AMERICAN UNIVERSITY OF BEIRUT

STUDY OF THE ROLE OF RB AND, THE COMBINED ROLES OF RB AND P53 PATHWAYS, DURING KIDNEY DEVELOPMENT

by

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AN ABSTRACT OF THE DISSERTATION OF

Carine Ali Jaafar for Doctor of Philosophy

Major: Cell and Molecular Biology

Title: Study of the role of Rb and, the combined roles of Rb and p53 pathways, during kidney development

Introduction: Establishment of renal nephron endowment occurs exclusively during embryonic development and is critical for proper renal function in the adult. Therefore, a better understanding of the signals and pathways that regulate the balance between nephron progenitor cell (NPC) proliferation and differentiation is essential. Studies of renal development in mice helped advance our understanding of the pathways governing some aspects of kidney development in humans given the relative conservation of the molecular mechanisms involved. The mammalian kidney maintains a population of self-renewing (Cited1+; Six2+) stem cells until termination of nephrogenesis. The Retinoblastoma protein, Rb, and the p53 pathways play key roles in the control of cell proliferation, senescence and apoptosis in many organs. Previous studies have uncovered a requirement for p53 in the regulation of renal development, specifically the maintenance of self-renewal capacity of the (Cited1+; Six2+) population and terminal differentiation in nephrons. However, it is still unknown whether Rb plays a role during kidney development. In the present study, we have examined and described for the first time a specific requirement for Rb during renal development, and the existence of crosstalk between the Rb and p53 pathways that are indispensable for proper kidney development and function in mice.

Methods: Using tamoxifen-inducible Nestin-CreER^{T2}-YFP/YFP mice, and, single or double Rb;p53 floxed/floxed ($^{fl/fl}$) mice, we induced conditional deletion(s) of Rb alone, p53 alone or both genes that are targeted to Nestin-positive cells and their progeny. Recombined renal cells include cap mesenchyme stem cells and progenitors and their progeny, endothelial cells in immature glomeruli, and podocytes of mature glomeruli. Tamoxifen was administered by oral gavage to pregnant females at E10.5 (the onset of nephrogenesis) and at E13.5. Embryonic kidneys were collected from single and double mutant embryos as well as wild type and heterozygote littermate controls at distinct developmental stages. Histological analyses and gene expression studies were conducted to characterize the renal phenotypes in the absence of Rb and/or p53.

Results: We assessed stem and progenitor cell proliferation, differentiation and survival in embryonic kidneys carrying single or combined deletion(s) in Rb and/or p53 at distinct stages during nephrogenesis. Our results showed that Rb negatively controls NPCs proliferation, and is needed for nephron survival around birth. Hence, compared with Rb littermate controls, loss of Rb let to increased and ectopic proliferation in the (Cited1-;Six2+) progenitor population, but without affecting self-renewal of the stem cell pool

(Cited1+; Six2+), nor subsequent nephron differentiation. However, this enhanced nephrogenesis was offset by a severely compromised survival in the tubule epithelia of the immature nephron at later stages in the absence of Rb. In $p53^{fl/fl}$ treated embryos, we identified multiple renal defects including renal hypoplasia, underdeveloped nephronic structures with severe nephron deficit, and expansion of glomerular Bowman's space in comparison with wild-type littermate controls. Unexpectedly, combined deletions of both Rb and p53 exacerbated the observed survival defects in Rb mutant embryos, as manifested by a more severe renal hypoplasia with almost complete loss of immature glomeruli at birth.

Conclusion: This is the first study to uncover a novel role for Rb in the control of NPC proliferation (rate of nephrogenesis), and a critical requirement for Rb in immature nephron survival around birth. It also highlights the existence of distinct as well as synergistic functions between the Rb and p53 pathways during kidney development, with direct implications on renal function. These findings about the roles of the Rb pathway and its downstream effector pathways will bring new insights on human renal development, and help understand better the pathogenesis associated with some cases of kidney disease and injury in humans.

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ABBREVIATIONS

AC3 - Active caspase 3

AKI - Acute kidney injury

aNSC - Adult neural stem cell

BCIP - 5-bromo-4- chloro-3-indolylphosphate

BMP - Bone morphogenic protein

BrdU - Bromodeoxyuridine

BSA - Bovine serum albumin

BW – body weight

Ca2+/CamKII – Calcium/ calmodulin-dependent protein kinase

CD - Collecting Duct

CDK - Cyclin-dependent kinase

CDKI - CDK inhibitor

ChIP - Chromatin immunoprecipitation

Cited1 – Cbp/p300-interacting transactivator with Glu/Asp-rich C-terminal domain 1

CKD - Chronic kidney disease

cKO - Conditional knock out

CM – cap mesenchyme

CS - Carnegie stage

CSB - Comma shaped body

Dchs1 - Dachsous cadherin-related 1

DCN - Decorin

DGC - Dentate granule cell

DIG - Digoxigenin

Dlx1 - Distal-Less Homeobox 1

DMEM – Dulbecco's modified Eagle's medium

DMSO - Dimethyl sulfoxide

E - Embryonic day

ERT2 - mutated estrogen receptor

Eya1 - Eyes absent 1 protein

FACS – fluorescence-activated cell sorting

FBS – fetal bovine serum

FGF - Fibroblast growth factor

fl/fl - floxed/floxed

FSGS - focal segmental glomerulosclerosis

GDNF - Glial cell-derived neurotrophic factor

GFP - Green fluorescent protein

H&E – Hematoxylin and eosin

IF – Immunofluorescence

IHC – immunohistochemistry

IM - Intermediate Mesoderm

IP – Intrapertoneal injection

iPSC - Induced pluripotent stem cell

Kif26b - Kinesin family member 26B

Mdm2 -Murine Double Minute-2

MEF - Mouse embryonic fibroblasts

MET - Mesenchymal-to-Epithelial transition

MET – mesenchyme to epithelial transition

MM – metanephric mesenchyme

MSC - Mesenchymal stem cell

MSCs -Mesenchymal stem cells

NBT - 4-nitro blue tetrazolium chloride

NC - Nephrogenic Cord

ND - Nephric Duct

NPC - Nephron Progenitor Cell

NuRD - Nucleosome remodeling and deacetylase

NZ – nephrogenic zone

OB- Olfactory bulb

OE - Olfactory epithelium

OSN - olfactory sensory neuron

Osr1 - Odd-Skipped Related Transcription Factor 1

P - Postnatal day

PA - Pre-tubular aggregates

Pax2 - Paired box gene 2 transcription factor

PBS – phosphate buffered saline

PCNA - proliferating cell nuclear antigen.

PCR - Polymerase chain reaction

PFA - Paraformaldehyde

Podxl - Podocalyxin like

qRT-PCR – quantitative real-time PCR

Rb - Retinoblastoma protein

RMS - Rostral migratory stream

Rpm - Revolutions per minute

RT-PCR – real-time PCR

RV - Renal Vesicles

RV – renal vesicle

Sall1 - Spalt Like Transcription Factor 1

SD - Standard deviation

SDS – sodium dodecyl sulfate

SGZ - Subgranular zone

Six2 - Sine oculis homeobox homolog 2

Slc12a3 - Solute Carrier Family 12 Member 3

Slc34a3 - Solute Carrier Family 34 Member 3

SPC - Stromal Progenitor Cell

SSB - S-shaped bodies

SVZ - Subventricular zone

TCF/LEF - T cell-specific transcription factor/lymphoid enhancer factor-1

UB - ureteric bud

WT1 - Wilm's tumor 1

YFP - Yellow fluorescent protein

CHAPTER I

I. INTRODUCTION

A. Kidney Development

1. Mouse kidney development

The mammalian kidneys are fully derived from the intermediate mesoderm (IM). As the IM extends along the rostral to caudal axis during embryogenesis, three pairs of kidneys form in a temporal sequence starting at embryonic day E8.5: the pronephros, the mesonephros and the metanephros (Davidson 2008). The first two structures are transient organs that degenerate, however, the metanephros, which develops at E10.5 near the caudal end of the IM, becomes the functional permanent kidney in the adult mouse (Mugford, Sipilä, Kobayashi, et al. 2008) (**Figure 1A, 1B**). During the process of metanephros formation, the dorsal IM known as the nephric duct (ND) (or Wolffian duct) secretes signals that induce the commitment of the undifferentiated ventral IM, known as the nephrogenic cord, at the level of the hind limb to form the metanephric mesenchyme (MM) (**Figure 1A**). Reciprocal signals emanating from the MM stimulate an outgrowth from the ND called the ureteric bud (UB) which invades the MM and initiates nephrogenesis (Grobstein 1955).

