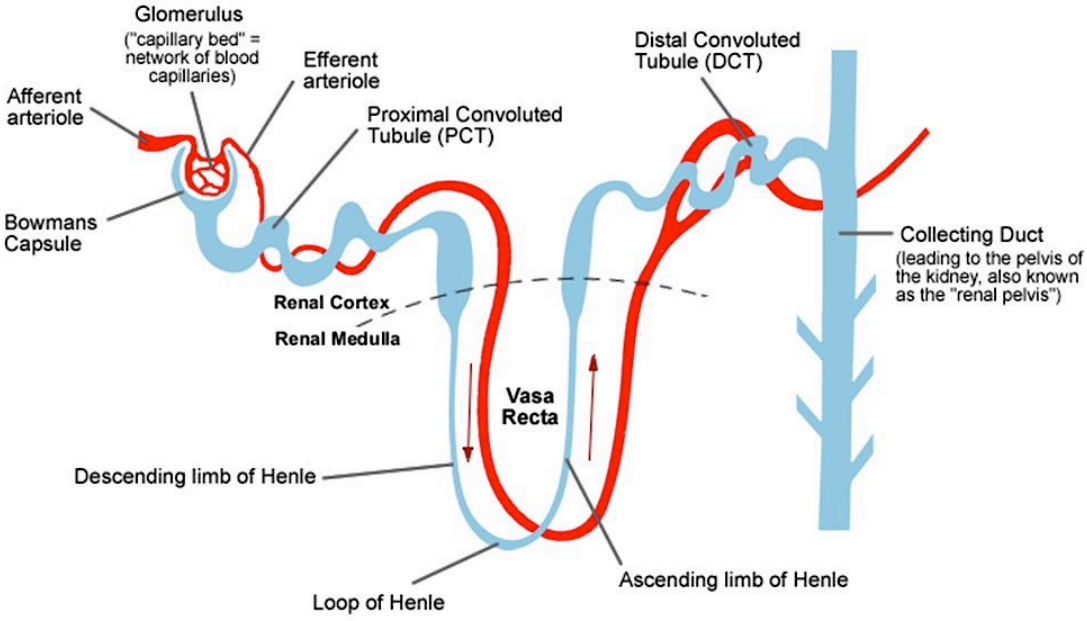
Supplementary Figure 1: Gross Anatomy of the Kidney. (A) The kidneys are protected by the ribs and located in the posterior abdominal wall in the retroperitoneal position. **(B)** The kidneys are further protected by connective tissue and fat layers. Image adopted from: https://opentextbc.ca/anatomyandphysiology/chapter/25-3-gross-anatomy-of-the-kidney/

The coronal section of the kidney reveals three distinct regions: the outer cortex, the inner medulla and the deeper renal pelvis, connecting the kidney to the ureter (Supplementary Figure 2). The renal cortex contains the cortical labyrinth and the medullary rays. Connective tissue extensions termed the renal columns radiate downward from the cortex to the medulla. They divide the medulla into two regions; the renal pyramids and the renal papillae. The renal columns provide the division of the kidney to 6-8 renal pyramids and serve as an entry and exit for the framework of blood vessels. At the tips of the renal pyramids are bundles of papillae called collecting ducts that drain excretory urine produced by the kidney's nephrons to the minor calyces. Several minor calyces form into larger calyces, termed major calyces. Major calyces then transport urine into the funnel shaped renal pelvis into the ureter for removal.



Supplementary Figure 2: Internal Anatomy of the Kidney. A coronal section of the kidney revealing various internal regions. Image adopted from: https://opentextbc.ca/anatomyandphysiology/chapter/25-3-gross-anatomy-of-the-kidney/

The functional units of the kidney are microscopic subunits called the nephrons that reside within the renal pyramids of the kidney. Their function is to cleanse the blood and form urine to be excreted. The nephron is composed of the renal corpuscle (located in the cortex) and the renal tubules (located from the cortex to the medulla). The renal corpuscle is composed of a glomerular tuft, a network of capillaries surrounded by podocytes that will filter the blood, in which the Bowman's capsule encapsulates them. The renal tubule is segmented into three distinct regions: proximal convoluted tubule, the loop of Henle, and the distal convoluted tubule (Supplementary Figure 3). Fluid will travel through the passageway of the renal tubules, in which the process of urine will form. The urine will drain into the collecting ducts and exit through the ureter.



Supplementary Figure 3: Diagram outlining the structure of the nephron in the mammalian kidney. Image adopted from: (Oni et al., 2008).

1.1.2 Normal Kidney Function

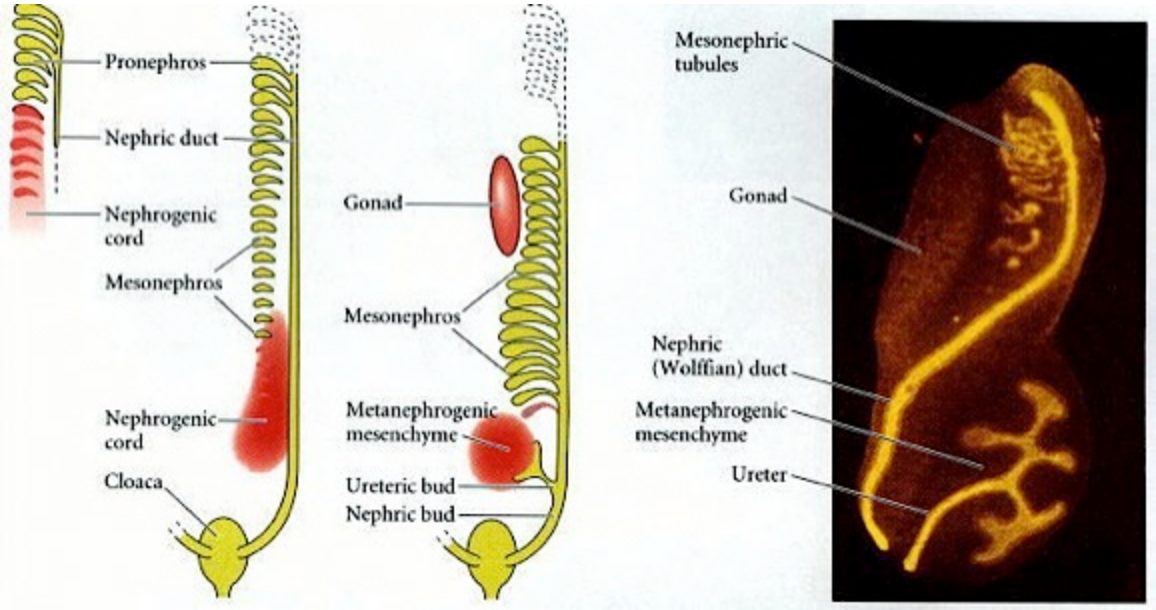
The kidney plays a vital role in the formation and excretion of urine from the body through the filtration of blood. By filtering the blood, the kidney regulates the water and soluble substances and reabsorbing what is needed. As mentioned above, the nephrons are the basic structural and functional unit of the kidney. Each nephron specializes in filtering, reabsorbing and secreting the necessary components to form urine. The process of urine formation begins when the afferent arteriole supplies blood to the glomerulus. The glomerulus is a capillary tuft that after receiving its blood supply from the afferent arteriole, it drives the force of fluid and solutes into the space of the Bowman's capsule. Before the fluid in the blood plasma can enter the interior of the Bowman's capsule, it must be filtered through a selective barrier. This selective barrier has three components: the diaphragms of the filtration slits, the thick glomerular basement membrane and inner visceral layer containing the foot processes of the podocytes. Once the fluid enters the luminal space of the Bowman's capsule it is called the glomerular filtrate. The glomerular filtrate then enters into the proximal tubule. Here, the glomerular filtrate is refined as molecules and water are exchanged between the lumen of the renal tubule and the peritubular capillaries that line the proximal tubule through the process of reabsorption and secretion. The solute concentration of the filtrate is further modified as it travels through the descending and ascending limbs of the Loop of Henle. Reabsorption continues through the peritubular capillaries when the filtrate enters the distal convoluted tubule. The peritubular capillaries secrete further toxins into the filtrate in the distal convoluted tubule. The filtrate then continues to travel from the distal convoluted tubule

into the collecting ducts for reabsorption of water into the blood. Lastly, the filtrate exits from into the minor calyces and through the ureter resulting in the exit of urine.

1.2 Kidney Development

1.2.1 Pre Metanephric Development

The development of the kidney progresses through three stages. The first two stages are transient and the third remains as a permanent mammalian kidney. Embryonic kidney development occurs on E8.5 in mice and day 22 in humans as the pronephric duct forms from the intermediate mesoderm. Cells of the pronephric duct migrate caudally and form the initial tubules in the anterior region. However, in mammalian kidneys, these ducts degenerate and the more caudal portions persist to form the excretory system. As the pronephric tubules degenerate, the central region of the nephric duct develops the initial kidney tubules. These tubules are referred to as the mesonephros. In some mammalian kidneys the mesonephros function in the filtration of urine, although for some it holds other functions such as the formation of the male gonads (Supplementary Figure 4).



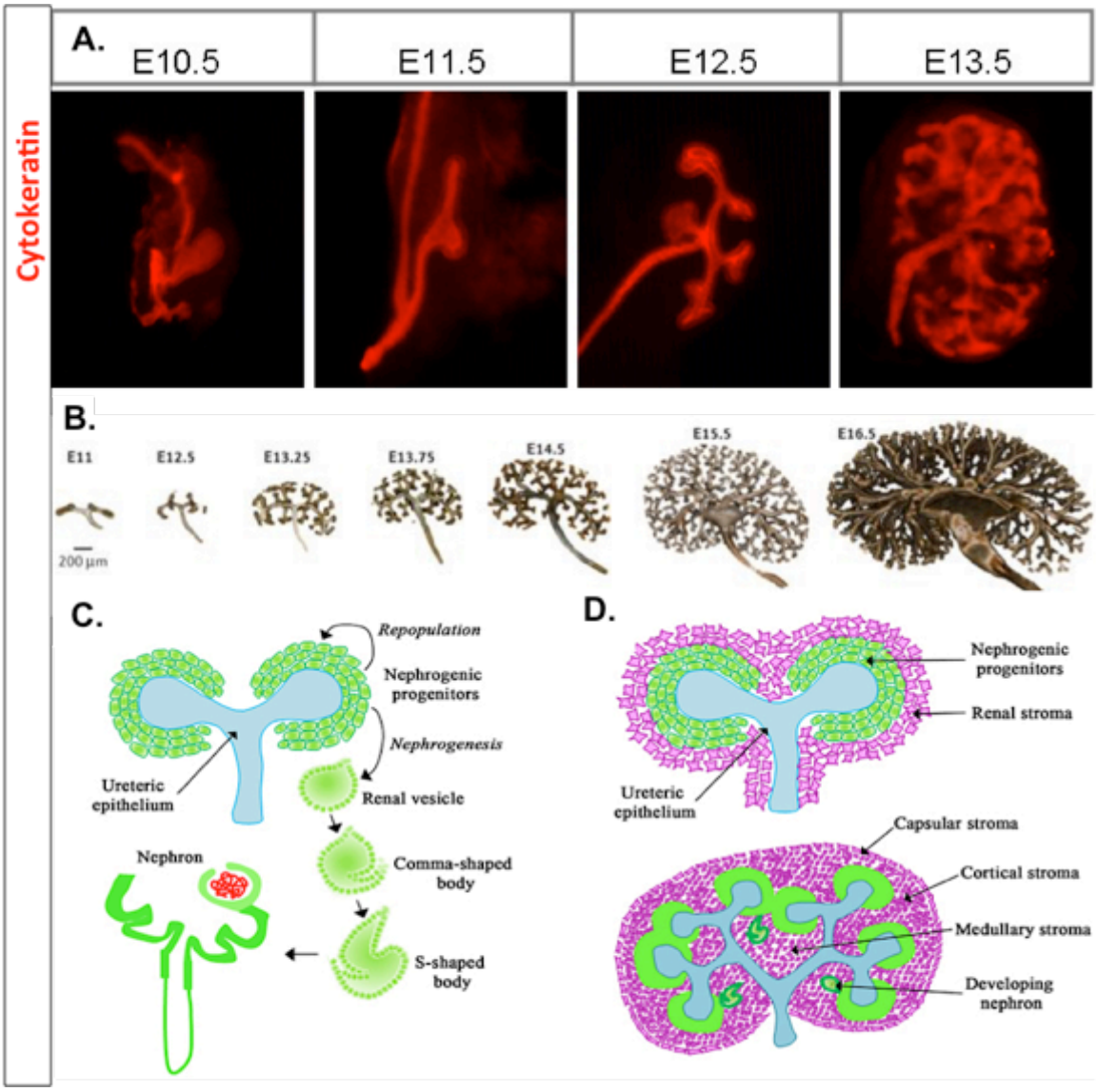
Supplementary Figure 4: Establishment of Wolffian Duct and Nephrogenic Cord. The Wolffian duct forms at E8.5, migrates caudally along the dorsal end of the body inducing the formation of mesonephric tubules. Pronephric tubules degrade via apoptosis. At E10.5, signals emanating from the metanephric mesenchyme cause an outgrowth off the Wolffian duct to form, called the ureteric bud. The ureteric bud then migrates into the Metanephric Mesenchyme. At E11.5 the ureteric bud has undergone branching morphogenesis to give rise to a T-shape branch. Image adopted: (Gillbert et al., 2000).

1.2.2 Mammalian Metanephric Kidney

The metanephrogenic mesenchyme (MM) arises in the posterior portion of the intermediate mesoderm developing the mammalian kidney. Development begins at E10.5 in mice and at 5 weeks gestation in humans. The mesenchyme then induces the development of a branch from each of the paired nephric ducts. These branches bifurcate to form the t-shaped ureteric buds. As the buds emerge from the nephric ducts, they invade the metanephrogenic mesenchyme. This invasion results in the mesenchymal cells to condense closer to one another adjacent to the ureteric epithelium (Gilbert et al., 2000). The ureteric bud and the metanephrogenic mesenchyme interact and reciprocate signaling molecules to induce the formation of the kidney. The ureteric bud branches elongate and undergo 10 cycles of branching morphogenesis in mice. These repeated cycles of ureteric bud elongation and bifurcation branching events will eventually generate the metanephric collecting duct system, renal calyx and ureter (Supplementary Figure 5A-B).

The interaction between the MM and UB also results in the aggregation of mesenchyme cells around the tips of the branches. These aggregated nodules are referred to as the cap mesenchymes. Each cap mesenchyme cell will either self-renewal or differentiate into a mature nephron. Those cells undergoing differentiation will condense further and form a pre tubular aggregate under the tips of the ureteric epithelium. The pre tubular aggregates will then undergo mesenchymal to epithelial transition. During epithelization, cells undergo morphogenesis and constriction of their apical surfaces to form a polarized spherical structure. This is the first stage in nephrogenesis that forms the renal vesicle. These renal vesicles subsequently differentiate into distinct morphological

structures, called comma-shaped, s-shaped, and developing nephron (Supplementary Figure 5C). Thus, these stages in nephrogenesis will ultimately form a mature nephron in the kidney. All nephrogenic stages occur in the nephrogenic zone in the cortex of the developing kidney (Supplementary Figure 5D).



Supplementary Figure 5: Morphology of Branching Morphogenesis and Nephrogenesis. (A-B) T-shape branch formation at E11.5. Branching morphogenesis results in continuous bifurcation branching of the ureteric epithelium giving rise to a branched collecting duct system. The pattern of branching morphogenesis is characterized by a swelling of each ureteric bud tip which is then remodeled to form two tips that extend and generate new branches. **(C)** Self-renewing and Nephrogenesis demonstrated with each stage and structure in the developing nephron (progenitors, renal vesicles, comma and s-shaped bodies, and a nephron). **(D)** Cap mesenchyme in the outer cortex of the kidney surrounded by adjacent renal stroma. Image adopted from: Dressler, 2009.

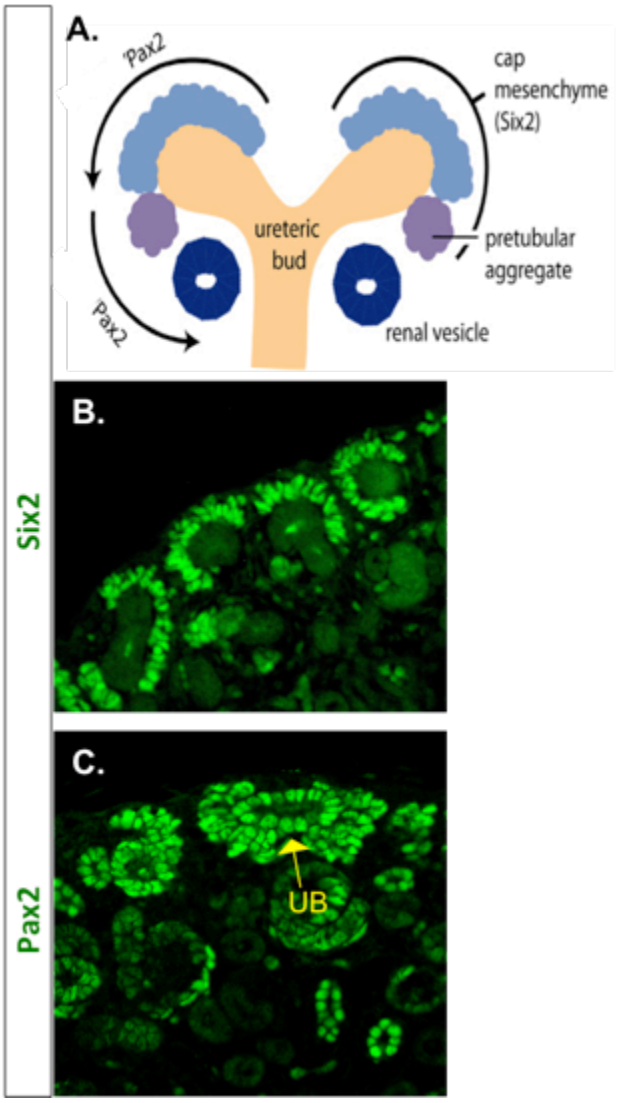
1.3 Cap Mesenchyme

1.3.1 Signaling in Nephron Progenitor Cells

The cap mesenchyme receives signals from the ureteric bud epithelium. These signals result in the cap mesenchyme to undergo two independent cell fates; self-renewal or nephrogenesis by initiating MET. In maintaining the CM pool of cells it ensures the continuation of UB branching morphogenesis in the developing kidney and a constant supply of cells to form new nephrons. However, once the progenitor pool gets exhausted in early postnatal development, nephrogenesis is completed. The nephron progenitors express many signaling molecules that are critical for the formation of the kidney. These factors include: *Eya1, Cited1, Hox11, Osr1, Pax2, Sall1, Six2,* and *Wt1* (Krause et al., 2015). The knockdown of these molecules results in abnormalities in kidney organogenesis. Sine oculis-related homeobox 2 (Six2) maintains the putative self-renewal potential of progenitors and prevents premature differentiation. It is expressed in all nephron progenitors however down regulated in pre-tubular aggregates (Supplementary Figure 6B). Studies have demonstrated that the depletion of Six2 results in the premature MET of all nephron progenitors to renal vesicles, causing early termination of nephrogenesis and a significant reduction in RVs (Self et al., 2006). Since, Six2 is expressed by every cap mesenchymal cell and plays a key role in halting ectopic pretubular aggregates from forming, it is thus a critical regulator for progenitor cell fate.