Ureteric epithelial cells present at the tip of the UB express the Ret receptors which respond to GDNF-secreted signal by the MM, thus inducing progressive UB branching within the MM and formation of the ureteric tree (Sainio et al. 1997; Costantini and Shakya 2006). MM cells condensed closest to the UB tip form the cap mesenchyme (CM), a unique subset of cells from which all epithelial segments of the nephron arise via a mesenchyme-to-epithelial transition (MET) (Costantini and Kopan 2010). GDNF

expression in the CM is regulated by a range of transcription factors including Eya1, Pax2 and Sall1 (Brophy et al. 2001; Bouchard 2004). Within each UB-CM niche, the generated epithelial structures known as renal vesicles (RV) undergo elongation and segmentation into comma-shaped bodies (CSBs) then S-shaped bodies (SSBs). The SSBs elongate further to establish a capillary loop with a vascularized proximal end, finally maturing to form the functional nephron (Bard et al. 2001). The most proximal component of the nephron that filters the blood is the glomerulus, which occupies the external cortex of the adult kidney and consists of a capillary tuft surrounded by interdigitating foot processes known as podocytes (Quaggin and Kreidberg 2008). In addition to the glomerulus, the mature nephron consists of a tubular epithelium that is essential for the fine-tuning and modification of the glomerular filtrate via tubule-specific transporters. This tubular epithelium is comprised of the proximal tubules, the loop of Henle running through the medullary region of the kidney, the connecting segment and the distal tubules that connect with the collecting duct system through which urine drains for disposal (Bulger and Hebert 1988) (**Figure 1**). Each of the segments in the body of the nephron is made of molecularly and anatomically distinct types of cells that carry out unique and specialized function(s) within the metanephric kidney. Despite of this functional diversity, these cells share a common precursor, which is the Nephron Progenitor Cell (NPC) (S. Boyle et al. 2008; Kobayashi et al. 2008).

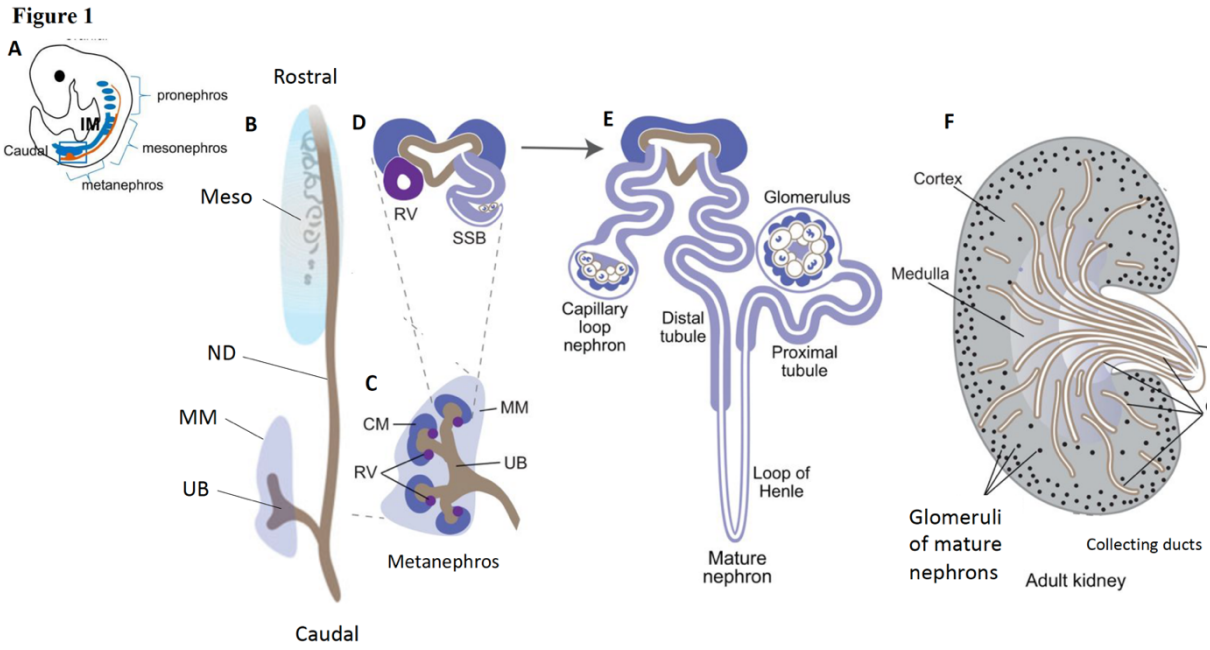

Figure 1. Schematic illustration of the mouse kidney development (**A**) a representation of a mouse embryo at E9.5. The intermediate mesoderm (IM) gives rise to the urogenital system including the kidneys. The dorsal IM (red line) running along the rostral to caudal axis, forms the nephric duct (ND). The ventral IM (in blue) forms the nephrogenic cord (NC). (**B**) The NC is induced to differentiate in response to signals from the ND, thus forming the three pairs of kidneys in a temporal manner: the pronephros, the mesonephros and the metanephros. At the level of the hind limb (square in **A**), the ND becomes a Ureteric Bud (UB) which evaginates through the metanephric mesenchyme (MM) (light blue in **C**) to initiate the formation of the metanephros (that later becomes the permanent adult kidney, **F**). The UB branches several times within the MM (**C**). The cells populating the region closest to the tip of the UB are known as the cap mesenchyme (CM) (shown in dark blue in **C**). These cells proliferate and are induced to epithelialize giving rise to the renal vesicles (RV) (shown in purple in **D**). Within each UB tip/CM niche, RV further elongates and segments into more differentiated structures, the S-shaped bodies (SSB). These undergo further elongation to form a capillary loop before establishing the mature nephron (shown in **E**) that is comprised of: the proximal tubules, Loop of Henle, distal tubules and the glomerulus (formed of a capillary tuft surrounding podocytes) (modified from (Takasato and Little 2015)).

2. Early specification of the Metanephric Mesenchyme

Over the past two decades, studies provided significant insights onto key developmental pathways underlying kidney formation and nephrogenesis. Fate-mapping studies identified Osr1 (Odd skipped related1) as the earliest transcription factor to be expressed by the IM progenitor cell population before E9.5 (James et al. 2006). The distinct lineages that constitute the metanephros including the nephric-ureteric lineage, the nephronic lineage and the stromal lineage, all originate from a common ancestral population of Osr1+ cells in the IM (Mugford, Sipilä, McMahon, et al. 2008). Nephric duct derivatives exclusively contribute to the collecting duct (CD) system, except for the connection zone where the distal tubules of the nephron fuses with the CD (Yu, Carroll, and McMahon 2002). Lineage labeling studies revealed spatial and temporal differences in the fate commitment of the three distinct lineages. Hence, cells occupying the anterior IM give rise to the nephric duct lineage before E9.5 while posterior IM cells commit to either the nephronic or the stromal lineage between E8.5 and E10.5. The latter lineages completely segregate and become distinct at a later age around E11.5. This time-point coincides with the onset of kidney development when stromal progenitors localize into the periphery of the MM while NPCs aggregate close to the UB tip forming the CM (Mugford et al. 2009). As the UB grows and branches into the MM, Osr1 expression becomes confined to the CM population and is required for the expression of specific genes necessary for the establishment and maintenance of the nephron progenitor population including: Eya1, Pax2, Sall1, Six2 and GDNF (Mugford et al. 2009). Loss of function studies showed that deletion of each of these transcriptional regulators during metanephric morphogenesis results in renal agenesis, highlighting their importance for kidney formation. Establishment of the specific roles

of these genes in the NPC was accomplished by using conditional NPC-specific knockout mouse models as will be discussed in detail in the next sections.

Once Osr1+ IM cells commit to the nephron lineage, they activate Six2 expression (Mugford et al. 2008). Thereafter, three major progenitor populations occupy the MM and give rise to distinct progeny. The first is the Six2+/Cited1+ nephron progenitor population which gives rise to epithelial components of the nephron (Kobayashi et al. 2008). The second is the Foxd1+ population of stromal progenitors which contributes to the mesangial, interstitial and pericytic components of the developing kidney (Humphreys et al. 2010). The third is the flk1+/CD146+ population of endothelial progenitors that generates the vasculature of the nephron and may contribute to support UB invasion (Gao et al. 2005) (**Figure 2**). Of note, another subpopulation of CD146-expressing cells originates from the Foxd1+ progenitors, indicating that distinct endothelial progenitors may arise from different ancestral lineages (Mukherjee et al. 2017).