In addition, Pax2 has been studied as a key transcription factor in orchestrating patterns of gene expression in specific cells during kidney development (Torban and Goodyer, 1998) (Supplementary Figure 6C). A complete knockdown of the *Pax2* gene

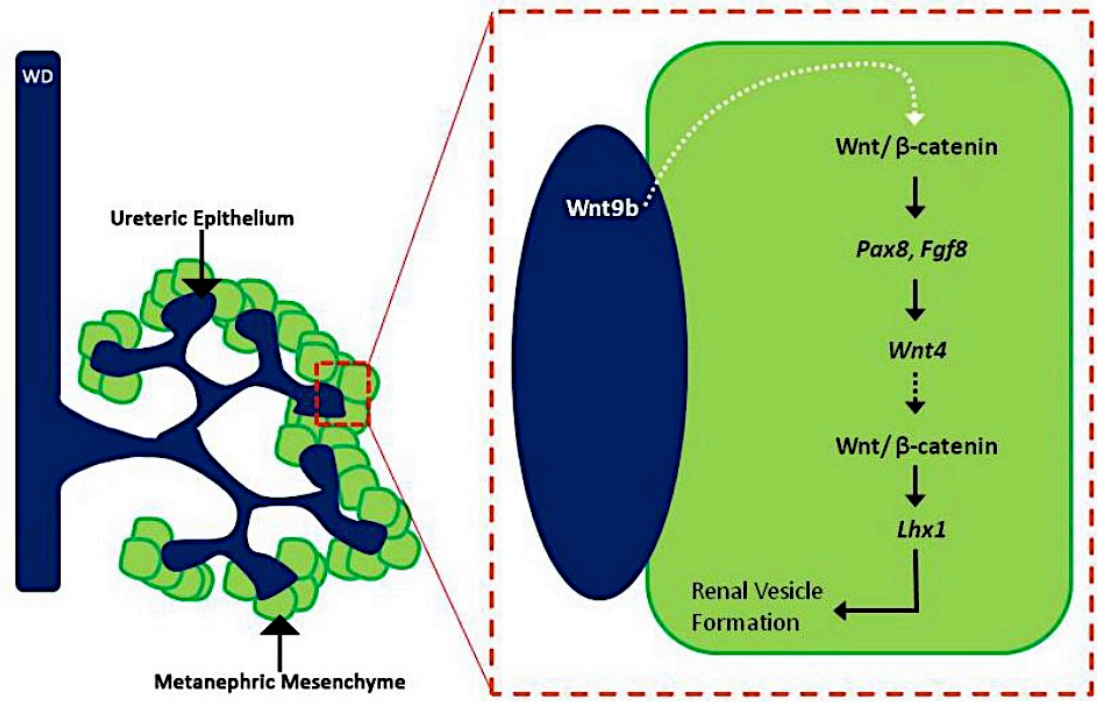
results in abnormal ureteric bud outgrowth and the inability to induce metanephric kidneys (Cai et al., 2005). During mammalian kidney development, Pax2 is first expressed in the nephric duct where it controls cell fate. It mediates the emergence of the UB into the adjacent lateral mesenchyme through activation of glial cell line-derived neurotropic factor (GDNF) in the mesenchyme and activation of the GDNF receptor (RET) in the ureteric epithelium cells (Brophy et al., 2001). Lastly, Pax2 plays a functional role in suppressing programmed cell death in the UB cells (Torban et al., 2000). Mice and humans lacking one allele of Pax2 have increases apoptosis of UB cells, decrease in UB branching and nephron number (Torban et al., 2000). Therefore, Pax2 has been demonstrated as an important transcription factor for normal kidney development.



Supplementary Figure 6: Cap mesenchyme signaling molecules. (A) Diagram depicting expression of Six2 and Pax2. **(B)** IF of E18.5 wildtype kidney expressing Six2 in the nephron progenitors in the outer cortex of the developing kidney. **(C)** IF of E18.5 wildtype kidney expressing Pax2 in the UB and CM.

1.3.2 Initiation of Nephrogenesis

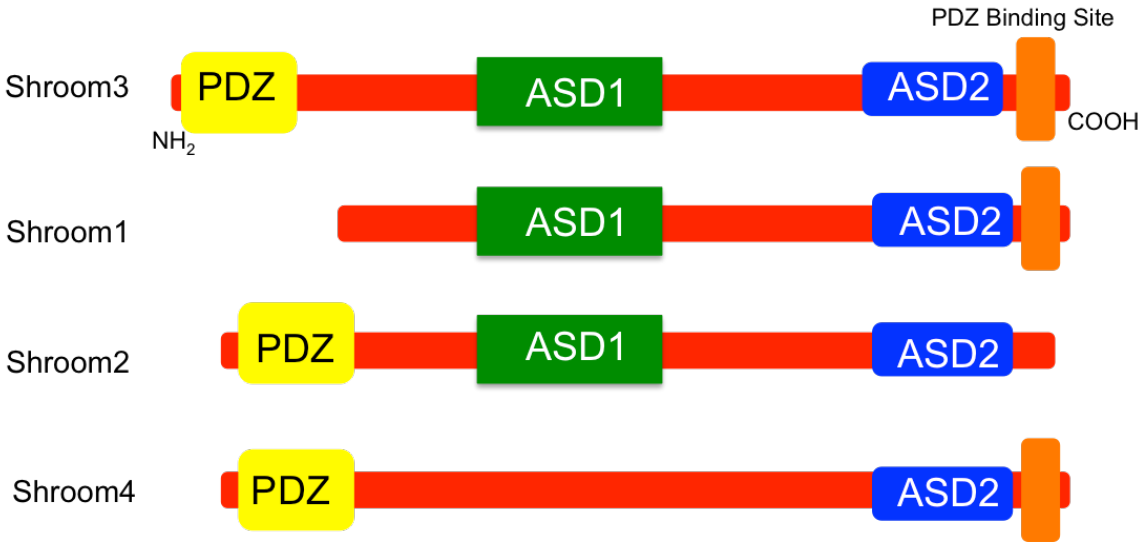
The start of nephrogenesis coincides with the inductive signaling of Wnt9b from the UB to the cap mesenchyme cells. Wnt9b induces a subset of mesenchymal cells to condense around the tips of the ureteric branches. The cap mesenchyme is composed of Six2-expressing nephrogenic progenitors, a subset of which will cluster to form a pre-tubular aggregate and express Wnt4 (Carroll et al., 2005). The identification of Wnt4, through the canonical Wnt9b/β-catenin signaling pathway, provided importance of Wnt signaling in kidney development. Like Wnt9b deficiency, in the absence of Wnt4 expression, nephron differentiation is prevented halting prior to pre tubular aggregate formation (Stark et al., 1994). In the case of Wnt4 deficiency, *Pax8* and *Fgf8* initially are expressed in the pre tubular aggregates, making them upstream targets of Wnt4 and downstream of Wnt9b (Stark et al., 1994). Studies have also provided evidence that *Lhx-1*-deficient embryos express Wnt4, however Lhx-1 is not expressed in Wnt4 and Fgf8 deficient mice (Peratoni et al., 2005). These findings provide a potential model for Wnt9b signaling for nephrogenesis (Supplementary Figure 7).



Supplementary Figure 7: Wnt/β-catenin signalling in nephrogenesis. Model of Wnt/β-catenin signaling in the metanephric mesenchyme in the control of Nephrogenesis In this model, Wnt/β-catenin signaling mediates Wnt9b signalling and activates the expression of key genes required in the early nephrogenic program. Image adopted from: https://macsphere.mcmaster.ca/bitstream/11375/17413/2/MSc%20Thesis-Hadiseh%20Khalili.pdf.

1.4 Shroom Family of Proteins

The family of Shroom's consists of four proteins in veretebraes. These actin binding proteins were previously named, Apx (Staub et al., 1992), Apxl (Schiaffino et al., 1995), Shroom (Hildebrand and Soriano, 1999), KIAA1202 (Hagens et al., 2006b) and recently renamed as Shroom1, Shroom2, Shroom3, and Shroom4; respectively (Hagens et al., 2006a). There are three domains in the Shroom3 family; the N-terminal PDZ domain, the central ASD1 domain, and the C-terminal ASD2 domain (Hildebrand and Soriano, 1999, Hagens et al., 2006) (Supplementary Figure 8). The PDZ domain interacts with various receptors to facilitate cytoskeletal architecture and polarity (Hildebrand and Soriano, 1999). The ASD1 domain directly mediates the interaction between Shroom and F-actin. Lastly, the ASD2 domain, high conserved amongst all Shroom proteins, binds to Rho kinases and is important in carrying out cell constricting activity (Hildebrand and Soriano, 1999). The functional differences in Shroom2, Shroom3 and Shroom4 are mediated by alterations in their subcellular localization. Their differences includes Shroom2 and Shroom4 being unable to induce apical constriction, even though they have the capacity to do so having the ASD2 domain. Shroom2 compartmentalizes to the cortical actin, while Shroom4 binds to a punctuate region of actin. Thus, Shroom3 is the only protein in the family that can induce apical constriction in epithelial cells (Dietz et al., 2006). Although the Shroom proteins use a common mechanism, their roles are determined by their temporal and spatial binding to different components of actin (Dietz et al., 2006).



Supplementary Figure 8: Domain structure of each Shroom protein.

1.4.1 Shroom3 Expression

Recent publications have begun to identify the important role of Shroom3 for tissue morphogenesis. Using gene trap mutagenesis, a mutation in Shroom3 caused exencephaly, acrania, facial clefting, and spina bifida, all of which can be attributed to failed neural tube closure (Hilebrand and Soriano, 1999). Mice carrying the gene trap mutation display neural folds that "mushroom" away from the dorsal midline (Hildebrand and Soriano, 1999). At the onset of neural tube closure, neural epithelial cells undergo apical constriction and form hinge point to ensure proper neural plate bending (Haigo et al., 2003). Hildebrand and Soriano observed that a complete knockdown of *Shroom3* results in failure of hinge point formation and defective neural tube closure. They identified that Shroom3 induces apical constriction in epithelized cells and is localized to the adherence junctions. However, Shroom3 has also been identified in not only the neural epithelia, but in in the developing gut, eye, lungs and somites (Hildebrand and Soriano, 1999, Sevilla-Perez et al., 2008). Shroom3's expression is required for cell shape changes and morphogenesis in these tissues as its knockout results in defective gut looping (Chung et al., 2010), and lens pit invagination (Plageman et al., 2010). In humans, a recessive missense mutation in SHROOM3 is associated with heterotaxy syndrome, suggesting it as a novel target for the control of left-right asymmetry (Tariq et al., 2011).

1.4.2 Shroom3 and the Kidney

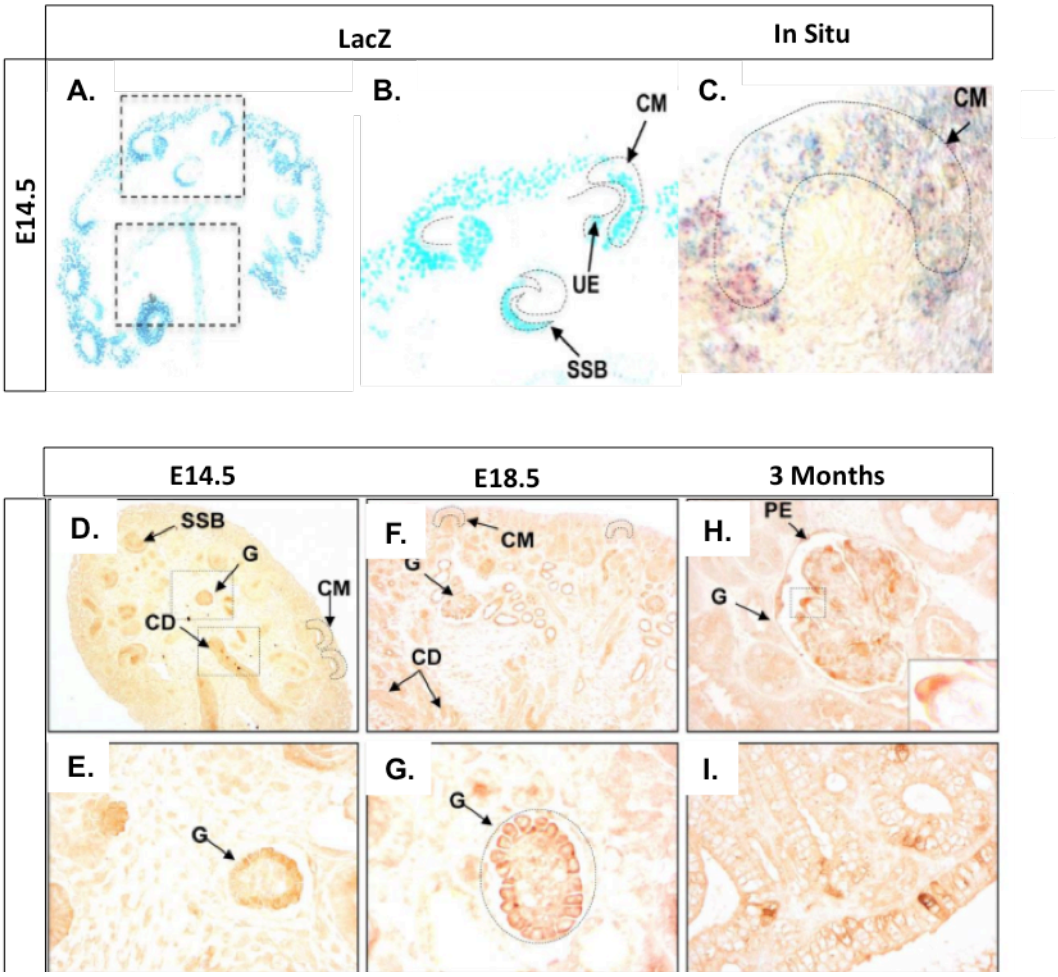
Genome wide associated studies (GWAS) have located regions in the genome associated with chronic kidney disease (CKD) (Kottgen et al., 2009). CKD is a heritable disease, and a major health concern for individuals as it causes significant morbidity and mortality. Much of the CKD heritability and risk loci remain vastly unknown (Yeo et al., 2014). The loci for CKD were identified using the glomerular filtration rate in the meta-analyses for study specific GWAS (Kottgen et al., 2009). Four loci were identified, one of which was a SNP at the eGFRcrea locus at the intronic SNP rs17319721. It was located in a highly evolutionary conserved region in Shroom3 on chromosome 4 (Kottgen et al., 2009). A study conducted by Yeo et al. identified 13 genetic variants in the Shroom3 gene that directly correlate with the onset of kidney disease in Fawn-Hooded Hypertensive (FHH) rats. Yeo et al. demonstrated that introgression of the wildtype Shroom3 gene onto the Fawn-Hooded Hypertensive (FHH) rat rescues glomerular defects. They generated a congenic rat strain called FHH.BN14, which encompasses the 14.50-21.40 Mbp region of Chromosome 14 of the Shroom3 gene onto the FHH rat model. When compared to the parental FHH rat, both homozygous and heterozygous FHH.Bn14 mice improved levels of acute kidney injury, glomerular sclerosis and did not have any foot processes fusion in the podocytes, as seen in the FHH rats.

A second study investigated the intronic SNP in Shroom3 in renal allograft recipients (Menon et al., 2015). They determined, in vitro, that the sequence containing the rs17319721 risk allele was a transcription factor 7-like 2-dependent enhancer element.

This enhancer element functioned to increase Shroom3 transcription and contribute to allograft fibrosis in conjunction with Wnt/β-catenin in renal tubular cells (Menon et al., 2015). This led them to further investigate associations of Shroom3 with glomerular/non-glomerular compartments in relation to CKD, in 2018. They identified the interaction of the Src-kinase FYN with Shroom3 via SH3-SH3 binding sites in podocytes. This interaction facilitated the regulation of FYN activation, nephrin phosphorylation, and actin organization. Thus, this resulted in altered podocyte morphology and migration (Wei et al., 2018). These studies provided an essential first step towards designing therapeutics for renal fibrosis by the inhibition of Shroom3.

A third study investigated the role and function of Shroom3 in the developing kidney (Khalili et al., 2016). The X-gal staining showed a distinct expression pattern throughout kidney development through endogenous Shroom3 expression. At E14.5, strong LacZ activity was observed in the condensing mesenchyme and the developing podocyte layer of the S-shaped body. In addition, post-natal mice demonstrated strong LacZ reporter activity in the glomerulus in a pattern consistent with the podocytes and modest expression levels in the parietal epithelial cells (Supplementary Figure 9A,B). Since LacZ reporter activity may not accurately represent the complete expression pattern of Shroom3, as these mice are mutants, immunohistochemistry was performed using an antibody specific to the C-terminus of the Shroom3 protein on WT kidney sections. Immunohistochemistry of E14.5 WT kidney demonstrated Shroom3 expression in the S-shaped body, glomerulus, and collecting duct system (Supplementary Figure 9C). Lastly, the study demonstrated Shroom3 mRNA was expressed in the condensing mesenchyme

in the outer marginal zone of the kidney, glomerulus, and collecting ducts (Supplementary Figure 9D-I). These studies suggest that Shroom3 is important in both nephrogenesis and branching morphogenesis.



Supplementary Figure 9: Shroom3 is expressed in developing kidneys. (A,B) X-gal staining of E14.5 kidney sections demonstrating endogenous shroom3 expression LacZ is expressed in the condensing mesenchyme, S-shaped body, glomeruli and collecting ducts (C) In-situ Hybridization of Wilm's Tumor-1 (blue), a marker of all nephron progenitors, and Shroom3 (red) demonstrating mRNA expression within the cap mesenchyme at E14.5. (D-I) Immunohistochemistry of Shroom3 expression in the glomerulus, s-shaped bodies and collecting ducts. (Khalili et al., 2016).

1.5 Overall Rationale

Despite the identification of Shroom3 in the kidney and its association with CKD through GWAS, its supportive role during kidney development is not well known. Since Shroom3 is a critical factor in actin localization in the podocytes and normal glomeruli development, it may be an important protein during proper nephron formation. However, understanding Shroom3's role during nephrogenesis has not been investigated, and it is not well known where Shroom3 is precisely located in the mesenchymal population of cells. Therefore, investigating the mesenchymal cells and the first stages of nephrogenesis can aid in understanding the molecular mechanisms by which Shroom3 mediates and/or regulates during kidney development.

2. HYPOTHESIS AND OBJECTIVES

2.1 Overall Hypothesis

The overall hypothesis of this study is that ***Shroom3 plays a role in nephron formation.***

2.2 Study Objectives

1. Characterize Shroom3 expression in the nephron progenitors

   Hypothesis: ***Shroom3 is expressed in some cells within in the cap mesenchyme and ureteric epithelium in early and late embryonic kidney development***

2. Characterize the nephron progenitors in Shroom3 wildtype and mutants

   Hypothesis: ***Shroom3 mutants will have abnormally organized nephron progenitors***

3. Determine if Shroom3 controls cell polarity in renal vesicles

   Hypothesis: ***Shroom3 mutants will have abnormally developed renal***

vesicles

3. MATERIALS AND METHODS

Mouse Strains

Animal studies were performed in accordance with Canadian Council for Animal Care and McMaster institutional guidelines (Animal utilization Protocol #10-08-55). Shroom3 heterozygous mutant mice Shroom3$^{Gt(ROSA)53Sor}$/J were gifted from Dr. Thomas Drysdale at the University of Western Ontario. These mice contain a gene trap cassette, SAβgalCrepA originally designed by Hildebrand and Soriano. This cassette is inserted between exon 3 and 4 of Shroom3 gene (Hildebrand and Soriano, 1999). The gene trap contains an adenovirus splice acceptor (SA) (Friedrich and Soriano, 1991), a bifunctional gene encoding a fusion between β-galactosidase (β-gal), Cre recombinase and an MC1 polyadenylation (pA) sequence (Thomas and Capecchi, 1987). Shroom3 heterozygous mice were crossed together to generate homozygous null mice.

PCR Genotyping

DNA was isolated by lysing mouse tails at 95°C in 50mM NaOH for 1 hour and then 0.5M Tris-HCl pH 8.0 was added into solution. The solution was mixed and centrifuged at 12,000 rpm for 3 minutes. PCR conditions for amplification of WT and mutant alleles were 95°C for 1 minute, 65°C for 1 minute and 72°C for 1.5 minute for 30 cycles. PCR conditions for amplification of LacZ gene were 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 1 minute for 35 cycles. The expected amplicon size for the LacZ gene was

389 bp. To confirm the presence of the *LacZ* allele, the forward primer 5'-GTTGCAGTGCACGGCAGATACACTTGCTGA-3', and reverse primer 5'-GCCACTGGTGTGGGCCATAATTCAATTCGC-3' were used.