Figure 2

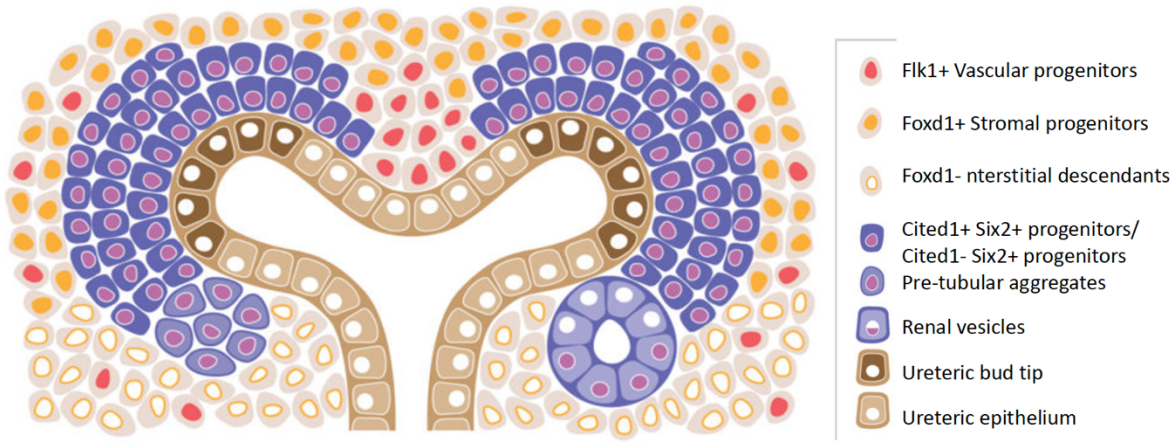

Figure 2. Schematic representation of the cellular compartments in the nephrogenic niche Illustration of the three distinct progenitor populations residing within the niche: Foxd1+ stromal progenitors, Flk1+ vascular progenitors and the Six2+ nephron progenitors (modified from (McMahon 2016)).

3. Human versus mouse kidney development

Research into renal morphogenesis using mouse as a model significantly contributes to our understanding of kidney development and disease in humans. However, anatomical differences between human and mouse kidneys are well illustrated. While the mouse kidney is unipapillate with one peripheral nephrogenic cortex, human kidneys are multi-papillate with 8-15 lobes, each consisting of a ureteric tree and medulla, surrounded by peripheral and interlobular nephrogenic regions (Little 2015). Human kidney development initiates around 4 weeks of gestation and ends at 35 weeks generating up to a million nephrons whereas in mice nephrogenesis starts at E10.5 and lasts until P4 forming around 16,000 nephrons. Furthermore, in the human kidney, UB primary invasion of the MM takes place at CS13 (Carnegie stage 13), and at CS16, the first UB branching event is reported with each branch surrounded by the CM. In mouse, UB branching begins at E12.5 (Little 2015). Clear organizational resemblances and differences are evident in the nephrogenic zones of human and mouse kidneys. For instance, in contrast to the mouse, the human kidney consists of interlobular regions that are first observed at CS19, in addition to the peripheral regions. These latter regions are nephrogenic niches that harbor SIX2+ NPCs in caps surrounding the UB tips. In mouse, the interstitial stroma surrounding the CM consists of Meis1+/Foxd1+ cells, likewise the interlobular regions in the human kidneys are demarcated with a similar Meis1+ interstitium. Furthermore, characterization of progression of nephrogenesis revealed temporal differences in the duration of transition from the PA stage to the SSB stage. This transition is shown to require 8 days in the developing human kidney, while SSB structures are first detected by E12.5 in mouse development, hence a duration of 24 hrs for PA to epithelialize and give rise to SSB (Short et al. 2014).

Of note, *Six1* is only transiently expressed in the mouse MM around ~E10.5 and is required for UB recruitment as well as inducing the expressions of *Six2*-related family members, which regulate MM survival. However, *SIX1* expression is maintained in the human kidney and plays overlapping roles with *SIX2* (O'Brien et al. 2016). To elucidate the discrepancy in Six1 expression and function between the two species, it remains to be investigated how SIX1 loss of function affect human NPCs development.

Cellular distribution and morphology of human kidney samples from week 11 and week 23 were compared to E15.5 and P2 mouse kidney tissues. While development of mouse and human kidneys proceeds at distinct time scales, cellular organization and tissue composition of the nephrogenic niches in both species follow similar developmental programs ending with a reduction of NPC endowment. For example, in the mouse, as the first branching events unfold, the number of NPCs per UB tip is reduced; this is followed by a plateau period where the NPC number per UB tip stays the same until birth when accelerated differentiation terminates nephrogenesis (Short et al. 2014). Similarly, in the developing human kidneys, the number of SIX1+ NPCs per UB tip decreases gradually between week 11 and week 15, then remains constant until week 17, and this is followed by a final reduction from week 18 until week 23. Moreover, the NPC population at week 11 are positioned above the ureteric epithelial tips and form a thick and large structure at this stage. At week 23, UB tips become narrower as the NPCs shift to the sides of the tip. These CM-UB tip architectural alterations that accompany progression of nephrogenesis between week 11 and week 23 are very similar to those that take place between E15.5 and P2 of mouse kidney development (Nils O. Lindström et al. 2018).

In this context, it should be noted that chronic kidney disease (CKD) in humans is linked to reduced number of nephrons that likely originates from defective kidney development (Boubred et al. 2013). Therefore, achieving a better understanding of the cellular and molecular processes governing kidney development will help uncover the mechanisms of disease pathogenesis and lead to the implementation of novel regenerative therapies.

B. Inductive signals and pathways that trigger nephrogenesis

Separation of the UB and the MM in the developing mouse kidney followed by co-culture experiments revealed a requirement for inductive signal(s) originating from the UB for proper nephron differentiation (Grobstein 1953). In fact, two major induction events are well defined and drive metanephric development: the first is the primary induction, which is the segregation of the CM (NPCs) and the stromal progenitors (as described in section **1.2.**), and the second is epithelialization of the CM to give rise to the nephron.

1. Wnt9b is a UB-derived inducer of nephrogenesis

In an attempt to identify factors derived from the UB that are responsible for mesenchymal-to-epithelial transition (MET), a series of experiments investigated the inductive features of the members of the Wnt family of secreted factors during metanephric development. As a result, Wnt4 deletion led to the formation of nephron deficient kidneys, rendering this factor a possible candidate. In fact, in Wnt4 knockout kidneys, the UB branches normally within the MM, however, MET is blocked, and no nephrons are formed (Stark et al. 1994). Moreover, Wnt4 expression is strictly localized to PA (the stage just before MET induction) and is absent from the UB, thus ruling it out from being the UB-derived inducer that triggers nephron epithelialization (Lombard

and Grobstein 1969). This finding shifted the focus to the other canonical Wnt family members that are expressed in the UB including Wnt7b, Wnt9b and Wnt11(Y. Wang, Zhou, and Liu 2018). Gene knockout studies revealed distinct and specific roles played by these genes in metanephric development; however, Wnt9b was shown to have a critical role as a nephron inducer due to several reasons. First, the loss of Wnt9b ablates Wnt4 expression and no PA or RV are formed, hence Wnt9b is upstream of Wnt4 during nephrogenesis. Second, Wnt9b is exclusively expressed in the UB, and not in the MM; in addition, it has the ability to trigger tubulogenesis in isolated MM (Carroll et al. 2005). Altogether, these data support the existence of a Wnt9b signal derived from the UB that is responsible for inducing nephrogenesis through initiating a Wnt4-dependent process in the CM.

2. Regulation of CM differentiation by canonical and non-canonical Wnt signaling pathways

Studies provided strong evidence for an essential role of the canonical Wnt9b/β-catenin signaling pathway in inducing epithelialization of the CM (J. S. Park, Valerius, and McMahon 2007). In this pathway, Wnt9b binds to and activates the frizzled receptors, thus blocking the β-catenin degradation complex from phosphorylating β-catenin and inducing its proteasomal degradation. This in turn leads to accumulation of intracellular β-catenin. High levels of β-catenin induce complex formation between β-catenin and the T cell-specific transcription factor/lymphoid enhancer factor-1 (TCF/LEF), thus modifying the transcriptional activity of TCF/LEF and activating the expression of Wnt target genes including Wnt4 and FGF8 (Nelson and Nusse, 2004). Of note, the FGF8 pathway was found to act upstream of Wnt4 and was also shown to contribute to the epithelialization of the CM. FGF8 functions in an autocrine manner within the pre-tubular aggregates (PA) to promote proliferation within the epithelial cells of RV. Its

sustained expression at later stages (CSB, SSB) is dependent on Wnt4 signal. Fgf8 loss of function blocks RV formation (Grieshammer et al. 2005).

Although β-catenin/TCF-dependent transcription is required for the priming of CM cells (formation of PA) and induction of Wnt4 expression (that triggers for MET), later studies using TCF-reporter mice revealed that the canonical Wnt pathway is not directly responsible for MET of the CM (progression of PA to RV) given that conditional deletion of β-catenin in NPCs blocks MET with no RV formation (J. S. Park, Valerius, and McMahon 2007). Indeed, activating β-catenin in Six2+ CM cells in Wnt4-/- or Wnt9b-/- kidneys allow NPCs to transit to PA, however, sustained and elevated levels of β-catenin blocked progression of differentiation to later stages and nephron formation (Schmidt-Ott et al. 2007; J. S. Park, Valerius, and McMahon 2007). These studies implicate the involvement of an independent pathway(s) that is needed to attenuate the level and response to β-catenin signal in order for progression of CM differentiation to occur.