Mouse Dissections and Fixation

Shroom3$^{Gt/+}$ x *Shroom3*$^{Gt/+}$ were crossed and plugs were checked between 7-9AM. Noon of the day of vaginal plug was considered to be embryonic day 0.5. Mouse embryos were resected from pregnant mice at E13.5 and E18.5 under an Olympus SZ61 microscope. Kidneys were dissected out of embryos using Dumont #5 INOX surgical forceps in cold Phosphate-buffered saline (PBS) pH 7.4. Once dissected samples were placed in 4% paraformaldehyde (PFA) for 24 hours at 4°C and then prepared for tissue analysis.

Histology

Whole embryos and resected kidney tissue were fixed in 4% paraformaldehyde for 24 hours at 4 °C. Embryos and kidneys were paraffin-embedded, sectioned to 4μm, and mounted on Superfrost™ Plus slides (Thermo Fisher Scientific, Waltham, MA) and laid flat to dry overnight at room temperature. Sections were deparaffinized using 3 xylene washes and rehydrated using graded ethanol washes (100%, 95%, 75%, 50%, H20). Tissue sections were used for Hematoxylin and Eosin staining (H&E), immunohistochemistry (IHC), and immunofluorescence (IF) as described below. Images were captured using either an Olympus BX60 or a Nikon 90i-eclipse upright microscope or Leica.

Hematoxylin and Eosin

Tissue sectioned slides were stained with Hematoxylin (Sigma, H3136) for 3 minutes and rinsed with deionized water, and then washed for 5 minutes with tap water. Kidney and embryo sectioned slides were then dipped 8-12 times in Acid ethanol to destain, and washed again twice in tap water for 5 minutes and once in deionized water for 2 minutes. Slides were then stained with Eosin (Sigma, E4009) for 1 minutes, and dehydrated using an ethanol gradient. Sectioned slides were mounted using permanent mounting medium (Vector Labs, H-5000). Slides were dried overnight in incubator. Images were captured using either an Olympus BX60 or a Nikon 90i-eclipse upright microscope or Leica ..

Immunofluorescence (Paraffin-embedded)

Tissue was deparaffinized, and antigen retrieval was performed for 4 minutes in 10mM sodium citrate solution pH 6.0 in a pressure cooker. Sections were washed in PBS 3 times. A hydrophobic barrier (Vector Labs, H-4000) created around the tissue and rewashed in PBS. Slides were incubated in blocking buffer (DAKO, X0909) for 1 hour at room temperature. Sections were then incubated with primary antibodies to Six2 (Proteintech Group, 1:200), Pax2 (Covance, 1:200), Par3 (Millipore, 1:100), and NCAM (Sigma, 1:200), overnight at 4°C. Tissue sections were then serial washed with PBS, and incubated with Alexa Fluor 488 or 568-conjugated secondary antibodies in blocking buffer (Invitrogen, 1:1000) for 1 hour at room temperature. Kidney sections were washed in PBS and stained with Dapi (Sigma, D9542; 1:1000 dilution) for 5 minutes and

coversliped using Fluoromount (Sigma, F4680). All IF images were captured with a high-resolution camera and associated software (Open-Lab; Improvision, Lexington, MA)

Immunofluorescence (Frozen)

Cryosectioned slides were used, previously sectioned by Hadiseh Khalili, in our laboratory that were stored at -80. Room temperature tissue sections were fixed in 4% PFA and then washed using PBS. Tissue was permeabilized using Trixton-X and then rewashed with PBS. Slides were incubated in blocking buffer for 1 hour in a humidified chamber at room temperature. Sections were then incubated with primary antibody Shroom3 diluted in blocking buffer (gifted by Dr. Plageman, 1:200) for 1 hour at room temperature. Slides were washed with blocking solution and incubated with Alexa Fluor 488 conjugated secondary antibodies (Invitrogen, 1:1000) for 1 hour at room temperature. Kidney sections were washed in PBS and stained with Dapi (Sigma, D9542; 1:1000 dilution) for 5 minutes and coversliped using Fluoromount (Sigma, F4680). All IF images were captured using Leica.

Immunohistochemistry

Paraffin embedded kidney sections were deparaffinzed and rehydrated using graded ethanol washes. Kidney sections were subjected to antigen retrieval in pressure cooker at 4 minutes in 10mM sodium citrate solution pH 6.0. Sections were washed in PBS, a hydrophobic barrier (Vector Labs, H-4000) drown to outline the tissue and rewashed in PBS. Endogenous peroxidase activity was quenched using 3% H2O2 for 10 minutes at

room temperature. Kidney sections were blocked in 10% horse serum in PBS for 1 hour at room temperature followed by blocking endogenous biotin binding activity using a biotin/avidin blocking kit (Vector Labs, SP-2001). Kidney sections were incubated with Shroom3 antibody (gifted by Dr.Plageman, 1:200) overnight at 4°C. Kidney sections were washed three times with PBS and incubated in biotinylated secondary antibodies (Vector labs, B-9500) diluted in blocking buffer for 1 hour at room temperature. Sections were washed in PBS and incubated with Vectastain elite ABC reagent for 30 minutes at room temperature (Vector Labs, PK7100). Kidney sections were washed with PBS and incubated in peroxidase substrate solution DAB (Vector Labs, SK4100) to develop desired stain intensity and slides were mounted using permanent mounting medium (Vector Labs, H-5000). Images were captured using either an Olympus BX60 or a Nikon 90i-eclipse upright microscope.

Cap Mesenchyme Area and Length Measurement

To determine the area and length of the cap mesenchyme for E13.5 and E18.5 kidneys three kidney sections each 60μm apart, were collected per kidney for analysis. E18.5 kidneys and E13.5 kidneys. were immuno-stained with Six2 (Proteintech Group, 1:200) and images from three different random regions were taken at 20X and 10X magnification respectively. The area and length were counted using ImageJ version 1.51 (2015). Using the known scale per 10x or 20x image, the scale was set prior to measuring the length and area of the cap mesenchyme. Using the Wand Tracing Tool, the inner cap

mesenchymal length and the full area of the cap mesenchyme was manually traced. Using the set scale, the length and area were measured. The area and length were measured for each 20X image and averaged per kidney section for 3 different E18.5 *Shroom3*^{WT} and 3 different *Shroom3*$^{Gt/Gt}$. The area and length were estimated for each 10X image and averaged per kidney for 3 different E13.5 *Shroom3*^{WT} and 3 different *Shroom3*$^{Gt/Gt}$.

Nephron Progenitor Count

To count the number of nephron progenitors in E18.5 *Shroom3*^{WT} and *Shroom3*$^{Gt/Gt}$ mice, three sections, fifteen sections apart, were collected per kidney for analysis. Kidneys were immuno-stained with Six2 (Proteintech Group, 1:200) and images were taken at 20X magnification in three randomly selected areas of the nephrogenic zone. The Six2+ nuclei were counted using ImageJ version 1.51 (2015). The images were adjusted in threshold by inverting them to black and white and removing any noise in the background. The threshold was adjusted separately per image. Images were then processed and water shed to separate any overlapping cells. Watershedding the cells allows for 1-pixel lines to separate the overlapping cells. Selecting analyze and then analyze particles, and then outline, the number of nephron progenitors were counted.

Renal Vesicle Count

To quantify the number of renal vesicles in E13.5 and E18.5 *Shroom3*^{WT} and *Shroom3*$^{Gt/Gt}$ mice, three sections, fifteen sections apart, were collected per kidney for analysis. Kidneys were stained with H&E and imaged at 10X magnification for E13.5 and 20X for E18.5 kidneys. The renal vesicles were counted based on the following criteria:

1) renal vesicles are in the outer marginal zone of the kidney, 2) Renal vesicles must be located in the "arm pit" of the UB, 3) They must have "pie" shaped cells in a spherical structure. The number of renal vesicles per section was averaged for each kidney from 3 different *Shroom3*^{WT} and 3 different *Shroom3*$^{Gt/Gt}$.

Statistical Analysis

The nephron progenitor count, area and length, and the renal vesicle count was analyzed using a two-tailed Student's t-test using GraphPad Prism software, version 6.0c (Graphpad, La Jolla, CA). $P < 0.05$ indicates statistical significance. When performing analyses in E13.5 kidneys, 3 different wildtype and 3 different mutants were used, with 3 non serial sections per kidney. In E18.5 kidneys, 3 different wildtype and 3 different mutants were used, with 3 non-serial sections with an average of three separate areas per section.

Ethical Statement

All animal studies were performed in accordance with animal care and institutional guidelines at McMaster University (Animal Utilization Protocol #10-08-55).

4. RESULTS

4.1 *Shroom3* knockout model

Our mouse model studies were performed utilizing heterozygous mutant mice (Shroom3$^{Gt(ROSA)53Sor}$/J) gifted to us by Dr. Thomas Drysdale at the University of Western Ontario. These mice were generated by inserting a gene trap cassette (SAβgalCrepA) inserted between exons 3 and 4 in the Shroom3 gene ending in a premature stop codon, which was originally designed by Hildebrand and Soriano. The gene trap has an adenovirus splice acceptor (S) (Friedrich and Soriano, 1991), a bifunctional gene encoding a fusion between β-galactosidase (β-gal), Cre recombinase and an MC1 polyadenylation (pA) sequence (Thomas and Capecchi, 1987) (Figure 1). Therefore, we crossed the Shroom3$^{Gt(ROSA)53Sor}$/J mice to generate knockout mutant Shroom3$^{Gt/Gt}$ mice. However, in order to identify (wildtype) *Shroom3*^{WT} and (heterozygous) *Shroom3*$^{Gt/+}$mice, PCR genotyping was performed on the tail DNA, to amplify the *LacZ* transgene, present in *Shroom3*$^{Gt/+}$ mice. To confirm the presence of the *LacZ* allele, the forward primer 5'-GTTGCAGTGCACGGCAGATACACTTGCTGA-3', and reverse primer 5'- GCCACTGGTGTGGGCCATAATTCAATTCGC-3' were used. *Shroom3*$^{Gt/Gt}$ null mutants exhibited severe exencephaly and spina bifida during embryonic development, which was not present in their wildtype and heterozygous

littermates. Lateral edges of the cranial neural folds appearing wavy and "mushroom" like was exemplified only in homozygous mutant mice during embryonic dissections (Figure 2).

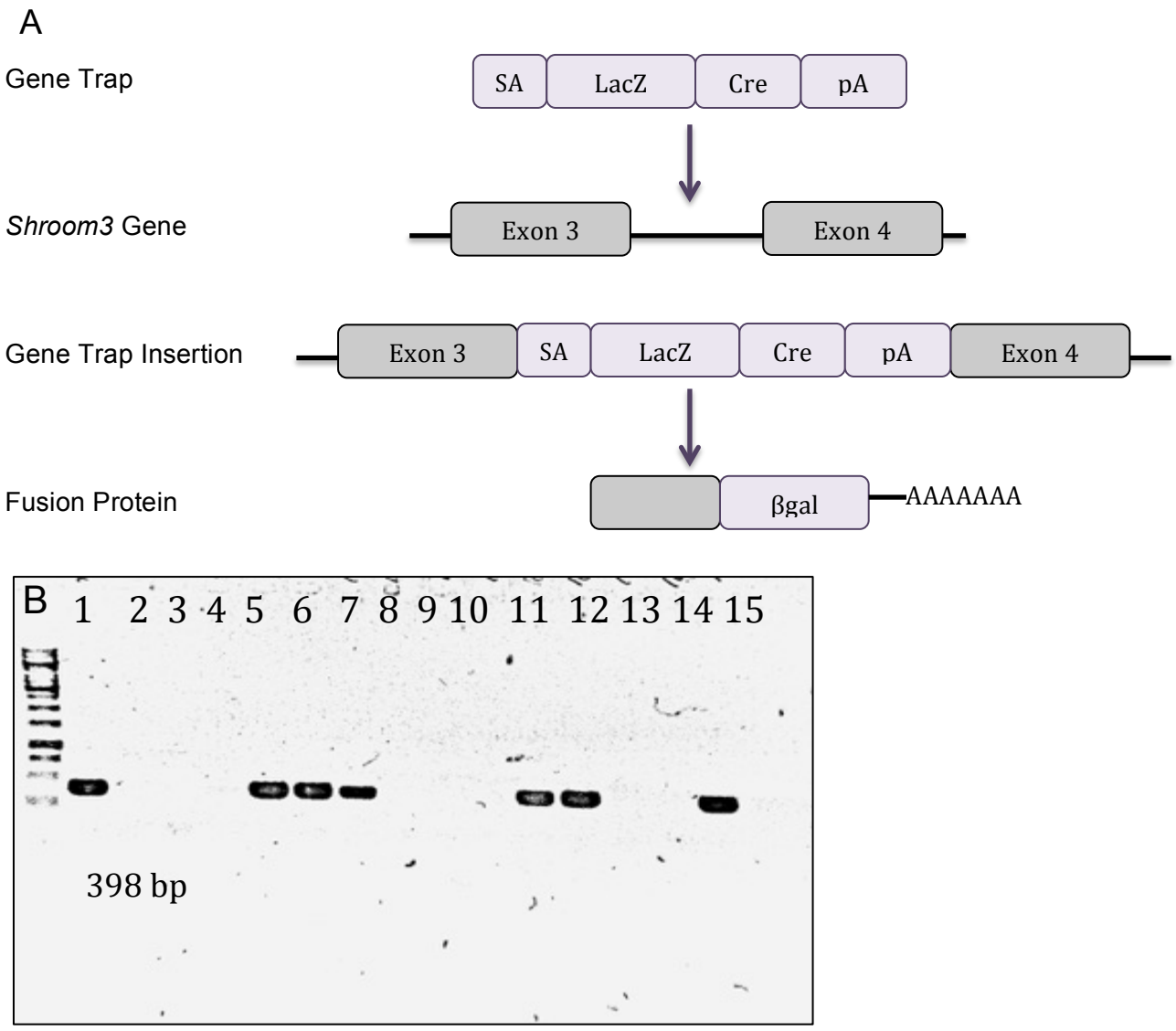
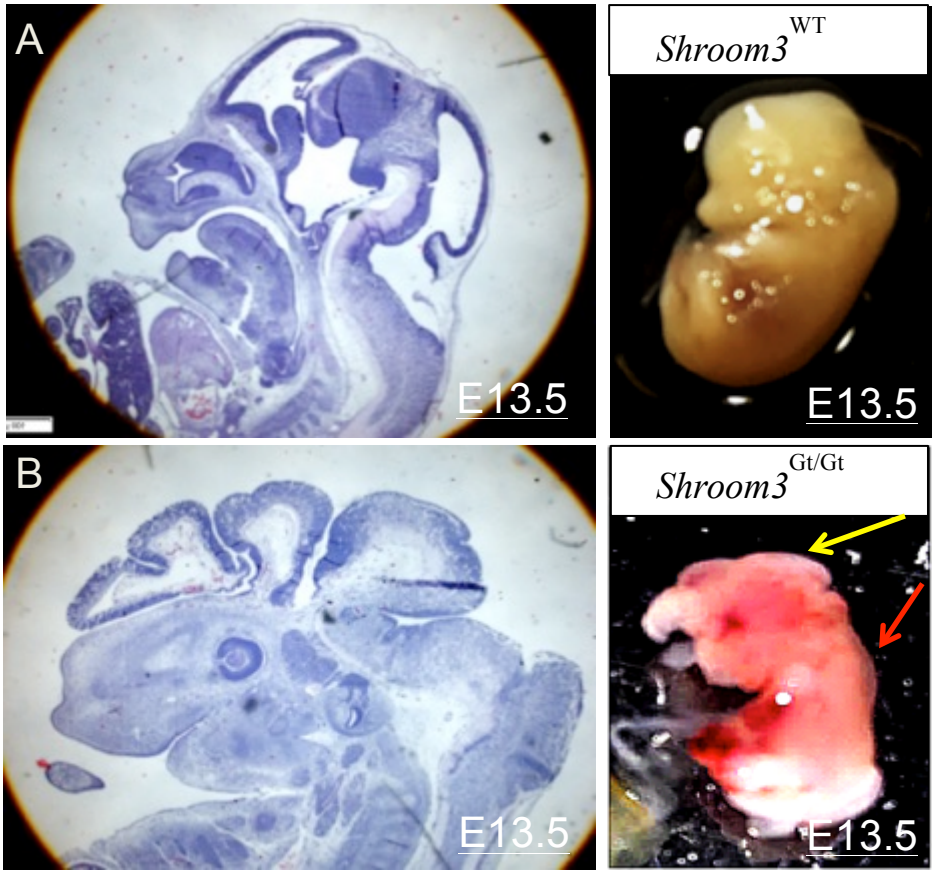


Figure 1: Shroom3 gene trap insertion. **(A)** The gene trap contains an adenovirus splice acceptor (S), a bifunctional gene encoding a fusion between β-galactosidase (β-gal), Cre recombinase and an MC1 polyadenylation (pA) sequence inserted between exons 3 and 4. **(B)** Genotyping of wildtype and heterozygote embryos using primers specific to heterozygote mice having the *LacZ* inserted allele (398 bp) (Bands 1, 5-7, 11-12, 15 show HET).

Figure 2: Identification of Shroom3^{WT} and Shroom3$^{Gt/Gt.}$



Identification of Shroom3^{WT} and Shroom3$^{Gt/Gt.}$. (A) H&E stain and gross anatomy of a *WT* E13.5 embryo demonstrating proper cranial development. **(B)** H&E stain of a mutant E13.5 Shroom3 embryo demonstrating cranial deformation. Gross anatomy of mutant E13.5 embryo. Yellow arrow indicating exencephaly. Red arrow indicating spina bifida.