In an attempt to delineate the exact mechanistic basis for the CM response to Wnt signaling, studies demonstrated that Wnt4 induces MET without activating TCF/LEF by treating rat MM with recombinant Wnt4 protein. Moreover, they showed that treatment with ionomycin, an intracellular calcium activator, partially compensates for the Wnt4-/- phenotype by triggering differentiation into RV and C-shaped bodies. The same study further showed that abrogating Wnt/β-catenin signaling by treating MM cultures with the specific inhibitor CT-HC-1 (dominant-negative inhibitor of β-catenin) did not appear to affect tubule formation *in vitro*. Therefore, the calcium influx that is induced by Wnt4 seems to be sufficient for promoting MET of the progenitor population

independently of the canonical Wnt/ β-catenin signaling pathway (Burn et al. 2011; Tanigawa et al. 2011).

In summary, the above data highlight a multi-step response governing differentiation of the CM, whereby 1) Wnt9b from the UB activates β-catenin in the adjacent CM cells which induces Wnt4 expression in these cells, and 2) in turn, Wnt4 signals through the Wnt-Ca2+/CamKII pathway to trigger epithelialization in parallel to a synchronized attenuation of β-catenin signaling (**Figure 3**).

Figure 3

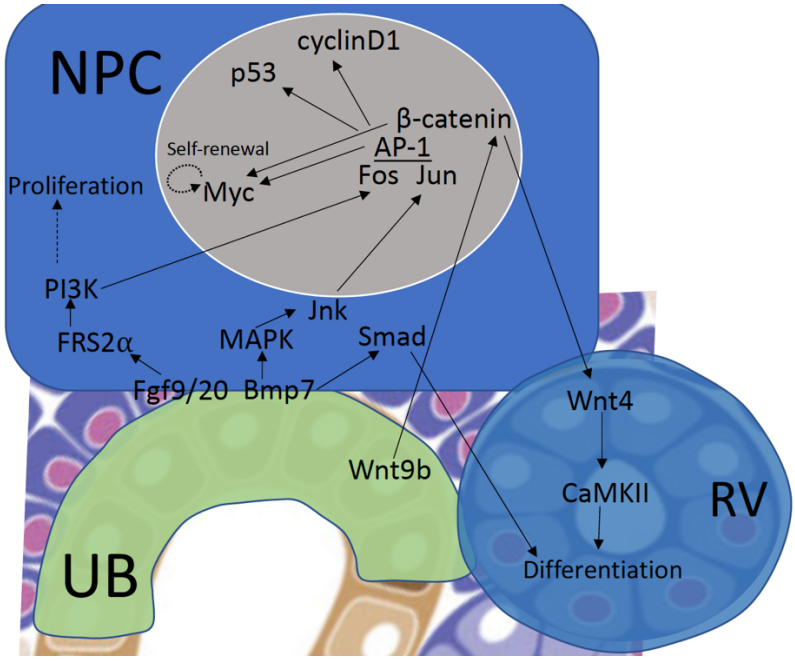

Figure 3. Schematic representation depicting signaling pathways regulating NPC proliferation and differentiation Bmp7 and Fgf9-20 signaling pathways converge at the level of the AP1 complex, which is comprised of Fos and Jun. In turn, the latter two transcription factors induce the expression of Myc, p53 and Cyclin D1, eventually regulating NPC proliferation. Alternatively, the Bmp7-Smad pathway can trigger NPC differentiation by promoting RV formation. Wnt9b signaling from the UB activates β-catenin expression in the adjacent CM, which triggers Wnt4 expression. Wnt4 induces NPC differentiation through the Wnt-Ca2+-CamKII non-canonical pathway (modified from (O'Brien 2019)).

C. Signaling pathways regulating CM maintenance and proliferation

Nephrogenesis is critically regulated by niche signals in the nephrogenic zone to ensure proper nephron endowment. Iterative rounds of epithelialization generate nephrons throughout the developmental period of the mouse kidney. Hence, part of CM is maintained and expanded to ensure enough NPCs are available to generate all the nephrons of the mature kidney over the entire period of development from E11.5 until P4. In this context, the FGF and BMP signaling pathways play critical roles within the nephrogenic niche to regulate growth and maintenance of the CM.

1. BMP signaling regulates NPC proliferation and survival

Bmp7 is secreted by the CM and the UB. Bmp7 mutant kidneys exhibit smaller kidneys caused by massive apoptosis of NPCs (Dudley, Lyons, and Robertson 1995; Luo et al. 1995). Similarly, conditional inactivation of Bmp7 in the CM at late stages of renal development results in elevated levels of cell death in the CM and NPC depletion, suggesting a critical requirement of Bmp7 in the maintenance and survival of the CM (Tomita et al. 2013). Bmp7 is found to act through two distinct downstream signaling pathways, MAPK/Jnk and Smad (Blank et al. 2009). Genetic deletion of downstream effectors of the BMP pathway such as Smads demonstrated their involvement in mediating the effect of Bmp7 (Ikeya et al. 2006). Bmp7 was shown to enhance proliferation of Cited1+ isolated cells *in vitro* through the MAPK-Jnk signaling pathway (Blank et al. 2009). In contrast, phosphorylation of Smads promotes the differentiation of these Cited1+ progenitors (Brown et al. 2013). Interestingly, Bmp7-Smad signaling was recently shown to contribute to postnatal epithelial regeneration in response to acute renal injury (Vigolo et al. 2019).

2. FGF signaling regulates NPC maintenance and survival

The FGF ligands, FGF9 and FGF20, are two essential factors of the nephrogenic niche that are secreted by the UB and contribute to the regulation of CM maintenance and survival. Loss of both ligands results in renal agenesis (Barak et al. 2012). NPCs express the FGF receptors, FGFR1 and FGFR2, which bind FGF9 and FGF20. Six2-Cre driven deletion of one of these receptors in NPCs did not cause any detectable renal defects suggesting redundancy in their functions. However, inactivating both receptors in NPCs compromises their survival and results in depletion of the Cited1+ self-renewing population (Di Giovanni et al. 2015). Moreover, the same study revealed that the FGF receptor's function is at least partially mediated through FSR2α (FGFR substrate 2α), the adaptor protein that links FGFR to downstream signaling molecules (Di Giovanni et al. 2015). Contribution of PI3K signaling downstream FGF- FSR2α is well supported; inhibition of PI3K/Akt pathway in kidney culture stimulate premature cell differentiation (Nils Olof Lindström, Carragher, and Hohenstein 2015). A study by Liu et al. 2017 showed that PI3K signaling plays an intermediary role in regulating cell autonomous self-renewal capacity of NPCs. In fact, young NPCs in the CM (at E13.5) have a higher glycolysis rate which maintains their stemness for a longer period of time compared with older NPCs (those harboring the CM at P0 or older). The PI3K-Akt pathway is proposed to induce a high glycolytic flux in NPCs to promote self-renewal. Accordingly, PI3K inhibition or decreasing glycolysis potentiates NPC differentiation (Jiao Liu et al. 2017).

3. Crosstalk between BMP and FGF signaling pathways in regulating CM proliferation

A model involving a crosstalk between the BMP and FGF signaling pathways in regulating NPC proliferation is well established (Muthukrishnan et al. 2015). Hence,

addition of BMP7 and FGF9 to isolated MM cells in culture increased cell survival compared to single treatments with either BMP7 or FGF9 alone, confirming the existence of a synergistic regulatory role between the two pathways in the CM (Barak et al. 2012; Dudley, Godin, and Robertson 1999). In fact, both pathways were shown to converge on activating components the AP-1 complex including FOS and JUN. Ras and PI3K which are downstream targets of FRS2α regulate FOS whereas JUN is a downstream mediator of the BMP7-MAPK-Jnk signaling pathway. The target genes that are transcriptionally regulated by AP1 include: *Myc, p53* and *cyclin D1.* Myc is shown to act cooperatively with β-catenin to promote transcription of genes, such as *Fam19a5*, that maintain NPC self-renewal (Pan, Karner, and Carroll 2017). The role of p53 in the developing kidney is well-established and discussed in **Section 9** below. How other AP-1 target genes such as Cyclin D1 are involved in the regulation of NPCs' proliferation warrant further investigation (**Figure 3**).

D. Regulatory role of stromal cells in NPC proliferation and differentiation

Stromal cells are characterized by the expression of Foxd1. Inactivation of Foxd1 expression leads to ectopic expansion of the nephron progenitor pool and blocks their differentiation (Hatini et al. 1996). A similar phenotype is detected upon Diphtheria Toxin-induced ablation of the stroma (Das et al. 2013). Studies have uncovered two mechanisms by which the stroma contributes to the regulation of NPC differentiation. Fetting et al. 2014 showed that the proteoglycan Decorin acts as an antagonist for BMP-mediated differentiation and a mediator of Foxd1 function in NPCs. In fact, Foxd1 inhibits Decorin given that this inhibition is lifted and Decorin is overexpressed in Foxd1-/- mutant mice. Consequently, BMP7-Smad signaling is attenuated in the nephrogenic zone, resulting in reduced NPC differentiation (Fetting et al. 2014).

The second mechanism by which stromal cells regulate CM epithelialization is through producing the atypical cadherin, Fat4, component of the Hippo pathway (Das et al. 2013). Foxd1-driven conditional deletion of Fat4 in the stroma results in NPC expansion and reduced differentiation (Das et al. 2013). Furthermore, Dchs1 and 2, protocadherins that bind Fat4, are localized on the CM-stromal interface supporting optimal spatial interaction to mediate Fat4 signaling. Deletion of Dchs1 or both Dchs1 and 2 results in accumulation of NPCs (Mao, Francis-West, and Irvine 2015). Studies investigating the downstream mechanism by which Fat4 and Dhsc1-2 cooperate to regulate NPC differentiation implicated the Yap-Taz transcriptional activators. Stroma-derived Fat4 induces differentiation by promoting the translocation of nuclear Yap-Taz to the cytoplasm in NPCs, thus allowing the expression of β-catenin pro-differentiation target genes (**Figure 4**). In contrast, Yap accumulation in the nucleus promotes the expression of pro-self-renewal genes in NPCs (Das et al. 2013).

In this context, the Sall1 transcription factor plays an important regulatory function given that Sall1 binding sites have been identified in the Decorin and Fat4 gene loci in stromal progenitor cells (SPCs) (Ohmori et al. 2015). One of the mechanisms by which the Sall1 transcription factor regulates NPC proliferation (cell non-autonomous manner) is by binding to the promoter regions of the Decorin and Fat4 genes in SPCs, thus decreasing Decorin transcription and increasing transcription of Fat4 (Ohmori et al. 2015). Accordingly, in Sall1-/- stroma, Decorin expression is upregulated and Fat4 expression is downregulated, which synergistically reduces NPC differentiation (**Figure 4**). Therefore, Sall1 expression in Foxd1+ progenitors inhibit NPC proliferation in a cell non-autonomous manner and is required for their maintenance (discussed in **Section 5c** below).

Figure 4

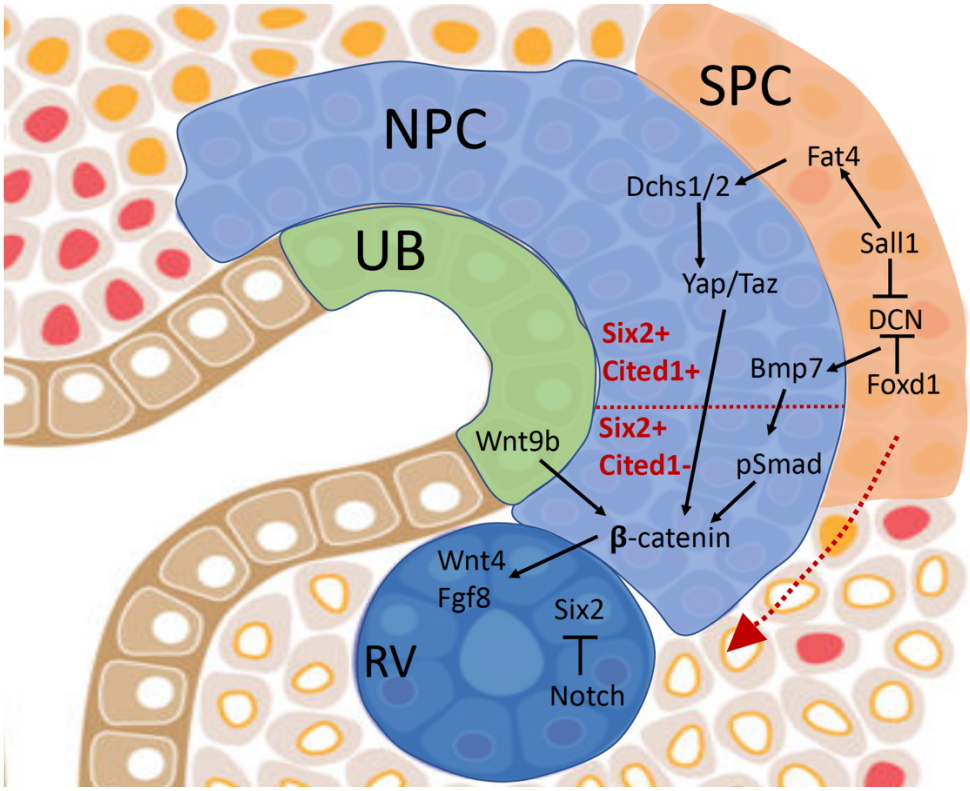

Figure 4. Schematic representation depicting regulatory signals and pathways governing NPC differentiation Dotted red line separate unprimed Six2+Cited1+ NPCs and primed Six2+Cited1- induced progenitors. Black arrows indicate positive signaling and stop sign indicates inhibitory signaling. The dotted arrow indicates differentiation. UB: ureteric bud NPC: Nephron Progenitor cell SPC: Stromal Progenitor cell RV: Renal Vesicle DCN: Decorin (figure modified from (O'Brien 2019)).

E. Transcription factors with critical roles in kidney development and nephrogenesis

1. Essential roles for WT1 and Gas1 during kidney formation

Wilm's tumor 1(WT1) transcription factor plays an important role during early MM specification. In fact, the MM lacking Wt1 expression undergoes apoptosis due to a failure to recruit the ureteric bud (Kreidberg et al. 1993). In this context, studies have shown that the WT1 regulates the transcription of several nephrogenic regulatory genes by binding to their promoters, including: Fgf20, Bmp7 and Sall1, thus demonstrating an essential role for WT1 in MM specification at E10.5 (Hartwig et al. 2010). In an attempt to decipher the disrupted pathways mediating the phenotype observed in WT-/- early MM, Motamedi et al. found that the abnormally increased BMP7-Smad signaling is the mechanism responsible for inducing severe death in the early MM. Moreover, treatment with FgF20 rescues the latter phenotype (Motamedi et al. 2014). These findings suggest that WT1 function in the specification of the early MM rather involves a complex crosstalk between multiple signaling pathways and implicates several downstream target genes.

Besides its role during early MM specification, WT-1 is highly expressed in S-shaped bodies and mature podocytes, and also plays a distinct role during late kidney development. WT1 indeed directly activates Nestin expression during development and in the adult kidneys (Wagner et al. 2006). Nestin-Cre mediated deletion of WT-1 resulted in severe reduction in nephron mass during late development, indicating a critical requirement for WT-1 in nephron differentiation (Berry et al. 2015). Moreover, using shRNA to knock-down WT-1, a study by Kann et al. 2015 showed a similar detrimental effect for the loss of WT-1 on nephron differentiation and numbers overall. However, the same study further revealed that this phenotype is linked to a modulatory

role of WT-1 in regulating NPC proliferation through Gas1 (Growth arrest-specific 1) rather than a direct control of nephron differentiation. WT-1 was shown to bind directly to the promoter region of GAS1 and activate its expression in NPCs. Indeed, Gas1 deletion caused a premature depletion of the nephron progenitor's pool by E17.5 leading to renal hypoplasia. Further investigation of the mechanism underlying Gas1 function linked its role in the control of CM proliferation to the activation of the PI3K/Akt pathway acting downstream of the FGF9-FGF20 axis. In summary, WT1 is needed for proper specification of the early MM and for the maintenance of CM proliferation and subsequent differentiation through its transcriptional control of GAS1 (Kann et al. 2015).

2. Indispensable regulatory roles for Six2 during kidney development

The number of NPC is under tight control and regulated by self-renewal during development. This ensures that a sufficient number of progenitor cells undergoes nephrogenesis to generate enough nephrons around birth before becoming completely exhausted by post-natal day 4 in mice, and around 35 weeks of gestation in humans (Little 2015). Proper nephron endowment is critical for normal kidney function in the adult and insufficient number of nephrons have been associated with high blood pressure and renal diseases in humans (Bertram et al. 2011).

The homeobox transcription factor, Six2, primarily maintains NPC self-renewal throughout kidney development until termination of nephrogenesis; its deletion causes premature CM depletion and ectopic epithelialization (Self et al. 2006a). The initial number of Six2-positive cells that form the murine CM is estimated to be around 10,000 cells at E11 (Kobayashi et al. 2008). Studies have shown that Six2 auto-regulates its own transcription by binding to its own promoter and to a proximal enhancer element

located in the *Six2* locus to support NPC self-renewal. Moreover, some of the intrinsic mechanisms by which *Six2* cooperates with other genes to control progenitor cell expansion have been uncovered. Hence, transcriptional profiling studies identified several Six2 target genes belonging to the regulatory networks that underlie the balance between maintenance of NPCs' self-renewal on the one hand, and, commitment to a nephrogenic fate on the other hand. In fact, Six2 forms a complex with the TCF/LEF factors and binds *cis*-regulatory elements adjacent to the *Fgf8* and *Wnt4* gene loci to silence their expressions, thus preventing NPC commitment. An additional regulatory role of Six2 cooperating with Wnt9b in NPCs self-renewal versus differentiation response was reported. Hence, Wnt9b was found to induce maintenance of self-renewal in NPCs expressing high level of Six2 whereas it triggers differentiation in NPCs with low *Six2* expression (Karner et al. 2011). In fact, high levels of β-catenin attenuate *Six2* expression, thereby allowing β-catenin to successfully bind to TCF/LEF and induce nephrogenesis. In contrast, when Six2 level is high, it competitively blocks β-catenin binding to TCF/LEF (J. S. Park et al. 2012). Six2 and its transcriptional co-activator Eya1 were shown to be co-expressed in the MM starting E10.5 and thought to interact in controlling NPC proliferation. As a result, temporal inactivation of *Eya1* attenuated *Six2* expression and was accompanied by ectopic nephrogenesis due to premature CM differentiation. Furthermore, Six2 was shown to induce Eya1 protein translocation to the nucleus where it modulates Myc phosphorylation state through its phosphatase activity, thus driving progenitor proliferation (Jinshu Xu et al. 2014). In summary, Eya1 enforces an undifferentiated state in the CM through its function as both a protein phosphatase and a transcriptional coactivator for Six2.