4.2 *Shroom3* expression in the cap mesenchyme and ureteric bud tips

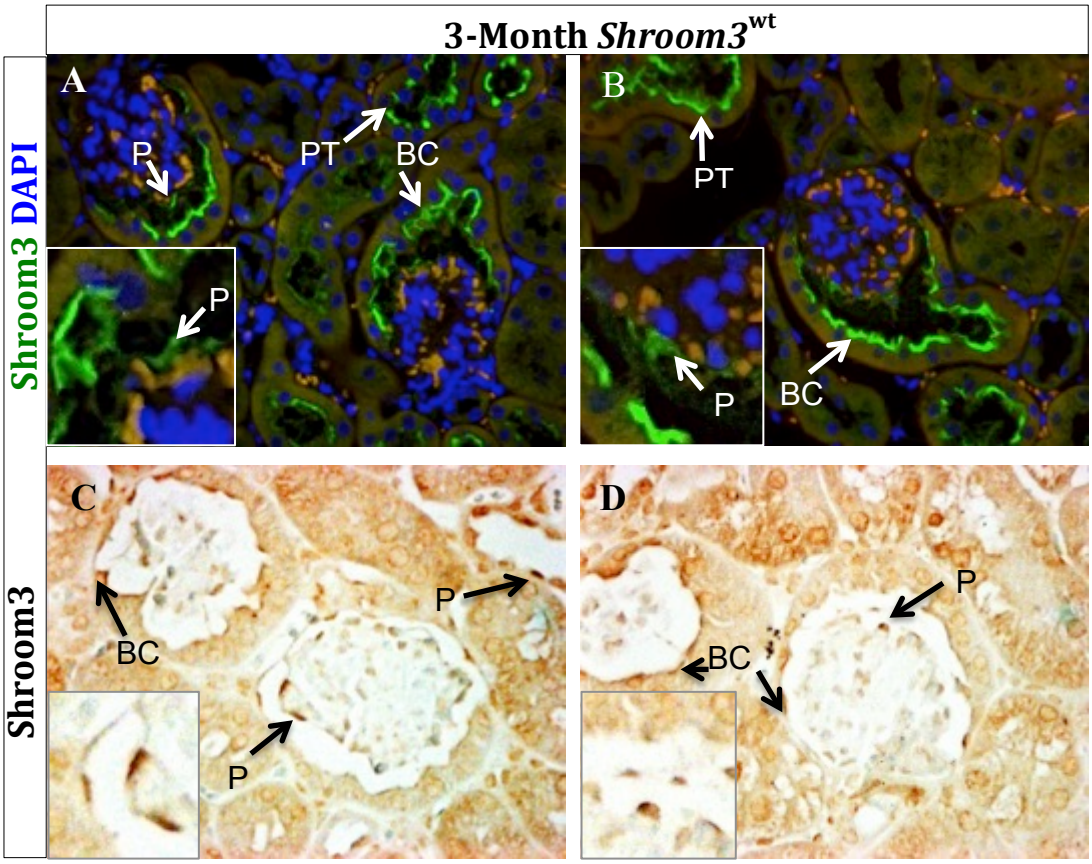
Previous data from our laboratory has shown that Shroom3 expression has a unique spatial and temporal expression pattern in the developing and mature kidney (Khalili et al., 2016). This was first demonstrated through X-gal staining of whole mount kidneys. The X-gal staining showed a distinct expression pattern throughout kidney development (Supplementary Figure 9). Most notably, *LacZ* activity was observed in the condensing mesenchyme. In addition, through in situ hybridization, Shroom3 mRNA was observed in the cap mesenchyme region of developing kidneys (Supplementary Figure 9). These studies demonstrated, for the first time, Shroom3 detection in the mesenchymal cell population (Haiseh et al., 2016). The cap mesenchyme is composed of two cell populations; the self-renewing cells and the nephron progenitors. However, the previous data was not able to determine whether Shroom3 is present in all cells within the cap mesenchyme or only some nephron progenitors. Therefore, the precise location of Shroom3 within the cap mesenchyme still needed to be investigated. While we have previously performed IHC and IF using Shroom3, this AB was from Santa Cruz and was no longer available. Therefore I used a new Shroom3 antibody from Sigma Aldrich and performed immunofluorescence and immunohistochemistry on 3-month-old postnatal kidney tissue. My initial goal was to verify previously reported Shroom3 expression patterns. After several trials and significant troubleshooting, I determined that the Shroom3 antibodies from Sigma did not identify Shroom3 expression. I next contacted Dr. Timothy Plageman since he developed a Shroom3 Ab in his laboratory, but it is not commercially available. This antibody was gifted to us and targets the amino acid

sequence CLLEGMRQADIRYVK on exon 5 of isoform 1 and 2 of the Shroom3 protein. Again, I verified this Shroom3 Ab using *WT* 3-month-old postnatal kidney tissue by IF. Shroom3 expression was observed in the glomerulus, specifically in the apical boarder of the Bowman's capsule and in the podocyte cell layer (Figure 3A-B). No significant expression was observed in the endothelial cells or in the mesangium. A strong Shroom3 expression pattern was also observed at the apical boarder of the proximal tubules, especially in the most proximal part of the proximal tubule. Shroom3 was not observed in distal tubules or collecting ducts. These results were consistent with Shroom3 staining patterns observed previously in studies with Shroom3 expression in postnatal kidney tissue. In addition, I also performed immunohistochemistry on 3-month-old kidneys using paraffin embedded tissue sections. IHC on *WT* 3-month-old kidneys demonstrated a similar pattern of expression in the Bowman's capsule and podocytes (Figure 3C-D). However, Shroom3 expression exhibited a stronger signal in the podocytes and weaker signal within the proximal tubules and Bowman's capsules.

To confirm the Shroom3 Ab expression patterns and confirm the antibody was working correctly, I performed IF on *Shroom3*$^{Gt/+}$ tissue (Figure 4). Heterozygotes should contain half the dose for Shroom3 when compared to wildtypes. Therefore if the antibody is working correctly and Shroom3 is not upregulated then half the amount of Shroom3 protein should be expressed in a similar pattern as the WT. As expected, Shroom3 was expressed in the proximal tubules, Bowman's capsule, and podocytes. Images were captured with identical exposure times and there was a significant reduction in immunofluorescence intensity in the heterozygotes than the wildtypes. This

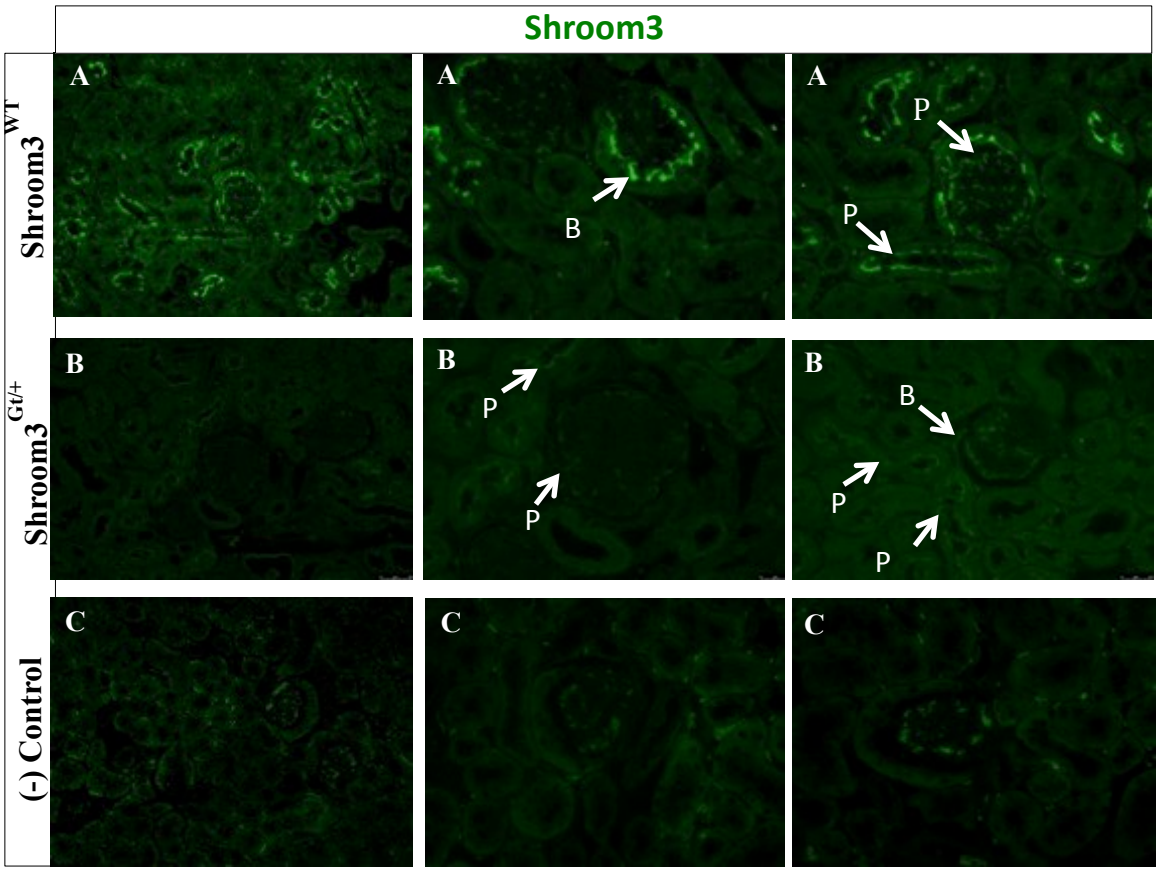
demonstrates that the heterozygotes are expressing Shroom3, but less protein is present. These studies support that the Shroom3 Ab generated in Dr. Plageman's laboratory identified positive Shroom3 expression within the kidneys.

Figure 3: Shroom3 expressed in the Bowman's capsule, proximal tubules and podocytes



Shroom3 expressed in the Bowman's capsule, proximal tubules and podocytes. 3 month old kidneys stained for Shroom3 using immunofluorescence and immunohistochemistry demonstrate signal in the Bowman's capsule, podocytes and proximal tubules. (A, B) Immunofluorescence of 3-month WT kidneys co-stained with DAPI (blue) and no-stain (Red). Insets demonstrating Shroom3 expression in the glomerulus. (C, D) Immunohistochemistry of 3-month WT kidneys. (P-podocytes, PT-proximal tubule, BC-Bowman's capsule).

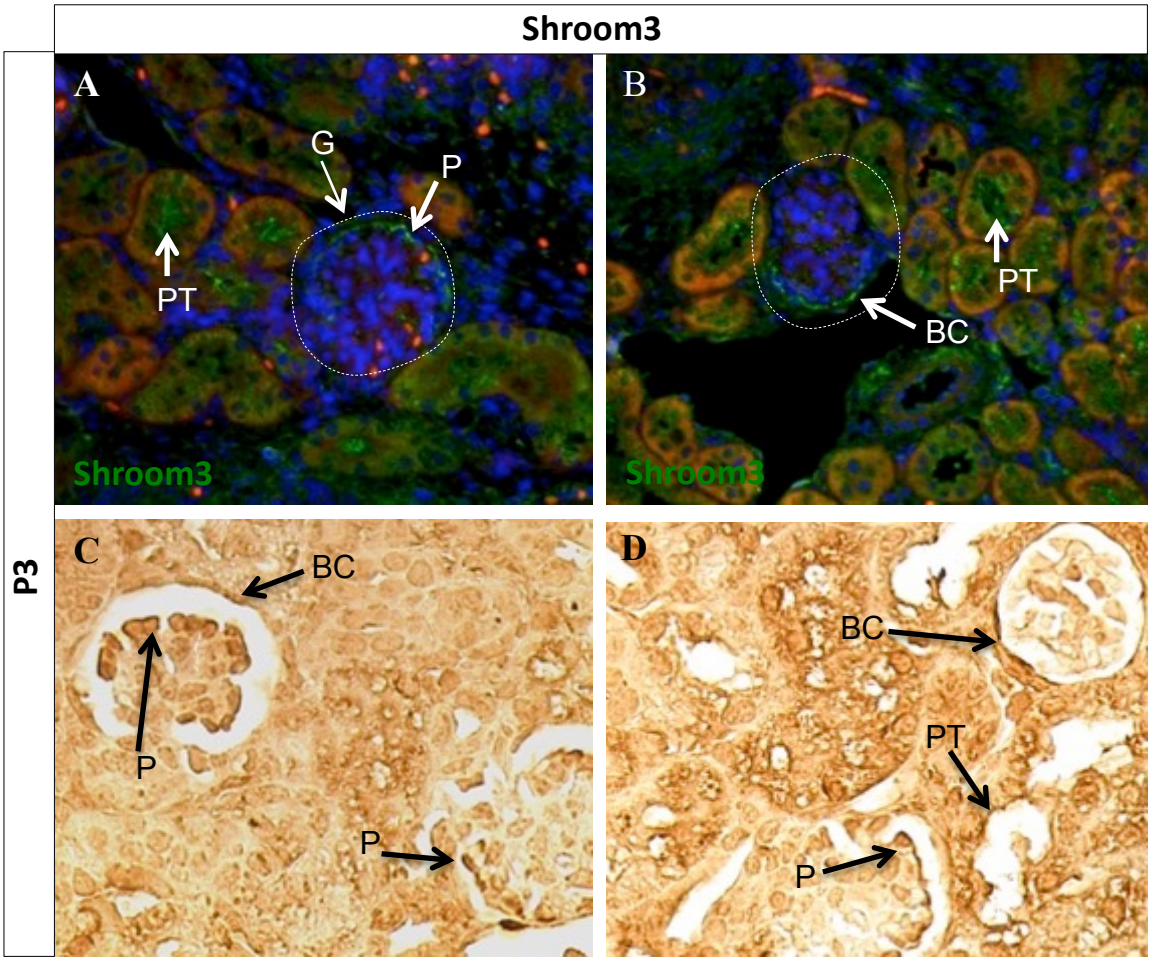
Figure 4: Shroom3 reduction in expression in Shroom3$^{Gt/+}$ kidneys.



Shroom3 reduction in expression in Shroom3$^{Gt/+}$ kidneys. (A, A', A") Immunofluorescence of Shroom3 staining in 3-month postnatal kidneys demonstrates signal detection within the proximal tubules, Bowman's capsule and podocytes. **(B, B', B")** Analysis of heterozygote (Shroom3$^{Gt/+}$) 3-month kidneys demonstrates presence, however weaker signal within the proximal tubules, Bowman's capsule and podocytes. **(C, C', C")** Negative control of *WT* 3-month kidneys. White arrows indicate positive staining.

I next performed immunofluorescence and immunohistochemistry on *WT* P3 paraffin embedded kidneys since the kidney is just completing development but also contains several mature structures. These results demonstrated a similar pattern of expression as observed in the 3-month-old animals. Specifically immunofluorescence detection of Shroom3 (green) was observed in the apical boarder of the Bowman's capsule and apical region of the proximal tubules. When analyzing the tissue in more detail, I observed expression in the foot processes in the podocytes in the filtration barrier (Figure 4A, B). I noticed this expression was less intense, than in the 3-month wildtype kidneys. Therefore, to get a better understanding of the localization of Shroom3 in P3 wildtype, I performed IHC. IHC of P3 demonstrated strong cytoplasmic brown staining in the outermost layer of the glomerulus. It also demonstrated significant brown staining depicting Shroom3 in the maturing podocytes. When looking closely, the podocytes are expressing Shroom3 in their foot processes (Figure 4C, D). In addition, Shroom3 expression was also observed in the proximal tubules, parallel with the IF Shroom3 expression in P3 wildtypes.

Figure 5: Shroom3 expressed in the Bowman's capsule, proximal tubules and podocytes in P3 WT kidneys.



Shroom3 expressed in the Bowman's capsule, proximal tubules and podocytes in P3 WT kidneys. (A-D) 3-day-old kidneys stained for Shroom3 using immunofluorescence and immunohistochemistry demonstrate signal in the Bowman's capsule, podocytes and proximal tubules**. (A, B)** Immunofluorescence of 3-day WT kidneys co-stained with DAPI (blue) and no-stain (Red). (G-glomerulus). **(C, D)** Immunohistrochemistry of 3-day WT kidneys.

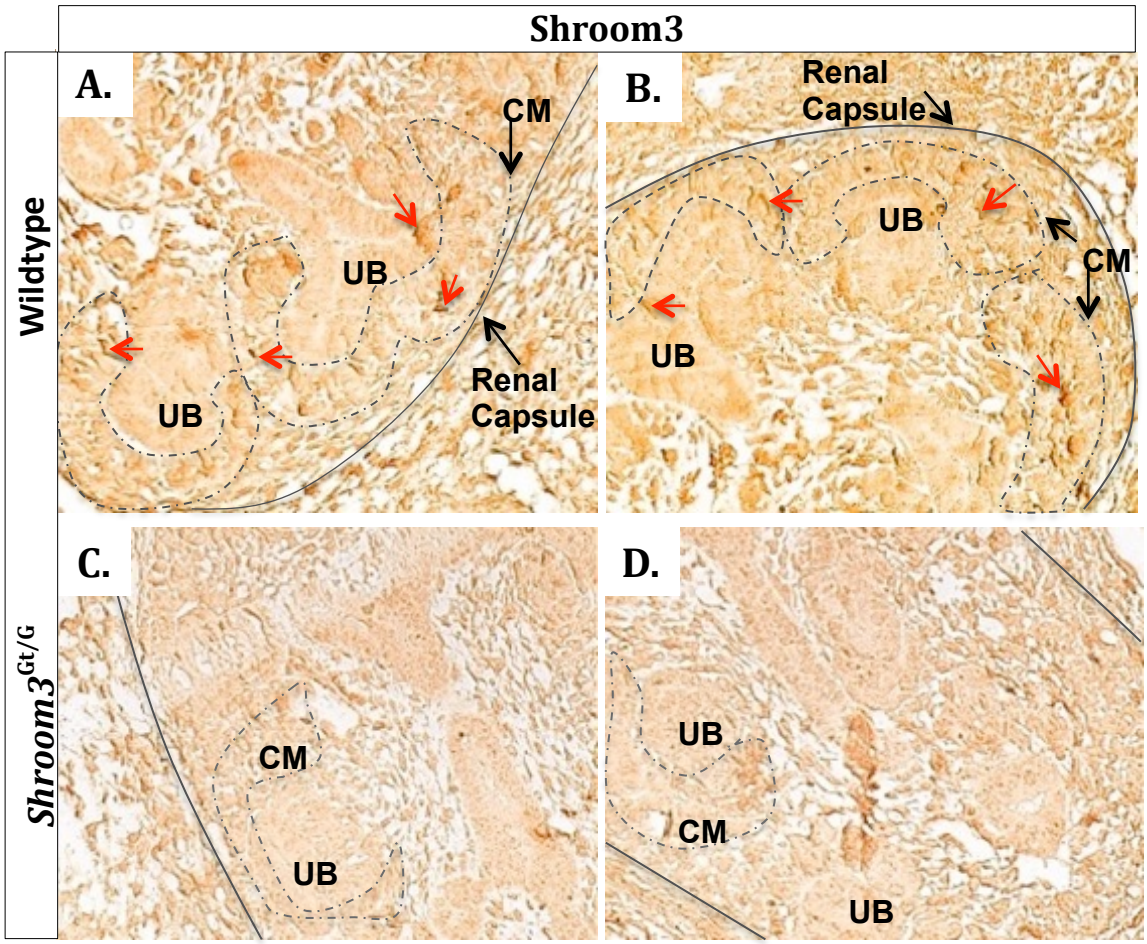
I next analyzed Shroom3 expression in embryonic kidneys by first performing immunohistochemistry. The initial analysis of E13.5 Shroom3 IHC staining pattern did not reveal any robust staining patterns. However, a close analysis of the nephron progenitor population revealed Shroom3 localized to a select few of the nephron progenitor cells that are primarily localized to the region where the pretubular aggregates and renal vesicles will form (Figure 6A-B). On close examination, the intracellular Shroom3 pattern was cytoplasmic and localized to one side of the cell. While I still was not convinced of this expression pattern, I next utilized Shroom3 null mutants (*Shroom3*$^{Gt/Gt}$*)* to determine if the expression was true staining or background. No expression of Shroom3 was present in the null mutants (Figure 6C-D). This verified that Shroom3 was in fact being expressed in wildtype E13.5 kidneys. The in situ hybridization experimental study (Supplementary Figure 9) demonstrated Shroom3 mRNA signal detection within the cap mesenchyme in the outer marginal zone of the kidney. However, when co localized with *WT1*, a marker of all nephron progenitors, Shroom3 mRNA was not present in every cell detecting *WT1*. These results were parallel to the expression pattern of Shroom3 being in a few cells in the mesenchyme. Therefore, IF evidence of Shroom3 and in situ hybridization of Shroom3 mRNA provides expression patterns of Shroom3 in the developing kidney on only one side of some mesenchyme cells.

Furthermore to confirm Shroom3 protein expression, I performed IF on E14.5 and E18.5 *Shroom3*^{WT} and *Shroom3*$^{Gt/Gt}$ frozen sectioned kidneys. I observed Shroom3 localization to the cap mesenchymal cells and ureteric bud cells at the tips adjacent to the cap mesenchyme (Figure 7). However, not every cell within the CM and UB expressed

Shroom3. This was consistent with the observations made in *WT* E13.5 IHC staining patterns. In summary, all experiments detecting Shroom3 in the embryonic kidney resulted in expression only in select cells within the nephron progenitors. This suggested Shroom3 is not present in every mesenchymal cell in throughout kidney development.

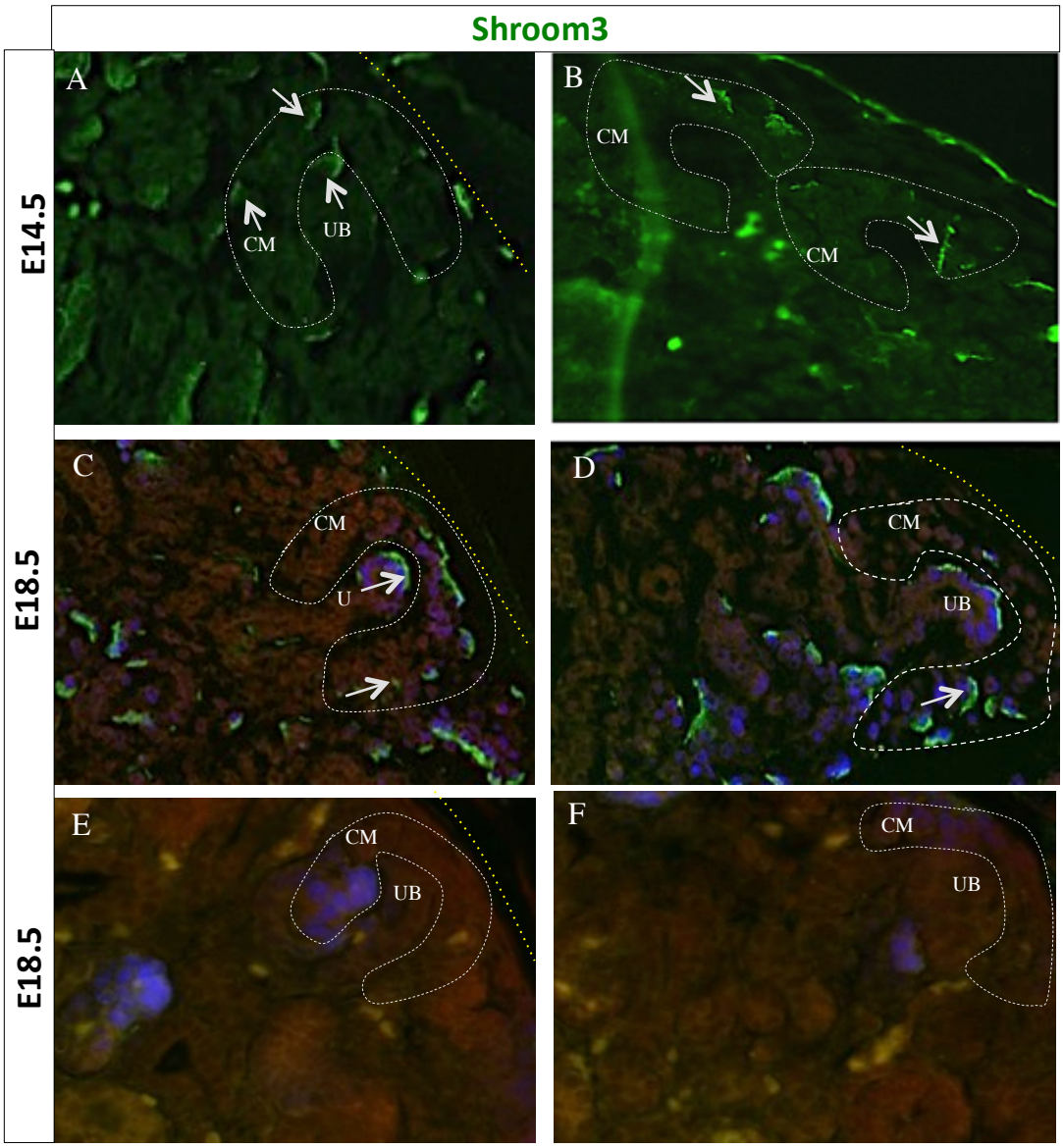
Selective expression of Shroom3 in the cap mesenchyme cells provides evidence that it may be playing an important role in nephron formation. Based on the functional role of Shroom3 in epithelial cells, I speculate that Shroom3 might induce cell movement and cell aggregation required to form the first stage of nephrogenesis. As the cap mesenchyme gives rise to nephron progenitors, perhaps it may be targeting cells preparing for cell aggregation and cell movement to form mature nephrons by aggregating them closer to the UB for proper Wnt9b induction. This brings up the various research questions for Shroom3's overall function and role in the embryonic kidney.

Figure 6: Shroom3 expression in some cells within the cap mesenchyme and ureteric epithelium.



Shroom3 expression in some cells within the cap mesenchyme and ureteric epithelium. (A, B) Analysis of immunohistochemistry of E13.5 *Shroom3 WT* kidneys stained for Shroom3 demonstrates positive protein detection within some cells of the cap mesenchyme and the ureteric bud epithelium. **(C, D)** No signal within mutant *Shroom*$^{Gt/Gt}$ kidneys. Red Arrows indicate positive Shroom3 expression. (UB- ureteric bud, CM- cap mesenchyme).

Figure 7: Shroom3 expression in the cap mesenchyme and ureteric bud epithelium in early and late kidney development



Shroom3 expression in the cap mesenchyme and ureteric bud epithelium in early and late kidney development. (A, B) Immunofluorescences of E14.5 kidneys show Shroom3 (green) in some cells of the cap mesenchyme and ureteric bud tips. **(C, D)** Immunofluorescence of E18.5 kidneys show Shroom3 (green) in some cells of the cap mesenchyme and ureteric bud tips. **(E, F)** E18.5 mutants show no Shroom3 expression. Dash lines indicate cap mesenchymes. White arrows- positive staining.

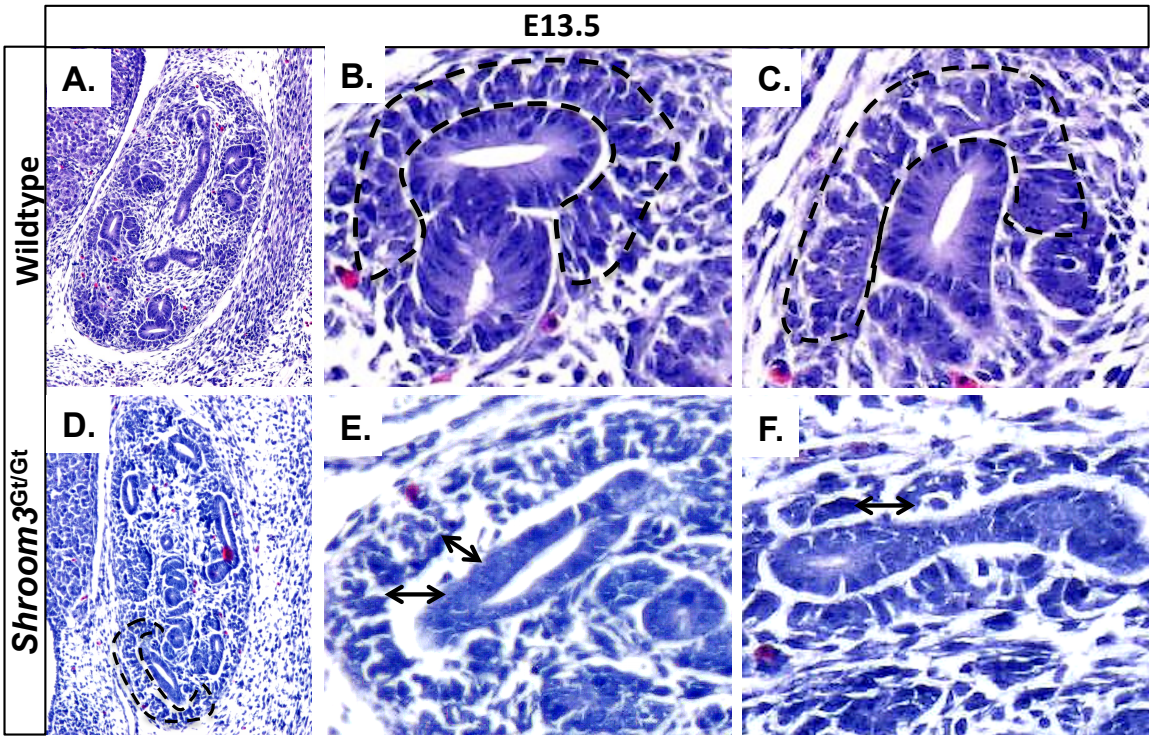
4.3 *Shroom3*$^{Gt/Gt}$ kidneys demonstrate abnormal clustering of nephron progenitors

To examine any renal abnormalities at a cellular level, histological analysis was performed on wildtype and Shroom3 mutant sectioned kidneys. During kidney development, undifferentiated mesenchyme cells aggregate and cluster close together, and surround the ureteric epithelium for reciprocal signaling and induction (Bard and Woolf, 1992). In wildtypes, the nephron progenitors are located at the cortex of the developing kidney capped around the UB tips (Figure 8A-C). However, *Shroom3*$^{Gt/Gt}$ mutants display several abnormalities within the organization of the nephron progenitors. First, *Shroom3*$^{Gt/Gt}$ nephron progenitors revealed to be loosely packed between one another (Figure 8F, F'). Second, *Shroom3*$^{Gt/Gt}$ mutants display regions of separation of nephron progenitors from the ureteric bud tips (Figure 8E, E'). Third, *Shroom3*$^{Gt/Gt}$ kidneys demonstrate abnormally larger areas of nephron progenitors than in wildtypes (Figure 8D, D'). While the nephron progenitors are also capped around the UB tips in *Shroom3*$^{Gt/Gt}$ mutants, the NPs are not nearly as condensed as they are in the wildtypes. *Shroom3*^{WT} kidneys demonstrate condensed NPs, however also demonstrate closely clustered NPs adjacent to the UB tips (Figure 8D-F).

To understand whether these abnormalities in the cap mesenchyme are limited to early developmental stages or if occur later in embryonic development, I analyzed the histology of E18.5 *Shroom3*$^{Gt/Gt}$ and *Shroom3*^{WT} kidneys. First, E18.5 *Shroom3*$^{Gt/Gt}$ kidneys demonstrated a severe NP to UB separation (Figure 9D-E). Second, *Shroom3*$^{Gt/Gt}$ kidneys displayed, again, loosely packed nephron progenitors around the UB tips. The kidney irregularities analyzed in *Shroom3*$^{Gt/Gt}$ mutants are not observed in *Shroom3*^{WT}

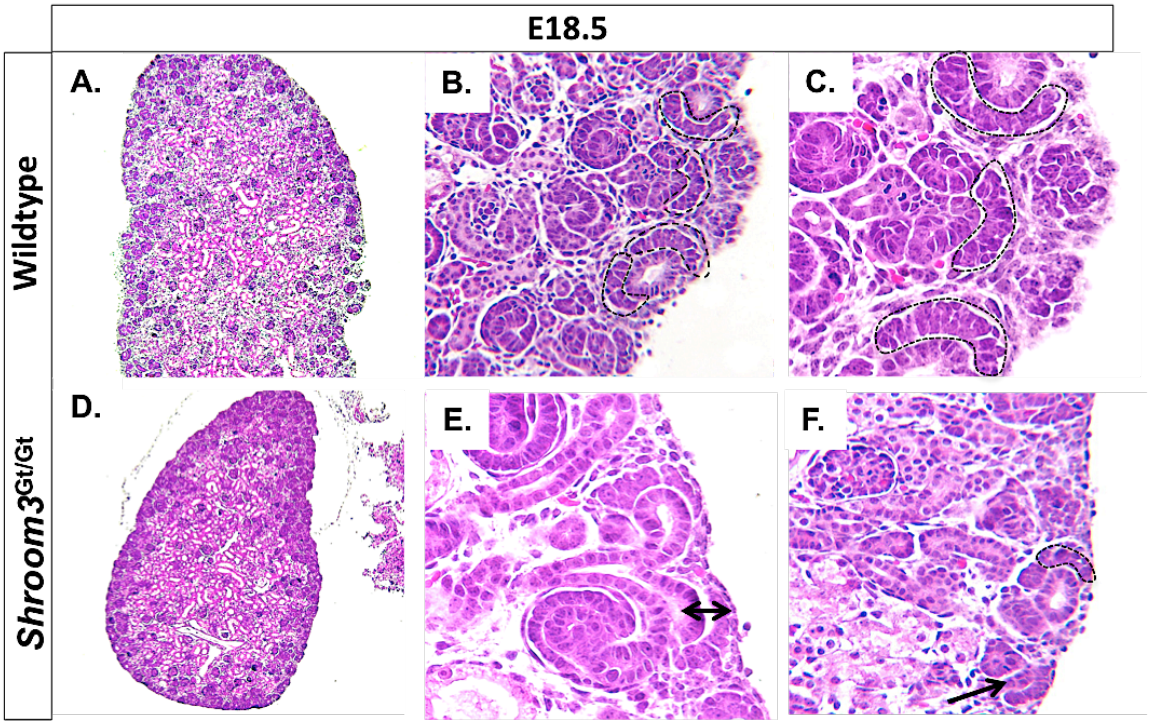
kidneys (Figure 9A-C). This suggests *Shroom3*$^{Gt/Gt}$ mice may have been having disruptions during nephrogenesis. Taken together, both E13.5 and E18.5 *Shroom3*$^{Gt/Gt}$ kidneys evidently demonstrate phenotypic abnormalities in nephron progenitors clustering and aggregation.

Figure 8: Abnormal nephron progenitor organization in Shroom3$^{Gt/Gt}$.



Abnormal nephron progenitor organization in Shroom3$^{Gt/Gt}$. (A-F) Histological analysis of nephrogenesis of wildtype and *Shroom3*$^{Gt/Gt}$ kidney tissue at E13.5 using Hematoxylin and Eosin staining. **(A-C)** E13.5 Shroom3 wildtype kidneys reveal nephron progenitors aggregated and capped around UB tips. **(D)** *Shroom3*$^{Gt/Gt}$ demonstrate large nephron progenitor expansion **(E)** Mutants have abnormal spacing between UB and nephron progenitors. **(F)** Mutants reveal loosely packed nephron progenitors. (black arrow).

Figure 9: Abnormal nephron progenitor organization in E18.5 Shroom3$^{Gt/Gt}$.

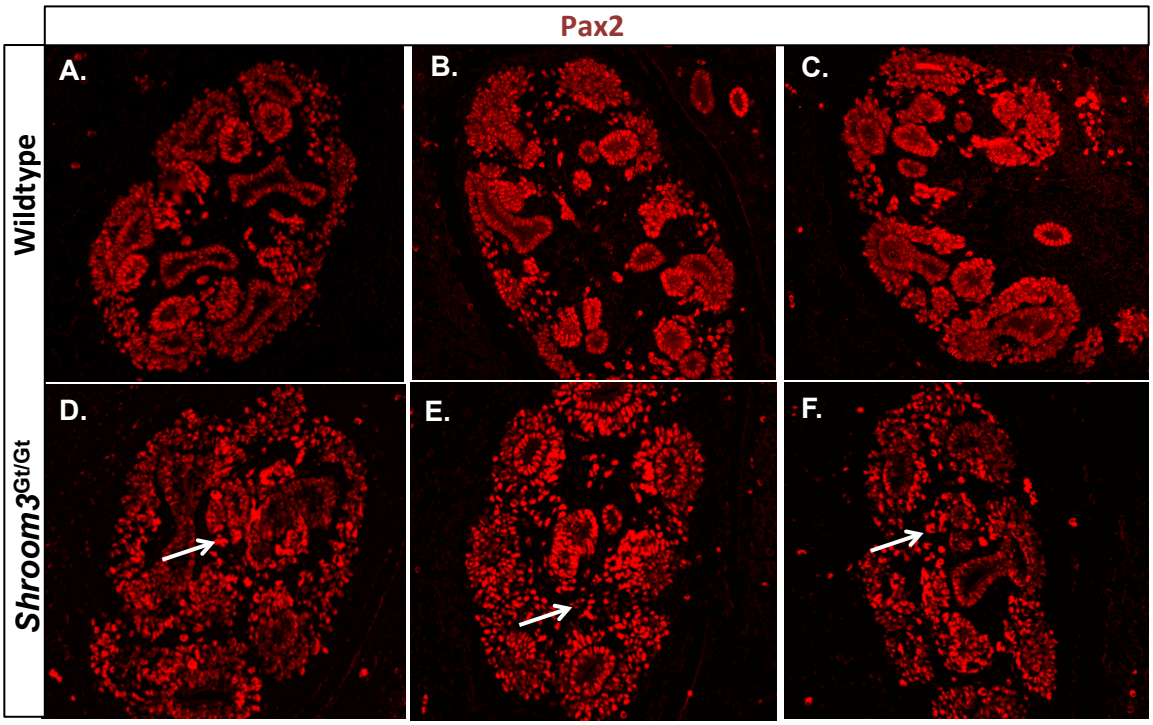


Abnormal nephron progenitor organization in E18.5 Shroom3$^{Gt/Gt}$. (A-F) Histological analysis of nephrogenesis in wildtype and *Shroom3*$^{Gt/Gt}$ kidney tissue at E18.5 using Hematoxylin and Eosin staining. **(A-C)** E13.5 Shroom3 wildtype kidneys reveal nephron progenitors aggregated and capped around UB tips. (black dashed arrow). **(D-E)** *Shroom3*$^{Gt/Gt}$ demonstrate have abnormal spacing between UB and nephron progenitors. **(F)** Mutants reveal less nephron progenitors. (black arrow).

4.4 Abnormal nephron progenitor organization in *Shroom3*$^{Gt/Gt}$

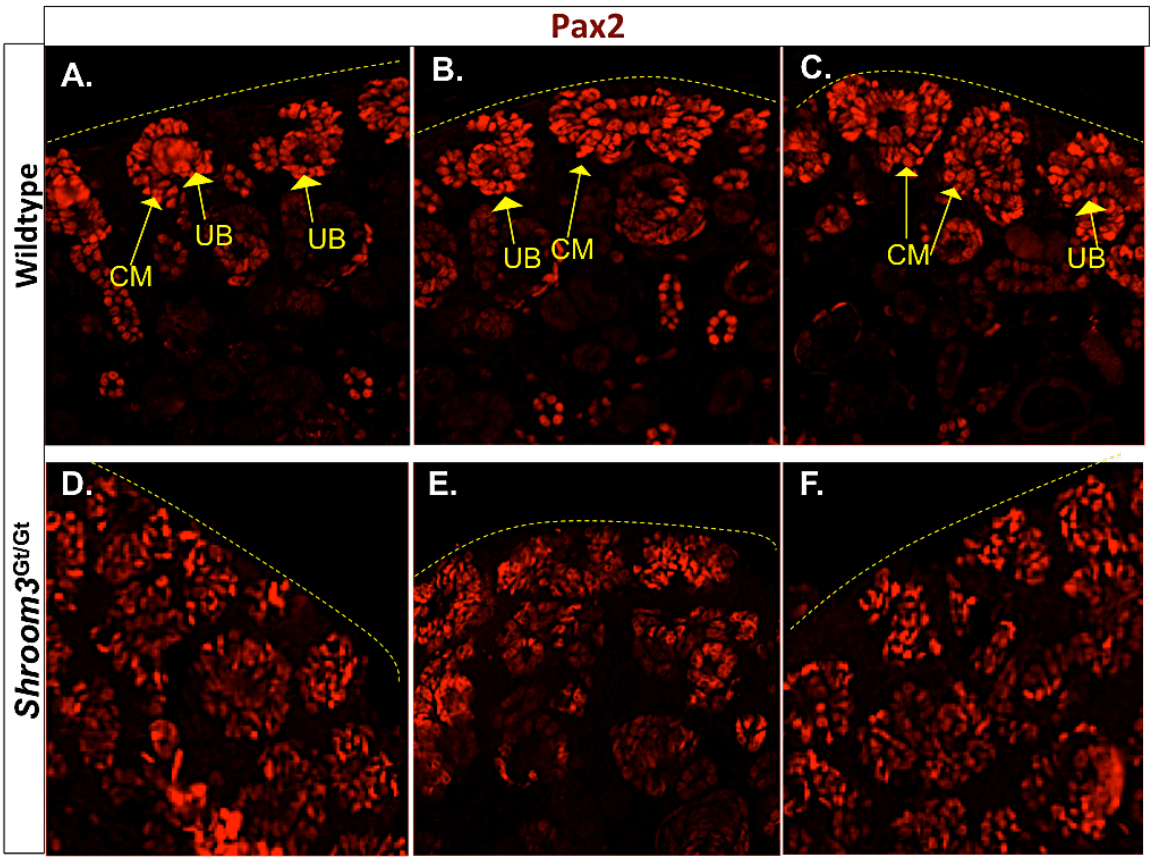
To build on the H&E data, I performed immunofluorescence to analyze the nephrogenic structures at E13.5 and E18.5 kidneys (Figure 10). I first used the antibody Pax2, a marker for the induced cap mesenchyme that identifies cells in aggregating pre-tubular cells, renal vesicles and ureteric bud epithelium. The cap mesenchyme and the ureteric bud tips make up the nephrogenic zone, located in the periphery of the developing kidney. This normal developmental pattern is observed in E13.5 and E18.5 wildtype kidneys (Figure 10A-C, 11A-C). The nephron progenitors are organized within the nephrogenic zone of the cortical region, with the ureteric epithelium extending from the cortex towards the center of the kidney. This was in contrast to E13.5 and E18.5 mutants, in which the organization of the nephron progenitors showed a stark difference. The results demonstrated that the organization of the nephron progenitors were not restricted to the cortex of the kidney, and expanded towards the medulla of the kidney (Figure 10D-F, 11 D-F). In addition, E13.5 and E18.5 *Shroom3*$^{Gt/Gt}$ kidneys also displayed nephron progenitors severely spatially detached from the tips of the ureteric epithelium (Figure 10D-F, 11D-F). Using Pax2 confirmed that *Shroom3*$^{Gt/Gt}$ kidneys, in early and late kidney development, are demonstrating nephron progenitor disorganization and localization in irregular patterns, not normally seen in kidney organogenesis. This analysis indicates that Shroom3 is playing a key role in nephron progenitor cell aggregation.