In contrast with Eya1's role, Notch signaling positively regulates CM differentiation. Hence, a Notch gain-of-function mutant displayed *Six2* downregulation and ectopic expression of *Wnt4* in the CM, thus promoting premature epithelialization and nephrogenesis (Chung et al. 2016). Loss of Notch signaling in the CM blocked differentiation and nephron formation on the other hand (Chung, Deacon, and Park 2017). Of note, the activation of Notch signaling in the CM of Wnt4-/-;Wnt9b-/- double null mice induced MET (S. C. Boyle et al. 2011); however, this was not the case when Notch signaling was activated in stromal cells (Jianing Liu et al. 2010). Therefore, the epithelialization process in response to Notch signaling is attributed to a distinct property of CM cells rather than a general effect.

Besides its interaction with Eya1, Six2 associates with Osr1 and both proteins act synergistically as a complex to drive transcriptional repression of target genes that are required for Wnt-dependent differentiation during metanephros development. Similar to Eya1 mutants, Osr1 conditional mutants recapitulated the phenotype seen after *Six2* deletion, therefore leading to premature progenitor depletion and renal hypoplasia in the CM (Jingyue Xu et al. 2014). Taken together, the above results highlight a central model by which Six2 interacts with various transcription factors and regulates the expressions of key downstream target genes in order to maintain a balance between progenitor proliferation (through its interactions with Eya1 and Osr1) and CM differentiation (via modulating Notch signaling).

Finally, fate-mapping studies have reported the expansion of the Six2+ population by 15 folds along the period of nephrogenesis, and identified this population as being the precursor to all epithelial components of the nephron. This renders Six2+ cells an attractive cell type to maintain or regenerate nephrons in the context of regenerative

medicine (Akio Kobayashi et al. 2008). While Six2 expression disappears shortly after birth with the subsequent differentiation of the remaining CM cells into nephrons, it was suggested that there is a possibility of restoring the CM state in adult epithelial cell(s) through the induced re-expression of important CM transcription factors such as Six2 (Hendry et al. 2013). The feasibility of this suggestion as well as the regenerative potential of generating such a multipotent progenitor population in the context of the adult kidney warrants further investigation. Nevertheless, preliminary data showed that the ureteric bud compartment, at least around P3, can still respond and interact with an embryonic CM (Hartman, Lai, and Patterson 2007).

3. Essential role of Sall1 during kidney formation

Sall1 is a transcription factor and member of the zinc-finger family that is essential for kidney development. The expression of Sal1 in the early MM is required for UB recruitment through a mechanism involving the expression of *kif26b*. In fact, it was shown that *kif26b* deletion downregulates the expression of integrin α8 in the MM surface; as a result, loss of interaction between integrin α8 and its ligand nephronectin around the growing UB disrupts primary CM induction signal that is initiated by UB invasion into the MM (Linton, Martin, and Reichardt 2007). As such, Sall1-/- mouse embryos exhibit renal agenesis (Nishinakamura et al. 2001).

In an attempt to study the role played by Sall1 later on during metanephric development, studies used Six2-Cre to induce a conditional deletion of Sall1 in the NPC population. As a matter of fact, Sall1 is expressed in both Six2+ progenitors and Six2- nascent nephrons (differentiating structures including RV and S-shaped bodies, as well as, tubules of the mature nephron). Therefore, loss of Sall1 in the CM led to progenitor pool depletion and cell death in the differentiating nephron structures. Microarray

analysis identified two categories of downstream target genes of Sall1: the target genes activated by Sall1 such as the pro-self-renewal genes including Six2, myc, Cited1 and Eya1. The second set of genes is comprised of pro-differentiation genes, that are repressed by Sall1 through its interaction with the NuRD (nucleosome remodeling and deacetylase) complex (Kanda et al. 2014; Basta et al. 2017).

Furthermore, CM-specific Sall1 knockouts phenocopies the Six2 deletion. Sall1 and Six2 share common target genes including Six2, Eya1 and Osr1. Sall1 activates the expression of these genes in order to maintain the pool of NPCs. However, as mentioned above, Sall1 is also found to function as a potent transcriptional repressor of differentiation genes through its interaction with the NuRD complex. Six2-Cre specific deletion of the Mi-2β domain of the NuRD complex resulted in hypoplastic kidneys and this phenotype was attributed to reduced Six2 and Cited1 expressions and a decrease in NPC proliferation (Denner and Rauchman 2013). To investigate the significance of the Sall1-NuRD complex interaction, a mutation in the Sall1 domain by which it interacts with the NuRD complex was generated. This disrupted Sall1-NuRD cooperation and resulted in accelerated differentiation. In turn, this caused premature depletion of the nephron progenitor pool leading to renal hypoplasia (Basta et al. 2017).

4. Role of Osr1 during renal formation

The Odd-Skipped Related Transcription Factor 1, Osr1, is essential for early MM specification as previously described. In Osr1 knockout embryos, the MM undergoes severe apoptosis as a consequence of disrupted UB invasion (Q. Wang et al. 2005). Moreover, NPC-specific Osr1 deletion caused premature progenitor differentiation. ChIP-seq studies revealed that Osr1 is a downstream target of Six2, and shown to

repress *Wnt4* expression in un-induced NPCs via binding and activating the TCF-Groucho transcriptional repressor complex (Jingyue Xu et al. 2014).

5. Role of Pax2 in the commitment to a nephronic fate

The Paired box gene 2 transcription factor, Pax2, is primarily required to restrict progenitor commitment to the nephron lineage by preventing interstitial cell fate (Naiman et al. 2017). Hence, loss of Pax2 in NPCs leads to a switch in their fate, thus promoting their differentiation into interstitial cell types instead of nephron cells (Naiman et al. 2017). Of note, potential lineage plasticity has been previously reported during early renal development whereby Foxd1+ stromal cells are found to slightly contribute to the Six2+ CM (Kobayashi et al. 2014).

F. Termination of nephrogenesis

In the mouse, nephrogenesis ceases around P4 when all NPCs have been exhausted through differentiation into nephrons that are responsible for the adult kidney function. The generation of the right number of mature nephrons is critical given that aberrant nephron endowment resulting from defective kidney development is associated with renal diseases in human. Concomitant with NPC depletion, *Six2* expression is significantly decreased between P1 and P4 while *Wnt4* expression is increased, thus supporting rapid NPC differentiation and nephron generation during the terminal rounds of nephrogenesis (Brunskill et al. 2011; Rumballe et al. 2011). Transcriptomic profiling studies of NPCs revealed downregulation of glycolysis genes during postnatal stages suggesting the involvement of cellular metabolic shifts in terminating nephrogenesis (Brunskill et al. 2011). Furthermore, it was shown that the progressive increase in the oxygen levels and the nutrient supply that is associated with elevated vascularization of the renal CM support progenitor differentiation (Rymer et al. 2014). Altered niche

morphology is also proposed to drive termination of nephrogenesis. In fact, a gradual decrease in the size of UB tips and the CM is observed as development progresses, and when the CM size decreases below a certain threshold, it induces a terminal wave of nephron induction followed by cessation of nephrogenesis (Zubkov et al. 2015). Finally, studies implicated a role for the BMP7 and FGF signaling pathways in the shift of the nephrogenic niche towards rapid differentiation and nephrogenesis termination. Hence, BMP-SMAD signaling was found increased between P1 and P4, which helped trigger a shift toward differentiation (Brown, Muthukrishnan, and Oxburgh 2015). High expression levels of FGF9 and FGF20 makes the niche in favor for an undifferentiated state of the CM (Barak et al. 2012). It is proposed that reduced FGF20 expression contributes to altering the aging niche in support of rapid differentiation (S. Chen et al. 2015; Oxburgh 2018). Hence, engrafting postnatal CM (late CM with postnatal NPCs) into a younger CM (e.g. E12.5) showed that older NPCs are more vulnerable to rapidly exit the progenitor pool and differentiate when placed in a younger niche (S. Chen et al. 2015). Moreover, transplantation of NPCs to an FGF20 deficient niche suggest a potential role of reduced FGF20 to the age-related alterations in NPCs (S. Chen et al. 2015).

G. Dynamic nature and plasticity of NPCs

Temporal and spatial profiling studies using high-resolution imaging and cell quantitation highlighted the dynamic nature of CM cells surrounding the UB tips across development (Lawlor et al. 2019; Combes et al. 2016; Short et al. 2014). In mouse, the rate of UB branching as quantified by the number of ureteric tips budding into the surrounding peripheral CM decreases with time until it stabilizes at E15.5, after which the branching events persists but at a slower rate. At the latter stage, two nephrons are

generated from each CM-UB niche, and this rate remains constant until nephrogenesis ceases (Short et al. 2014). Moreover, the ratio of NPCs/UB tip cells (estimated to be 2:1) in a given niche is dependent on the cell cycle and proliferation rates of NPCs. In addition, within the same niche, regionally specified Six2+ NPCs are reported to exhibit distinct cell cycle rates. Hence, slow-cycling cells expressing high levels of Six2 occupy more peripheral regions closer to the cortex and are considered the self-renewing population, while fast- cycling cells expressing lower levels of Six2 are located lateral to the UB tip and represent the progenitor population that is preferentially induced to differentiate (Short et al. 2014). Live time-lapse imaging of NPCs in kidney explants indeed showed that these cells exhibit a highly dynamic spatial and temporal distribution, and are capable of re-arrangement around the UB tip in response to attenuations in UB branching. Hence, with each round of UB branching, NPCs re-arrange in response to cues within the nephrogenic niche with a potential involvement of the repulsive Robo2-Slit1 signaling in the regulation of this process (Wainwright et al. 2015). Moreover, NPC migration involves changes in cellular adhesion and requires a combination of attractive and repulsive guidance cues to direct their movement. Studies have shown that loss of Kif26b (an intracellular motor protein of the large kinesin family) (refer to **section 2.2.3**), and, p53 (refer to **section 2.2.4**) resulted in defective adhesion and failure of NPCs to successfully attach to the UB, thus highlighting the function of these proteins in controlling NPC motility (Yuwen Li et al. 2015; Uchiyama et al. 2010). NPC plasticity is proposed in a study using the Wnt4-Cre inducible mouse line, where GFP is fused to CreERT2 and is under the control of the Wnt4 promoter. This study reported re-entry of primed Wnt4 lineage cells to the progenitor domain where they cease expressing Wnt4 and re-shift their transcriptional

identity to the gene profile of cells in their uninduced state. Furthermore, this study proposed a mechanism governing NPC commitment by which random cell migration is suggested to affect the duration of exposure to inductive zone-specific cues and this establishes whether the cells progress to differentiate or migrate back to the cap. In other words, if the uninduced cell gets exposed to differentiation cues long enough before it migrates back to more apical zones of the cap, it exits the NPC pool and undergoes differentiation. However, if it spends a short time in the induction zone and migrated back quickly, it will remain within the NPC pool (Lawlor et al. 2019). In summary, the described plasticity and dynamic nature of NPCs is important to ensure proper maintenance of an adequate number of undifferentiated CM cells located around each UB tip, which in turn is needed for optimized completion of UB branching and nephrogenesis (Combes et al. 2016).

H. Characterization of Nestin expression in the developing and the adult kidney

1. Nestin expression in the mouse kidney

Nestin is an intermediate filament protein widely known as a neural stem and progenitor cell marker but was later shown to be also expressed in renal progenitors. A study by Dubois et al. 2006 was the first to report Nestin expression in the developing and adult kidneys by examining Cre-mediated recombination in Nestin-Cre1 transgenic mice (Dubois et al. 2006). Further evidence from cell lineage studies using the same Nestin-Cre1 line revealed specific Nestin expression throughout the MM compartment excluding UB structures in mice. Hence, Nestin expression was detected in Cited1+ CM progenitors starting at E11.5 as well as in CD31+ and Flk1+ endothelial cells of the immature glomeruli during development (J. Chen et al. 2006). After birth, Nestin

expression becomes restricted to the podocytes and associated with CDK5 in mature glomeruli in the adult kidney (J. Chen et al. 2006; Bertelli et al. 2007a).

2. Nestin expression in the human kidney

Nestin expression is detected in a variety of cells belonging to distinct lineages in the developing human kidney including the renal vesicles, the podocyte layer as well as endothelial cells of S-shaped bodies and mesangial progenitors (Bertelli et al. 2007a). Moreover, it was proposed that, under damage conditions, the induced increase in Nestin expression stimulates mesangial cell proliferation (Daniel et al. 2008) and promotes proximal tubule cell migration during the repair process (Wen et al. 2012). Indeed, a more recent study reported the presence of a reservoir of Nestin+ mesenchymal stem cells (MSCs) in the postnatal kidneys. These Nestin+ cells were shown to have a reparative potential when injected into murine models of acute ischemic kidney injury (M. H. Jiang et al. 2015).

I. Role of the p53-Mdm2 pathway during kidney development

Previous studies demonstrated that a tight control of p53 level of expression is essential for proper renal development (reviewed in (S. S. El-Dahr et al. 2014)). The regulation of p53 expression and therefore its activity is primarily under the control of its negative regulator Murine Double Minute-2 (Mdm2), which binds to and restrains p53 transcriptional activity.

1. Consequences associated with Mdm2 loss or p53 overexpression in the developing kidney

Cell-type specific functions of p53 and Mdm2 in the metanephric mesenchymal and nephric duct lineages during kidney development were thoroughly investigated. For instance, specific deletion of Mdm2 in the ureteric lineage severely affected renal formation. Thus, $UB^{mdm2-/-}$ kidneys were hypo-dysplastic and displayed abnormal UB

branching as well as a reduced number of UB tips associated with decreased expression levels of key nephrogenic genes including Wnt9b, Lhx1 and Pax2. Consequently, this decrease in Wnt signaling between the UB and CM resulted in a three-fold increase in apoptosis in the CM and disruption of nephrogenesis (Hilliard et al. 2011). In comparison, specific deletion of Mdm2 in NPCs caused embryonic lethality, whereby $Six2^{mdm2-/-}$ kidneys were hypo-dysplastic with reduced CM size hosting less Six2+/Cited1+ self-renewing NPCs, and showed severely reduced expressions of Sall1, Eya1 and Bmp7 (S. S. El-Dahr et al. 2014). The phenotypic defects in both studies described above were associated with elevated levels of p53 given that p53 deletion in both the ureteric bud and in NPCs ($UB^{mdm2-/-}$ and $Six2^{mdm2-/-}$) restored postnatal survival as well as a normal renal phenotype in these mice (Hilliard et al. 2011; S. S. El-Dahr et al. 2014). Consistent with Mdm2 loss of function, the generation of transgenic mice overexpressing p53 resulted in hypoplastic kidneys, nephron deficiency as well as UB differentiation defects (Godley et al. 1996). In conclusion, these studies clearly indicate that inhibition of p53 function by Mdm2 and the fine-tuning of p53 protein levels during distinct stages of renal development is required for normal kidney morphogenesis and maintenance of the self-renewing Six2+ population inside the CM.

2. Effects of p53 loss on renal morphogenesis

p53 germline deletion is associated with an incompletely penetrant renal phenotype that is dependent on the mice genetic background, and is most frequently manifested by hypoplasia, nephron deficit and formation of double ureters (Saifudeen et al. 2009; Y. Li et al. 2015). Among the key mechanisms that mediate the function of p53 in the developing kidney is the upregulation of Pax2 expression, a key regulator of nephron differentiation and UB branching. Thus, in p53-/- kidneys, Pax2 is significantly

downregulated resulting in nephron deficit (Saifudeen et al. 2012). In another study, Six2-Cre driven deletion of p53 in NPCs led to the formation of smaller kidneys, nephron deficit, and dispersed cap mesenchymal cells as early as E13.5. Strikingly, $Six2^{p53-/-}$ exhibited progressive depletion of the Cited1+/Six2+ population with a prolonged cell cycle, suggesting a decrease in their self-renewal capacity without any obvious increase in apoptosis (Y. Li et al. 2015). A pronounced loss of cellular adhesion is also reported in the p53 mutant CM and is linked to a severe reduction in NCAM expression. This led, as a result, to the loss of the CM-stromal interface, and is believed to affect the response to extrinsic regulatory signals generated from the surrounding extracellular matrix within the niche and that are essential for NPC maintenance. Additionally, NPCs lacking p53 failed to differentiate normally as shown by the decrease in their E-cadherin and FGF8 levels of expression. Subsequent transcriptome analyses reported altered expressions of important metabolic genes, therefore attributing the renal phenotype to reduced metabolic fitness that compromised self-renewal in the population of Cited1+;p53-null stem cells (Y. Li et al. 2015).

In summary, NPC depletion was shown to be a consequence of both loss and gain of p53 function, however, distinct mechanisms seem to be responsible for this depletion in each case; on the one hand, reduced proliferation and increased apoptosis when p53 is overexpressed, and, disrupted cellular metabolism following loss of p53 on the other hand.

Of note, the role of the p53-Mdm2 pathway in the stroma is not yet investigated. Given the complex interplay of growth and differentiation signals between the CM, UB and the stroma, it is of great importance to characterize the renal phenotype when p53 is

deleted in the stromal lineage including endothelial, interstitial and glomerular mesangial cells.