Figure 10: Abnormal nephron progenitor organization in Shroom3$^{Gt/Gt}$



Abnormal nephron progenitor organization in Shroom3$^{Gt/Gt}$. (A-F) Analysis of nephrogenesis of wildtype and *Shroom3*$^{Gt/Gt}$ kidney tissue at E13.5 using immunofluoresence microscopy. **(A-C)** Wildtypes demonstrating the cap mesenchyme and the ureteric bud tips make up the nephrogenic zone, located in the periphery of the developing kidney. **(D-F)** *Shroom3*$^{Gt/Gt}$ reveal nephron progenitors in irregular pattern not restricted to nephrogenic zone of kidney indicated by white arrows.

Figure 11: Disorganization of nephron progenitors in Shroom3$^{Gt/Gt}$.



Disorganization of nephron progenitors in Shroom3$^{Gt/Gt}$. (A-F) Analysis of nephrogenesis of wildtype and *Shroom3*$^{Gt/Gt}$ kidney tissue at E18.5 using immunofluoresence microscopy. **(A-C)** Wildtypes demonstrate normal nephrogenesis. (CM – Cap mesenchyme, UB – ureteric Bud). **(D-F)** *Shroom3*$^{Gt/Gt}$ mutants display irregular pattern of nephrogenic structural development. Nephron progenitors are spatial detached from UB tips in mutants. Dashed arrow indicates renal capsule.

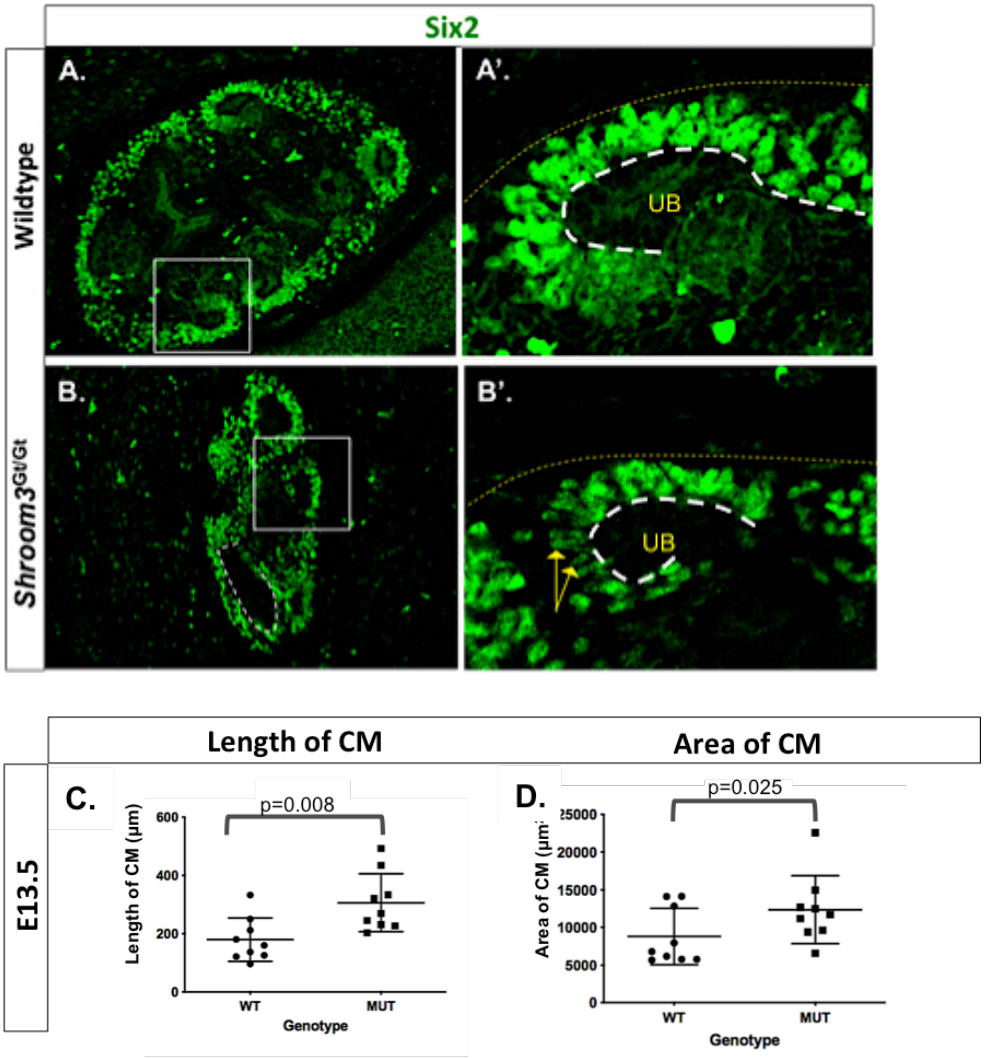
4.5 *Shroom3* mutant kidneys demonstrate abnormal nephron progenitor cell clustering

Previous studies have investigated the role of Six2 in maintaining the progenitor population, and in its absence resulting in premature and ectopic differentiation from mesenchymal cells to epithelia (Self et al., 2006). To determine whether nephron progenitor are organized and maintained normally in *Shroom3* mutant kidneys, I analyzed the expression pattern of Six2 at early and late kidney development. Normal developmental pattern was observed in E13.5 and E18.5 wildtype kidneys. The condensed Six2+ cells, marking the entire cap mesenchyme, were in the outer cortex in the periphery of the kidney (Figure 12A-A', 13A-B). In addition, the wildtypes demonstrate the mesenchymal progenitor population is in a consistent "U" shaped pattern in the nephrogenic zone. Our results show that this consistent clustered pattern of mesenchyme cells was not observed in *Shroom3*$^{Gt/Gt}$ mutants (Figure 12B-B', 13C-D). First, in E13.5 *Shroom3*$^{Gt/Gt}$ mutants, the Six2+ cells were not confined to only nephrogenic zone but were located in the medulla of the kidney (Figure 12B). Second, loosely packed nephron progenitors were observed, with distinct spatial separation between the Six2+ cells (Figure 12B'). Third, *Shroom3*$^{Gt/Gt}$ mutants demonstrated a larger area of mesenchymal progenitors (Figure 12B). This irregular expansion of the mesenchyme was significantly increased in E13.5 *Shroom3*$^{Gt/Gt}$ mutants than in wildtypes. (wildtype mean= 310.5 versus mutant mean=186.6, $p<0.008$) (Figure 12C). The area of Six2+ cells was also dramatically increased in mutants than in wildtypes (wildtype mean= 8976 versus mutant mean=12287 $p<0.025$) (Figure 12D). These results show evidence that nephron

progenitors are not clustering as closely in *Shroom3*$^{Gt/Gt}$ mutants than in wildtypes in early stages of kidney development (Figure 12).

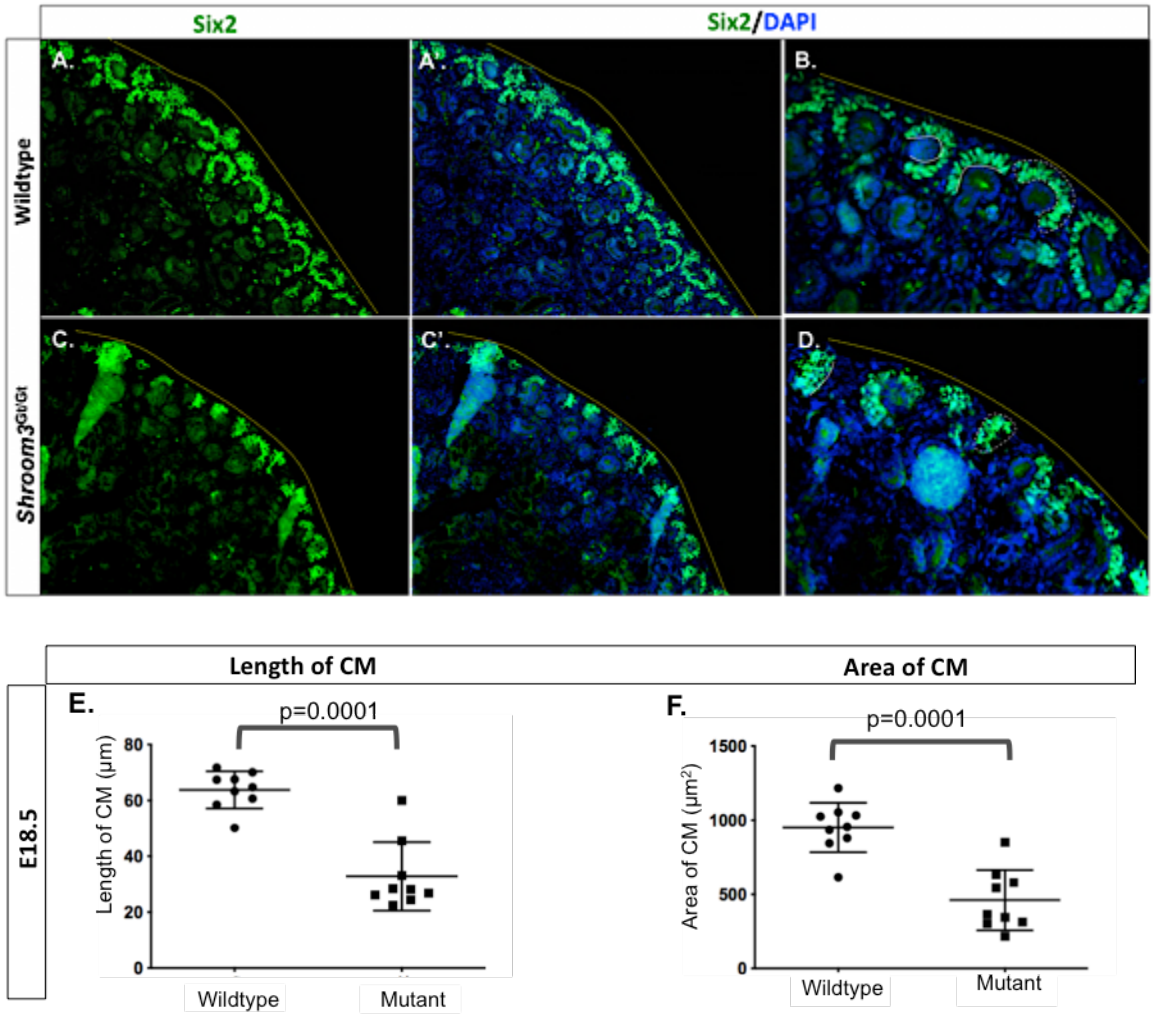
Interestingly, we also observed abnormal mesenchymal progenitor patterning in late embryonic development. In contrast to E13.5 mutants, the results demonstrated that E18.5 *Shroom3*$^{Gt/Gt}$ mutant cap mesenchymes were actually shortened in length around the UB tips (wildtype mean= 63.5 versus mutant mean=35.4, $p<0.001$) (Figure 13E). In addition, we analyzed and measured the area of the Six2+ cells per cap mesenchyme, and these results revealed that E18.5 *Shroom3*$^{Gt/Gt}$ kidneys had a significantly decreased area (wildtypes mean=856 versus mutant mean= 461.8, $p<0.001$) (Figure 13F). This approach confirmed that the nephron progenitor pool of cells is drastically reducing in late embryonic kidney development in E18.5 *Shroom3*$^{Gt/Gt}$ mutants. It demonstrated that the reduction in clustering and aggregation in early embryonic development seen in *Shroom3*$^{Gt/Gt}$ kidneys leads to the decrease in the cap mesenchyme region. Close examination of E18.5 *Shroom3*$^{Gt/Gt}$ mutants expressing Six2 revealed what looked like missing regions of mesenchymal progenitors (Figure 13D). In addition, the Six2 cells did not cap the ureteric bud tips in a consistent pattern (Figure 13C-D). As such, these findings demonstrate the important role Shroom3 plays in aggregating the nephron progenitors and perhaps maintaining their proliferation.

Figure 12: Abnormal nephron progenitor aggregation in E13.5 *Shroom3*$^{Gt/Gt}$



Abnormal nephron progenitor aggregation in *Shroom3*$^{Gt/Gt:}$ (A-B) Immunofluorescence using Six2 antibody to mark nephron progenitors in E13.5 *Shroom3*^{WT} and *Shroom3*$^{Gt/Gt}$ kidneys. **(A, B)** In contrast to wildtype, E13.5 *Shroom3*$^{Gt/Gt}$ kidneys demonstrate nephron progenitors not confined to only the nephrogenic zone but were located in the center of the kidney. White boxes represent insets. **(A')** High power image of E13.5 wildtype represents condensed nephron progenitors. **(B')** High power image of E13.5 mutant demonstrates loosely aggregated cells. (Yellow arrows – individual nephron progenitors, White dashed line – cap mesenchyme, Yellow dashed line – renal capsule). **(C, D)** Quantification of CM length (wildtype mean= 310.5 versus mutant mean=186.6, $p<0.008$, n=3), and quantification of CM area (wildtype mean= 8976 versus mutant mean=12287 $p<0.025$, n=3) demonstrates significant increase in *Shroom3*$^{Gt/Gt}$.

Figure 13: Abnormal nephron progenitor aggregation in E18.5 *Shroom3*$^{Gt/Gt}$

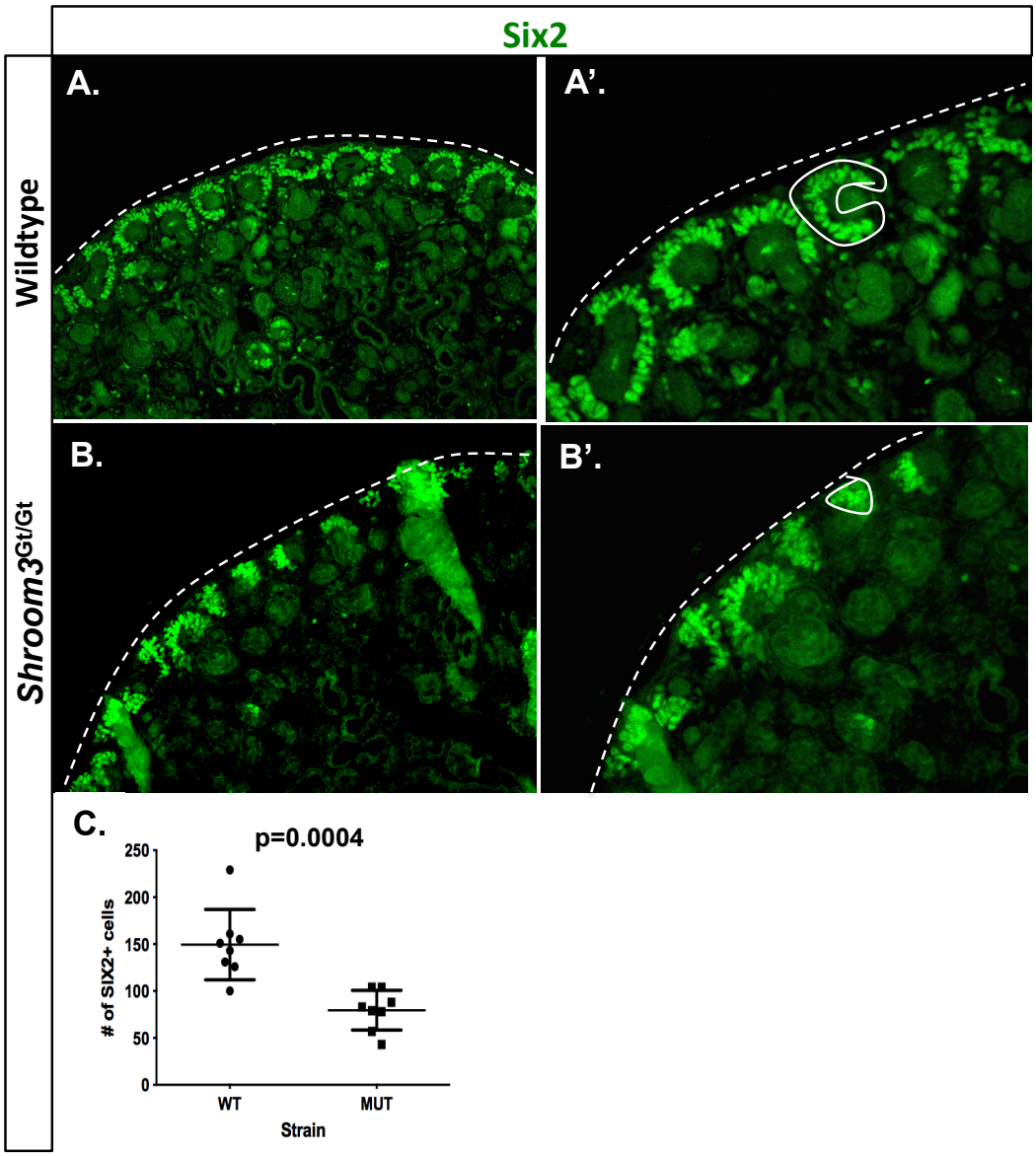


Abnormal nephron progenitor aggregation in E18.5 *Shroom3*$^{Gt/Gt}$. (A-D) Immunofluorescence analysis using Six2 (green) antibody and DAPI (blue) on E18.5 *Shroom3*^{WT} and *Shroom3*$^{Gt/Gt}$ kidneys. **(C-D)** *Shroom3*$^{Gt/Gt}$ mutants exhibit missing regions of mesenchymal progenitors and progenitors not capping the ureteric bud tip in a consistent engulfing pattern. **(E)** Quantification of CM length (wildtype mean= 63.5 versus mutant mean=35.4, $p<0.001$, n=3) and quantification of CM area (wildtypes mean=856 versus mutant mean= 461.8, $p<0.001$, n=3) represents a significant decrease in *Shroom3*$^{Gt/Gt}$

4.6 *Shroom3* mutants depict a reduction in nephron progenitors

Previous work has demonstrated that a loss in the mesenchymal pool of Six2 positive cells results in premature nephrogenesis, and this ultimately leads to severely hypoplastic and non functional kidneys following birth (Self et al., 2006). Since Six2+ expression in late embryonic kidney development demonstrated reduced mesenchymal length and cap mesenchyme area, we sought to determine whether the amount of nephron progenitors were in fact reduced in late embryonic development. To initiate our analysis, we utilized E18.5 *Shroom3*^{WT} and *Shroom3*$^{Gt/Gt}$ kidneys expressing Six2 (Figure 14A-B). The Six2 expression represents all mesenchymal cells in the kidney, defined to the outer cortex in the nephrogenic zone. To quantify the progenitors, ImageJ software was used to count all Six2+ expressing cells. This technique provided us with a non-bias progenitor count as it quantified only cells expressing Six2 with significant amount of intensity, and did not account for background noise (see materials and methods for the quantification protocol). As expected, wildtype mice showed dramatically more mesenchymal progenitors than in *Shroom3*$^{Gt/Gt}$ kidneys since their cap mesenchyme length and area was much larger than mutants (wildtype mean= 150 versus mutant mean=77 $p<0.0004$) (Figure 14C). This analysis indicates that potentially premature nephrogenesis is taking place as a result in the reduction of mesenchyme cells in late stages of development.

Figure 14: *Shroom3*$^{Gt/Gt}$ demonstrate a reduction of nephron progenitors in late kidney development.



***Shroom3*$^{Gt/Gt}$ demonstrate a reduction of nephron progenitors in late kidney development. (A-B)** Immunofluorescence of E18.5 *Shroom3*^{WT} and *Shroom3*$^{Gt/Gt}$ kidneys expressing Six2 (green) antibody. (White dashed line – renal capsule, White line – CM). **(C)** Quantification of Six2+ cells kidneys (wildtype mean= 150 versus mutant mean=77 $p<0.0004$, n=3) demonstrates a significant loss of nephron progenitors in late kidney development.

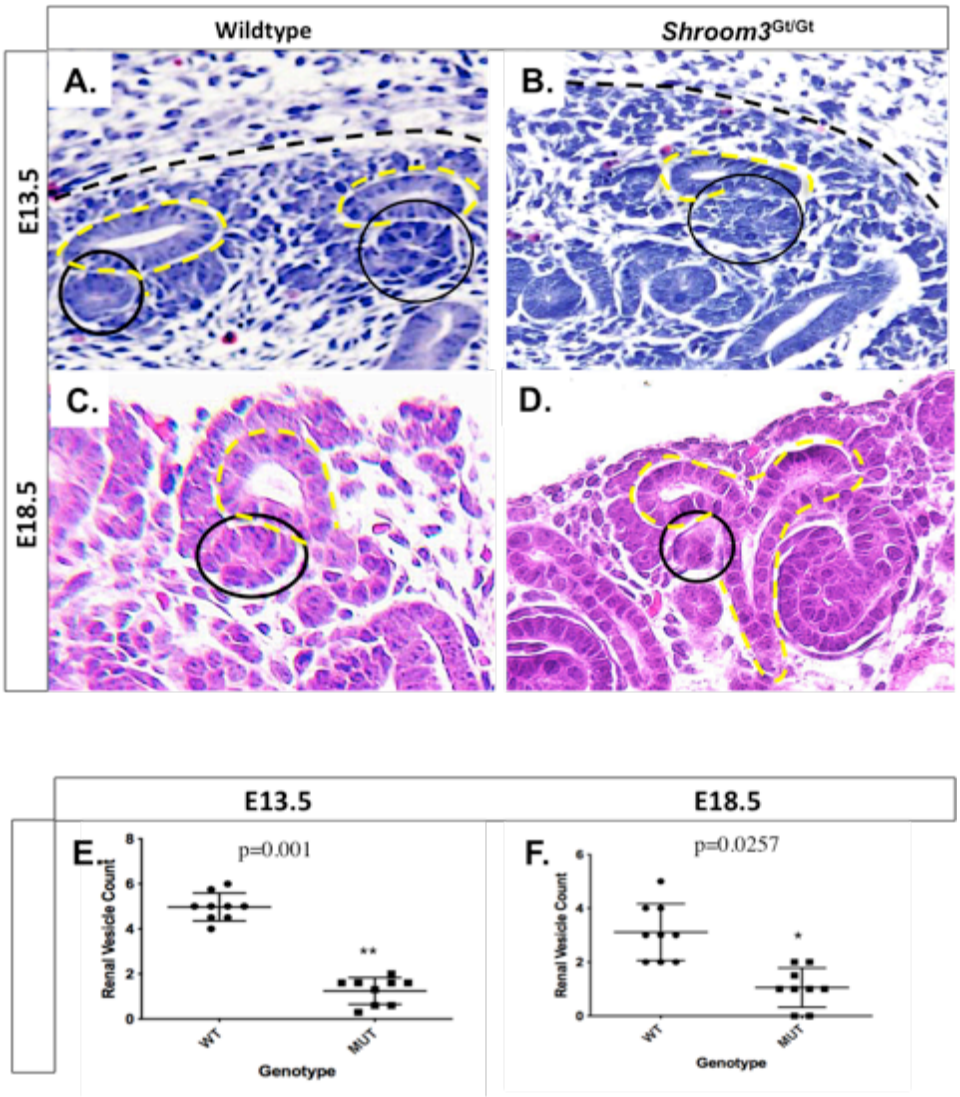
4.7 Reduction of renal vesicles in Shroom3 mutants

Cells within the cap mesenchyme that are set to form mature nephrons, undergo a mesenchymal to epithelial transition. These nephron progenitors undergo tight aggregation and cell morphogenesis. They form wedge shaped cells into a spherical structure labeled as a renal vesicle (Yang et al., 2013). This depicts the first structure of nephrogenesis. Therefore, to analyze the formation of renal vesicles in Shroom3 mutants, I performed H&E staining on E13.5 and E18.5 kidneys (n=3). In order to identify the renal vesicles, I had to set out a list of criteria. The criteria were as follows: 1) renal vesicles are in the outer marginal zone of the kidney, 2) Renal vesicles must be located in the "arm pit" of the UB, 3) They must have "pie" shaped cells in a spherical structure with a central lumen. When examining the H&E in E13.5 wildtypes, an average of 2-4 renal vesicles were observed per kidney section (Figure 14A, E). When I followed the criteria for locating properly forming renal vesicles, I was only able to observe an average of one per mutant kidney. My quantitative analysis determined a significant reduction in renal vesicles in E13.5 *Shroom3*$^{Gt/Gt}$ kidneys, than in *Shroom3*^{WT} (Figure 14B, E). This suggests that Shroom3 may be an important factor in allowing these nephron progenitors to undergo epithelization and develop into maturing nephrons.

To determine whether there was a significant reduction in late kidney development, E18.5 kidneys were analyzed. I performed H&E staining on *Shroom3*^{WT} and *Shroom3*$^{Gt/Gt}$ kidneys. Our analysis revealed an average of 4 renal vesicles present per kidney section, imaged at 20X field of view (Figure 14C, F). During my analysis, I was able to easily identify the renal vesicles. However, during my analysis of *Shroom3*$^{Gt/Gt}$

kidneys, I only observed an average of 1-2 renal vesicles (Figure 14D, F). We observed that there was a significant reduction in RVs in mutants, than observed in wildtypes. Further, at E18.5, mutants were displaying abnormally forming renal vesicles (Figure 14D). These improper RVs suggest, again, that Shroom3 may be playing a key role in proper cell movement and cell aggregation of the NPs. If disrupted, NPs will not be able to aggregate close to one another and properly apically constrict to form normal RVs. Therefore, we need to further analyze the organization of the renal vesicles, and exactly how these abnormally RVs are forming.

Figure 15: *Shroom3*$^{Gt/Gt}$ mutants exhibit a reduction in renal vesicles.



Shroom3$^{Gt/Gt}$ **mutants exhibit a reduction in renal vesicles. (A-D)** Histological analysis of nephrogenesis in wildtype and *Shroom3*$^{Gt/Gt}$ kidney tissue at E13.5 and E18.5 using Hematoxylin and Eosin staining. In contrast to *Shroom3*^{WT}, mutants display irregular shaped renal vesicles. (Black line – renal vesicle, Yellow dashed line – ureteric epithelium, Black dashed line – renal capsule). **(E)** Quantification of renal vesicles in E13.5 kidneys (wildtype mean – 5, versus mutant mean – 1.2, p=0.001, n=3) demonstrate a significant reduction. **(F)** Quantification of renal vesicles in E18.5 kidneys (wildtype mean – 3, versus mutant mean – 1, p=0.0257, n=3) demonstrate a significant reduction.

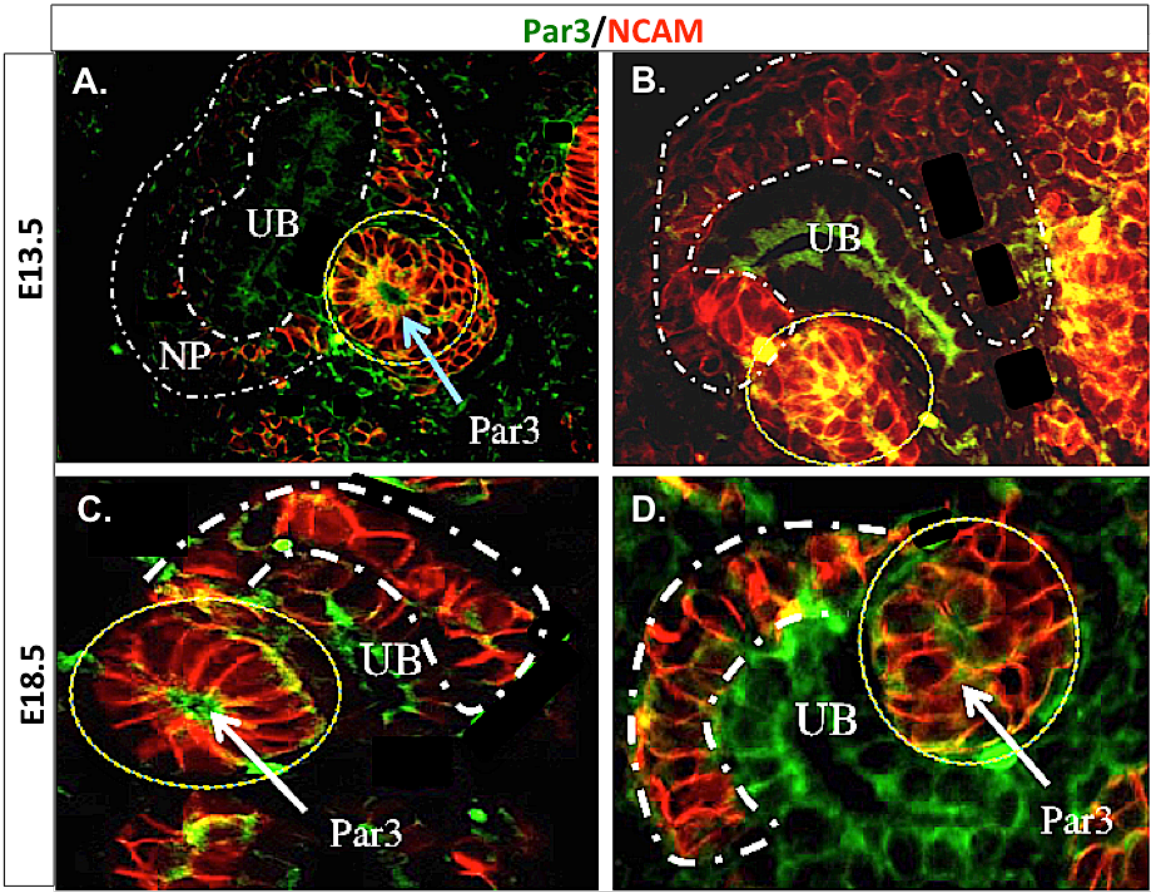
4.8 Renal vesicle cells are disorganized in Shroom3 mutants

To analyze the organization of renal vesicle epithelized cells, I performed IF using Par3 and NCAM on E13.5 and E18.5 *WT* and *MUT* kidneys. Par3 is an adapter protein involved in cell polarization processes and plays a functioned role in the formation of epithelial cell junctions. It will be used as an apical polarity cell marker. NCAM is a cell adhesion molecule that will depict the nephron progenitors and renal vesicles. First, I co-stained E13.5 wildtypes with NCAM and Par3 via immunofluorescence (Figure 16A). The IF analysis revealed renal vesicles forming wedge shaped cells. These cells formed a spherical structure with a central lumen. However when in E13.5 mutants, I observed some renal vesicles that were disorganized (Figure 16B). These renal vesicles lacked a presence of a central lumen. The Par3 apical expression was not uniformly seen within the lumen, but present throughout the renal vesicle. This was never observed in *Shroom3*$^{Gt/Gt}$ kidneys. The observations demonstrated the lack of properly organized renal vesicles in the absence of Shroom3 in early kidney development.

To analyze renal vesicle formation in late kidney development, we utilized E18.5 kidneys via IF. E18.5 wildtypes demonstrated uniformly forming renal vesicles with "pie" shaped cells stained using NCAM (red). These renal vesicles had a clearly formed central lumen depicted by the Par3 IF stain (green). No evidence of disruption in renal vesicle formation was observed in wildtypes (Figure 16C). I then performed IF on E18.5 mutant kidneys using Par3 and NCAM (Figure 16D). Shroom3$^{Gt/Gt}$ renal vesicles were observed to have irregularities in proper formation. The cells within the renal vesicle were not uniformly "pie" shaped and not forming a round structure. In addition, Par3 staining

was throughout the renal vesicle with staining throughout. Our results demonstrate that both early and late renal vesicle organization is disrupted. Since renal vesicles require cell morphology and organization to form their structures, Shroom3 may be a key protein in allowing these renal vesicles to form the first stage of nephrogenesis. If absent, these renal vesicles may not have the key regulator to form these cell shape changes and cell migrations.

Figure 16: Disorganized development of renal vesicles in *Shroom3*$^{Gt/Gt}$ kidneys.

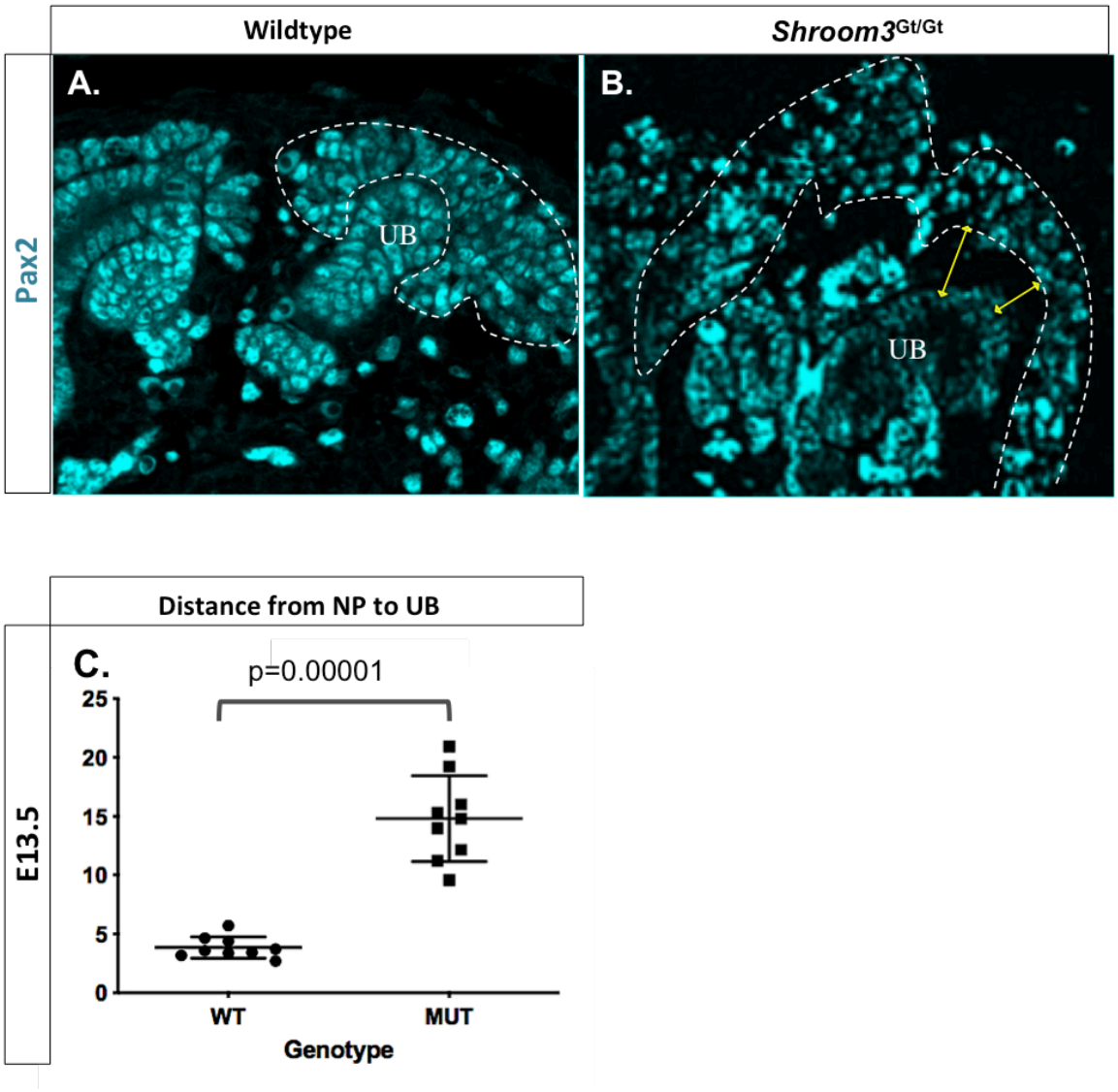


Disorganized development of renal vesicles in *Shroom3*$^{Gt/Gt}$ kidneys. (A-D) Immunofluorescence expression analysis using Par3 (green) and NCAM (red) antibodies in E13.5 and E18.5 *Shroom3*^{WT} and *Shroom3*$^{Gt/Gt}$ kidneys. **(A,C)** Wildtypes demonstrate normal formation of renal vesicles. An evident central lumen (green) and apically constricted cells (red) are observed. **(B, D)** *Shroom3*$^{Gt/Gt}$ kidneys reveal irregular development of renal vesicles. No central lumen is observed as Par3 is expressed throughout whole renal vesicle, and NCAM highlights non-apical constricted cells. (White dashed line – CM, Yellow dashed line – renal vesicle).

4.9 Shroom3 mutants display NP to UB detachment

Nephron progenitors organize in a distinct niche around the ureteric buds tips in a coherent manner to communicate and receive the necessary signals for cell fate. Previous studies have shown that proper NP niche interactions are critical in sustaining NP maintenance and organization (Ihermann-Hella et al., 2018). Therefore we sought to determine whether Shroom3 plays a role in maintaining nephron progenitor and UB contact. We used Pax2, a marker for the nephron progenitors and ureteric bud epithelium, to mark any changes between both structures. By performing immunofluorescence, E13.5 Shroom3 wildtype kidneys (n=3) had a tightly condensed cap mesenchymal pool of cells adjacent to the ureteric epithelial branch tips (Figure 17A). In contrast to Shroom3 mutants having a larger NP to UB tip separation (Figure 17B). Therefore, we quantified the distance between the UB and the nephron progenitors and mutants indeed had a significantly increased spatial separation than wildtypes (wildtype mean=4.68 versus mutant mean=14.89, $p<0.0001$) (Figure 17C). This finding demonstrates Shroom3's activity in potentially maintaining the correct nephron progenitor location in the developing kidney.

Figure 17: Irregular nephron progenitor niche separation from ureteric epithelium.



Irregular nephron progenitor niche separation from ureteric epithelium. (A,B) Immunofluorescence expression analysis using Pax2 (cyan) antibody in E13.5 *Shroom3*^{WT} and *Shroom3*$^{Gt/Gt}$ kidneys. In contrast to wildtypes, *Shroom3*$^{Gt/Gt}$ mutants demonstrate nephron progenitors spatial detached from the basement membrane of the ureteric bud epithelial cells. (White dashed arrow- CM, Yellow arrows – spatial distance of NP from UB). **(C)** Quantification of nephron progenitors to ureteric bud epithelia (wildtype mean=4.68 versus mutant mean=14.89, $p<0.0001$, n=3) represents a significant increase in mutants in comparison to wildtypes.

5. DISCUSSION

5.1 Overall Findings

Kidney development is defined by two major processes including nephrogenesis and branching morphogenesis. Studies in mouse and rat models have demonstrated key regulatory factors, transcription factors, and signaling pathways that play important roles in the development of mature nephrons. Despite these studies, key molecules and proteins involved in nephrogenesis and their functions are still poorly understood. Previous experimental studies have, for the first time, identified the presence of Shroom3, in the embryonic and adult kidney (Khalili et al, 2016). In addition, Shroom3 mutants have been linked to having abnormal glomerular development, and these abnormalities observed during development manifest into postnatal complications such as proteinuria (Khalili et al., 2016). My studies extend these findings by two fold. First, by localizing the spatial Shroom3 expression pattern in the cap mesenchyme and second by demonstrating Shroom3 mutants show abnormal aggregation of nephron progenitors during the earliest stages of nephron development. Overall, this suggests that Shroom3 may be playing a role in the movement and organization of nephron progenitors. Without proper organization, inductive signaling molecules may not be received. As a result, unregulated nephrogenesis may lead to the presence of abnormally developed nephrons. These abnormally developed nephrons are linked to the development of CKD. Thus, the intronic SNPs in the Shroom3 allele, previous associated with CKD (Menon et al., 2015), may be

correlated with the transcriptional and/or interaction with necessary nephrogenic signaling pathways needed for normal kidney development.

5.2 Shroom3 expression in the kidney

Understanding the temporal and spatial expression pattern of Shroom3 within the kidney will lead to a better understanding of Shroom3's function and role. In the study conducted by Yeo et al., they were able to detect Shroom3 expression in zebrafish in the tubules and pronephric glomeruli via ISH. In addition, Lee et al. were able to detect Shroom3 transcripts in the pronephric kidney in Xenopus embryo. Studies have also suggested that Shroom3 is expressed in the proximal tubule of the human kidney (Menon et al., 2015). Khalili et al. were the first to define the spatial and temporal Shroom3 expression pattern in the developing and mature kidney. Their initial studies showed endogenous gene expression in all of the cap mesenchyme, the glomeruli, parietal epithelium of the Bowman's capsule, the collecting ducts and proximal tubules. These findings are in parallel to the observations made in my study, which found the Shroom3 was expressed in the cap mesenchyme, the Bowman's capsule and podocytes in the glomeruli, and the proximal tubules in postnatal mice. Although Khalili et al. were able to demonstrate Shroom3 expression in the kidney, they were unable to locate where in the cap mesenchyme Shroom3 was located.

Nephron progenitors are localized in the cap mesenchyme, and are programmed to either self renew or differentiate into epithelialized structures (Liu et al., 2017). Each subset of nephron progenitor cells reside in a niche, and those undergoing differentiation remain in a separate compartment (Liu et al., 2017). As kidney development progresses

more nephron progenitor cells exit the self-renewing niche and proceed to differentiation (Liu et al., 2017). My work determined that Shroom3 was present in only a few cells within the cap mesenchyme in E13.5, E14.5, and E18.5 wildtype mice via immunofluorescence. Our expression analysis in the embryonic *Shroom3*$^{tg/+}$ kidneys demonstrates that Shroom3 expression is in the differentiating nephron progenitor compartment. We believe that Shroom3's expression is within the cells that are adjacent to the pretubular aggregates that are preparing to undergo epithelialization to form renal vesicles. Therefore, based on its location this suggests that Shroom3 is being turned on just prior to the first stage of nephrogenesis. Since the functional role of Shroom3 is currently limited to the polarized epithelial cells (Hildebrand, 2005) it may be that Shroom3 is functioning to polarize the nephron progenitors. In addition, another role for Shroom3 in the nephron progenitors is Shroom3 may be preparing these NPs to undergo cell morphogenesis and form into the first stage of nephrogenesis, a renal vesicle. Since a strong induction of Wnt9b causes nephron progenitors to undergo nephrogenesis, Shroom3 may be mediating the aggregation of these cells to receive the appropriate amount of signal. Ultimately, this may give reason as to why Shroom3 is only expressed in a few progenitor cells during kidney development.

When analyzing my IHC and IF on *Shroom3*$^{tg/+}$ embryonic Shroom3 expression patterns, I demonstrated an interesting intraceullar localization in that Shroom3 appeared to localize to consistently to only 1 side of the cell. This is an interesting finding since metanephric mesenchymal cells are not yet polarized. Therefore, it raises the question why Shroom3 is defined to a distinct region? It is well defined that Shroom3 plays a role

in apical constriction in epithelial cell morphogenesis through its recruitment to actin on the apical membrane (Nishimura et al., 2008). Thus, in the case of mesenchyme cells, Shroom3 is preparing the cells for nephron progenitor apical constriction to allow the cells to for renal vesicles.

5.3 Nephron progenitor disorganization in Shroom3 mutants

During the first steps of nephrogenesis the loosely packed cap mesenchyme cells first begin to aggregate and tightly align around the UB tips in a highly unified manner. This is likely achieved via changes in the actin cytoskeleton and interactions with the extracellular matrix, the neighboring stromal cells and/or the basement membrane of the ureteric bud epithelium. Our findings demonstrate nephron progenitor disorganization in *Shroom3* mutants in early and late kidney development. This disorganization may suggest the role of Shroom3 and its downstream targets in mediating communication between the NP niche and its surroundings for proper clustering of nephron progenitors. These results suggest that the architecture in the renal mesenchyme stems from the regulated aggregation of the UB-adjacent NP population. Although the key regulators in mesenchymal cell aggregation have not been fully understood, our studies suggest that Shroom3 may be an essential regulator mediating this mesenchymal cell clustering. In addition, Shroom3 may be functioning to mediate cell movement, through its interaction with the actin cytoskeleton.

Several studies have assessed Shroom3's role in cell movement. In a study conducted by Ernt et al., zebrafish lateral line (LL) cell migration was disorganized upon knockdown of Shroom3. Enrt et al. believe that the cell migration defect in Shroom3

knockouts was due to the down regulation of the G-protein coupled receptor gene *cxcr7b* in the Fibroblast growth factor (FGF) signaling pathway. These findings are in parallel to the observations we made in our study, which found that cells in the cap mesenchymal region also did not migrate appropriately in Shroom3 null mutants. Shroom3$^{Gt/Gt}$ mice had nephron progenitors not assembled in close proximity to one another, and were not clustered as tightly next to the ureteric bud epithelium, in comparison to wildtypes. We believe that this irregular pattern in Shroom3 mutants is due to the down regulation of downstream targets of Shroom3, such as Rho-kinase and Non-muscle myosin II (NMII), which play a key role in the movement of cells. We propose that Shroom3 may be the mediator in movement leading to cell aggregation in the nephron progenitors, and is probably coordinating the recruitment of Rho-kinase and activation of NMII. Rho-kinase phosphorylates NMII resulting in activation. Without the activation of NMII, the cell is unable to move and constrict the actin filaments. Therefore, cells without Shroom3 will not be able to activate this cascade and provide the necessary cell clustering adjacent to the UB. As such, we believe that Shroom3 null mutant NPs are unable to move and thus having the inability to aggregate. However, the Shroom3 signaling pathway needs to be investigated further in NPs, as it's downstream targets are still unknown in the mesenchyme. And such, Shroom3's downstream targets may also account for the disorganization of all the nephron progenitors. Since, Shroom3's expression is only in a few cells, it may be responsible for the activation and transcription of downstream targets necessary for nephron progenitor organization through proper cell-to-cell communication.

5.4 Reduced nephron progenitor population

The nephron progenitors are segregated into cells that will undergo differentiation and cells that will proliferate to maintain the population. In early kidney development, nephron progenitors will have similar cell counts in self-renewing versus those that will undergo nephrogenesis (Liu et al., 2017). However, as development progresses, the proliferating progenitors will decrease and the remaining cells will all differentiate until postnatal day 3, where no more proliferation occurs. This balance between both nephron progenitors is critical in developing an adequate amount of nephrons for normal kidney function. My results demonstrate a normal number of nephron progenitors at E13.5. But the number of progenitors is significantly reduced by E18.5 in *Shroom3*$^{Gt/Gt}$ kidneys. This reduction can be explained by either a reduction in prolifetation in self-renewing cells and/or an increase in apoptosis. However, without knowing the mechanism and function of Shroom3 in the progenitors, it remains unknown as to why the cells are decreasing in late kidney development.

There have been a number of growth factors that have been studied in regulating this balance in the cap mesenchymal pool including the FGFs, bone morphogenic proteins (BMPs), and Wnt sequestered molecules (Carroll et al., 2005; Dudley et al., 1995; Greishammer et al., 2005; Luo et al., 1995; Parantoni et al., 2005). In a study conducted by Blank et al., they determined that the maintenance of the nephron progenitor pool is

dependent on BMP7 signaling. They observed that BMP7 null mutants experience a significant and rapid loss in progenitors. In addition, the absence of FGF8 results in cell death in the periphery of the kidney in the metanephric mesenchyme (Greishammer et al., 2005). This abnormal cell death is presumably accounting for reduced kidney size and death following birth in FGF8 mutants (Greishammer et al., 2005). However, it is still unclear how FGF signaling promotes cell survival during kidney development. Therefore, Shroom3 may be interacting with various growth factors to maintain the nephrogenic pool of cells, and if lost, can severely impact the amount of proliferating self-renewal and/or differentiating progenitors.

However, various studies have demonstrated Wnt sequestered molecules mediating nephron progenitor maintenance. Nephron progenitor induction is required by the sequential activity of two main molecules: Wnt9b and Wnt4 (Carroll et al., 2005; Stark et al., 1994). Wnt9b is sequestered from the ureteric bud tips to the adjacent nephron progenitors (Carroll et al., 2005). It then induces certain nephron progenitors aggregate and express Wnt4 through the Wnt4/β-catenin pathway. Although the signaling pathway of Wnt4 has been determined, Wnt9b's has not. It has been proposed that Wnt9b is the key factor in determining the balance between proliferation and differentiation in nephron progenitors (Carroll et al., 2005). This was demonstrated in Wnt9b knockout mice, in which they experienced a peripheral mesenchyme and ureteric epithelium, however no nephrons or intermediate precursors of nephrogenesis (Carroll et al., 2005). In addition, studies have investigated how Wnt9b/ β-catenin cooperates with Six2 in determining nephron progenitor fate (Karner et al., 2011). Thus, the reduction of nephron

progenitors observed in Shroom3 mutant kidneys may be a result of inability to appropriately respond to sequestered Wnt9b signal induction. Therefore, nephrogenesis is disrupted and the amount of proliferating progenitors may be reduced, although our understanding of the molecular mechanisms that regulate the nephron progenitor expansion and differentiation is still unknown.

It is most likely a combination of these mechanisms, rather than just one, that regulates and contributes to the development and maintenance of the nephron progenitor pool of cells. The strongest evidence supports that FGF8 may be contributing to the overall reduction in nephron progenitors and thus an overall decrease of renal vesicles. First, Ernt et al. demonstrated that Shroom3 is a transcriptional target of the FGF signaling pathway in posterior neuromasts in zebrafish. We propose that perhaps Shroom3 may be downregulating the transcriptional activity of a receptor gene necessary in the FGF signal pathway. As mentioned above, in the absence of Fgf8, null mice experience cell death in the nephron progenitors in the periphery of the kidney. Although we did not observe Shroom3 mutant cell death, we did note a marked decrease in nephron progenitors. In addition, Ernt et al. also observed Fgf8 null mice not progressing past the formation of S-shaped bodies during nephrogenesis, in contrast to our Shroom3 knockout mouse model that had maturing nephrons. However, Fgf8 null and Shroom3 null mice did both experience a reduction in the periphery of nephron progenitors. These observations raise the possibility that, in vivo, Shroom3 may be a target for Fgf8 signaling activity.

Alternatively, reduction of cells in the cap mesenchyme in the nephrogenic zone may be due to the in ability to receive adequate Wnt9b sequestered signals. Since Wnt9b

knockouts do not undergo nephrogenesis (Carroll et al, 2005), we believe that *Shroom3*$^{Gt/Gt}$ kidneys are still receiving a Wnt9b signal due to the presence of renal vesicles and due to the formation of aggregates. However, it may be that Shroom3 regulates a separate set of progenitor specific factors that interact or can alter the response of Wnt9b/β-catenin signaling. The mechanism may then dampen or amplify the response to Wnt9b. This may be plausible since Shroom3 mutants are still expressing Six2. It has been proposed that Six2 cooperates with Wnt9b/β-catenin directly or indirectly to drive expression of target genes (Karner et al., 2011). Therefore, if the Wnt9b/β-catenin signal is being altered, it may be contributing to either the premature nephrogenesis of progenitors or the inability for nephron progenitors to proliferate and maintain the pool. Understanding the pathway of Shroom3 within the nephron progenitors will shed more light role it plays during kidney development.

5.5 Reduction of renal vesicles in Shroom3 mutants

After the nephron cells aggregate, the cells that will differentiate into the nephrons organize into a round sphere to form the renal vesicle. I demonstrated that the number of normally forming renal vesicles is significantly decreased in early and late kidney development in Shroom3 mutants. This disorganization may be contributing to the loss of proper aggregation of cells through increase in apoptosis or reduction in proliferation. As mentioned above, without proper mesenchymal cell clustering, Wnt9b induction of nephrogenesis may be disrupted. If a weak Wnt9b signal is received due to improper aggregation, fewer progenitors will be able to undergo epithelization for renal vesicle

formation. As a result, fewer progenitors will form spherical renal vesicles. Therefore, Shroom3 may be playing a role in NP aggregation and downstream maintaining the adequate production of renal vesicles.

In addition, we observed a significant reduction in progenitors in late embryogenesis. A decrease in progenitors in late development may therefore be directly contributing to the quantity of renal vesicles being produced. Khalili et al. observed a significant reduction in the amount of glomeruli in E18.5 Shroom3 mutants in comparison to wildtypes. Thus, a significant reduction in renal vesicles in late kidney organogenesis may be directly resulting in the reduction in glomeruli, as previously published.

5.6 Shroom3$^{Gt/Gt}$ abnormal renal vesicle polarization

Shroom3$^{Gt/Gt}$ kidneys exhibit abnormally forming renal vesicles in early and late kidney development. There are a few explanations that may account for the irregularity in renal vesicles. Shroom3 has been known to control apical constriction in epithelial cells. Shroom3 controls cell morphology by interacting directly with the components of the cytoskeletal machinery (Hildebrand, 2005). It is recruited to the apical junction of cells via its interaction of its centrally located domain ASD1 with F-action. ASD2 domain of Shroom3 binds directly to the SBD domain of Rho-kinase. This interaction regulates both apical localization and activity of Rho kinase. Rho then phosphorylates MLC directly and indirectly by inhibition of myosin phosphatase, which results in the activation of myosin

II and actomyosin contraction required for cellular morphogenesis. In terms of renal vesicles, they possess an apically constricted surface forming a spherical structure during the early stages of nephrogenesis. I believe that in the absence of Shroom3, nephron progenitors designated to undergo mesenchymal to epithelial transition will lack the necessary machinery to properly apically constrict. As a result the formation of the renal vesicle will be disorganized and lack the proper structure. Here, we show that Shroom3 mutants in E13.5 and E18.5 kidney development have renal vesicles with non-apically constricted epithelial cells lacking polarization. Therefore, it is likely that Shroom3 is playing an important role in the proper constriction of cells during nephrogenesis.

5.7 Increased spatial separation between nephron progenitor and ureteric bud tips in Shroom3 mutants

Shroom3 mutants exhibited a disruption in the nephron progenitor and UB tip basement membrane contact. The interaction between mesenchymal progenitors and the ureteric bud epithelium is required for successful kidney formation (Halt and Vanino, 2014). During normal kidney development, canonical Wnt signaling activates differentiation, while Six2 maintains the progenitor pool (Kiefer et al., 2012). To ensure all nephrons are formed and normal nephron development is produced, these opposing signals help mediate these processes. Studies have demonstrated that in the absence of Wnt9b, nephrogenesis fails, while overexpression of Wnt signaling drives commitment to differentiation (Halt and Vanino, 2014). Therefore, this tight regulation of Wnt9b is critical in balancing progenitor fate in cooperation with Six2. Carroll et al. demonstrated that progenitor cell fate is determined based on strength of Wnt9b received. He observed

that the, in cooperation with Six2, stronger Wnt9b signal induces differentiation, while weaker proliferates self-renewing cells. Since Shroom3 mutants are demonstrating separation in NP and UB tips, we propose this is interfering with a response to the amount of Wnt9b signal. This inadequate Wnt9b induction may then be accounting for the regression observed Shroom3 mutant kidneys in late development. Although we did not quantify the amount of Six2 being expressed in Shroom3 mutant nephron progenitors, we did observe its presence. Therefore, we postulate that an important function of Shroom3 may be to orient and migrate the nephron progenitors towards the ureteric epithelium to receive the necessary Wnt9b signal for differentiation or self-renewal.

5.8 Shroom3 Association with Kidney Disease

Genome wide associated studies (GWAS) have located regions in the genome associated with chronic kidney disease (CKD) (Kottgen et al., 2009). CKD is a major health concern for individuals as it causes significant morbidity and mortality. However, much of the CKD heritability and risk loci remain vastly unknown (Yeo et al., 2014). Understanding Shroom3's potential role in nephron formation is critical in understanding it's association with CKD. The loci for CKD were identified using the glomerular filtration rate in the meta-analyses for study specific GWAS (Kottgen et al., 2009). Four loci were identified, one of which was a SNP at the eGFRcrea locus at the intronic SNP rs17319721. It was located in a highly evolutionary conserved region in Shroom3 on chromosome 4 (Kottgen et al., 2009). However, they have failed to identify the underlying causative mechanisms for these SNPs. Boger et al. have identified the same

intronic SNP in Shroom3 associated with a reduced glomerular filtration rate as Kottgen et al. This SNP, was also the second most significant SNP, *UMOD* being the number one, for albuminuria. Therefore, uncovering these variants, such as Shroom3, has made advances in understanding the genetic components contributing to the onset of diseases such as CKD.

Recent publications have begun to identify the potential role of Shroom3 in renal pathogenesis. A study conducted my Yeo et al. identified 13 genetic variants in the Shroom3 gene that directly correlate with the onset of kidney disease in Fawn-Hooded Hypertensive (FHH) rats. While this study demonstrated an important role for Shroom3 in the kidney, it did not provide evidence for early developmental defects in kidney development. Furthermore, it did not provide any evidence for the identification and localization of Shroom3 within the embryonic kidney. Therefore, the role of Shroom3 still needs to be investigated in the kidney prior to nephrogenesis, and whether Shroom3 regulates the stages in nephrogenesis through the same mechanisms observed in the known epithelialized cells.

A second study investigated the role and function of Shroom3 in the developing kidney (Khalili et al., 2016). The study demonstrated that Shroom3 null mutants experienced collapsed and degenerating glomeruli, and retraction of podocyte foot processes. In addition, heterozygous Shroom3$^{+/-}$ exhibited no overt glomerular pathologies at 3-months of age. However, pronounced proteinuria and development of glomerulosclerosis was observed at 1 year of age. These findings suggested that Shroom3

contributed to the development of the podocytes and formation of proper glomeruli in the developing kidney. Although these studies introduced Shroom3 as a key predictor for the onset of proteinuria and disruptions in podocyte formation, they did not highlight a role for Shroom3 during nephrogenesis. Therefore, analyses of early and late stages of nephrogenesis need to be investigated and whether these abnormal glomeruli are stemming from early-disrupted nephrogenic structures during embryogenesis.

5.9 Conclusion

Here we present the expression analysis of Shroom3 in the condensing mesenchyme during embryonic kidney development. Shroom3 is specifically expressed in only a few select cells in the renal mesenchyme. Our studies of knocking out Shroom3 in the developing kidney have demonstrated that Shroom3 is required for adequate aggregation and organization of nephron progenitors towards the ureteric bud epithelium. In the absence of Shroom3, nephron progenitors and renal vesicles are significantly reduced. In addition, Shroom3 mutants develop abnormally forming renal vesicles. This suggests that potential mutations in the *Shroom3* SNP may be directly cause abnormalities in kidney development by reducing the amount of Shroom3 present in the kidney. A decrease in Shroom3 activity may therefore be reducing the amount of functioning nephrons in the mature kidney. A reduction in nephrons may then lead an increased susceptibility to chronic kidney disease.

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