J. Role of the Retinoblastoma protein, Rb, and the Rb-E2F pathway during development

1. Classical role of Rb in the control of cell proliferation at the G1-S phase checkpoint

The Retinoblastoma gene, Rb, is the first known tumor suppressor gene to be identified in human (Huang et al. 1988) and found dysfunctional in many pediatric and adult human cancers (Dunn et al. 1988). The Rb protein is very well-known for its regulatory role in controlling cell cycle progression at the G1-S phase restriction point through its specific interaction with members of the E2f family of transcription factors (Sherr 1994; Hamel, Gallie, and Phillips 1992).

Inactivation of Rb involves cyclin-dependent kinases (CDKs) and cyclin complexes (Genovese et al. 2006). These complexes modulate the phosphorylation status of Rb whereby CDK4/6-cyclin D complexes bind and phosphorylate Rb, then additional phosphorylation by the CDK2/cyclin E complex renders Rb in a hyper-phosphorylated and inactive state. Hypo-phosphorylated/active Rb remains associated with E2F transcription factors, thereby favoring degradation of cyclin E and blocking S phase entry (Serrano, Hannon, and Beach 1993; Kitagawa et al. 1996). Additional cell cycle regulation is mediated via the INK4 and CIP/KIP families of inhibitors including p16 and p21, respectively.

2. Family protein members: p107 and p130

The Rb protein family constitutes of three members: pRb (less commonly known as p105), p103 (or Rb2) and p107 (or Rb1*) (*Hannon et al. 1993; Li et al. 1993; Zhu et al. *1993).* Rb proteins share sequence homology in their pocket domains by which they

bind E2f transcription factors (Ewen et al. 1991; Hannon, Demetrick, and Beach 1993; Li et al. 1*993;* Trimarchi and Lees 2002*)*. However, they were shown to differ in their binding affinity to E2fs. For example, Rb interact with E2fs 1-5, however, p107 and p130 form complexes only with E2f4 and E2f5 (Lees et al. 1993; Hijmans et al. 1995; Moberg, Starz, and Lees 1996; Dyson 1998; Trimarchi and Lees 2002*;* Kong et al. 2007). In vivo studies showed that these different complexes contribute to timed regulation of S-phase entry. Rb proteins operate in a sequential manner during the cell cycle: p130 associates with E2fs 4 and 5 in G0, p107 binds E2f4 in G1, however, Rb complexes with E2f4 in the S phase (Shirodkar et al. 1992; Cobrinik et al. 1996; Dyson 1998; Dimova and Dyson 2005). Rb also interacts with E2f1-3 to control entry into S-phase as previously stated. E2f6-8s are Rb-independent repressors (de Bruin et al. 2003; Logan et al. 2004; Logan et al. 2005; Maiti et al. 2005; Christensen et al. 2005). The Rb family members were shown to exhibit different functions in general, however, studies in Rb mutant mice demonstrated also overlapping and partially redundant roles among them in specific contexts.

3. Distinct and context-specific roles of Rb beyond cell cycle control

Numerous studies uncovered various regulatory roles of the Rb-E2F pathway that extend beyond its traditional function in cell cycle control to include direct regulation of cell differentiation, migration and survival, specifically of progenitor cell populations in various tissues. Hence, phenotypic studies revealed a wide range of context-dependent regulatory functions played by Rb during embryogenesis and postnatal tissues as summarized in **Table1**. For instance, in the developing retina, loss of Rb resulted in defective differentiation of cholinergic neurons, a phenotype that was shown to be directly mediated by a specific interaction of Rb with E2f3 and independent from its

role in cell cycle control (Danian Chen et al. 2007). In addition, *in vitro* studies showed that Rb controls cellular quiescence in skin precursor cells and satellite cells that are involved in muscle repair post-injury. In satellite cells, loss of Rb was shown to cause an expansion of the myoblast progenitor pool (Hosoyama et al. 2011) along with inhibition of differentiation (Huh et al. 2004). Moreover, conditional deletion of Rb in stratified epithelia of the skin resulted in hyperkeratosis (thickening of the outermost layer of the epidermis) in addition to severe perturbations in terminal epidermal differentiation. Of note, the severity of these defects was increased following co-deletion of p107 (a member of the Rb family of pocket proteins), indicating a partial compensatory role of this pocket protein in the absence of Rb (Ruiz et al. 2004). Similar to its role in the skin, Rb was shown to be required to maintain cell quiescence in many systems such as in MEFs (Sage et al. 2003), muscle cells (Pajcini et al. 2010) and adult cortical neurons (Matthew G Andrusiak et al. 2012). As such, the inactivation of Rb is sufficient to drive cell cycle re-entry in these various tissues. Additionally, Rb was found to suppress re-programming of mouse embryonic fibroblasts into induced pluripotent stem cells through a mechanism that is independent from cell-cycle regulation. Hence, Rb is shown to promote the association of repressive chromatin on pluripotency genes, including: Sox2 and Oct4 (Kareta et al. 2015).

During brain development, the Rb-E2F pathway was shown to play important roles in the control of progenitor proliferation as well as in the onset of neuroblast differentiation and migration such as in the telencephalon via the direct regulation of *Dlx1* and *2* transcriptional activities. In the latter example, the absence of Rb led to increased expression level of the transcriptional repressor E2f-7, which transcriptionally repressed *Dlx*1/2 gene expression, blocking terminal differentiation (Ghanem et al.

2012). Using telencephalon-specific Rb mutant mice, Ferguson et al. assessed the role of Rb during cortical neurogenesis and showed that Rb-cKO (conditional knock out) mice exhibited enhanced neurogenesis that is manifested by ectopic proliferation of committed neuroblasts as well as cortical lamination defects (Ferguson et al. 2002). Similarly, conditional Rb deletion in the retina caused ectopic division of differentiating precursors (Danian Chen et al. 2004). Furthermore, Rb was shown to play an essential cell-autonomous role in tangential migration of cortical neuroblasts by regulating the expression of neogenin (M. G. Andrusiak et al. 2011). In the developing hippocampus, Rb plays a consistent role as in the cortex, hence its loss resulted in dentate granule cell (DGC) expansion (Vandenbosch et al. 2016). Furthermore, our laboratory showed that loss of Rb induced enhanced and 'ectopic' proliferation of immature olfactory sensory neurons in the olfactory epithelium, and severely compromised their survival and terminal maturation (Jaafar et al. 2016). Other studies show that Rb is required for survival of specific cell types such as ganglion and photoreceptor cells in the developing retina (Danian Chen et al. 2004). Hence, a conditional Rb deletion in the developing cerebellar vermis precursor cells caused increased cell proliferation and apoptotic cell death affecting specifically granule cell precursors (Marino et al. 2003). Taken together, the above studies demonstrate tissue-specific and direct requirement(s) for Rb in the regulation of various cellular functions that extend beyond cell cycle control.

In the adult brain, our laboratory uncovered a critical requirement of Rb during adult neurogenesis in the adult subventricular zone (SVZ) lining the lateral ventricle and the subgranular sone (SGZ) inside the dentate gyrus in the hippocampus. Concordant with its role in the developing cortex, Rb does not control self-renewal of adult neural

stem cells (aNSCs); however, it specifically regulates neuronal progenitor proliferation in the SVZ and the rostral migratory stream (RMS) (Naser et al. 2016). Unexpectedly, Rb conditional deletion (using Nestin-CreERT2-YFP; $Rb^{floxed/floxed}$) in activated Nestin+ stem and progenitor cells did not seem to affect neuroblast differentiation and migration, however, it severely compromised long-term survival of newborn adult neurons in the olfactory bulb (OB) (Naser et al. 2016). We obtained similar results in the adult SGZ where loss of Rb was associated with increased proliferation of immature dentate granule neuroblasts, and negatively impacted short-term survival of newborn neurons (Vandenbosch et al. 2016).

Given all of the above, it is clear that, besides its main role in cell cycle progression (cell division), Rb plays a wide range of other regulatory roles governing progenitor cell differentiation, maturation and/or survival in diverse tissues in a context-specific manner (**Table 1**). However, whether Rb is required for proper kidney development, including nephrogenesis has not been addressed to date.

K. Crosstalk between the Rb and p53 pathways in several contexts

The cell cycle regulatory pathways under the control of Rb and p53 are inter-dependent; for instance, damage-induced p53 activation leads to inhibition of cyclin dependent kinase 2 (Cdk2)/cyclin E complex through the action of its downstream effector p21. In turn, this p53-mediated Cdk inhibition by p21 prevents Rb phosphorylation, thus preserving its association with E2F transcription factors and thereby arresting the cell cycle (Sherr and McCormick 2002). Indeed, several studies suggest the presence of strong crosstalk between the Rb and p53 pathways in the regulation of cell cycle processes including cell proliferation, cell cycle exit and survival. **Table 2** summarizes the studies characterizing the phenotypic defects resulted from dual inactivation of Rb

and p53 in distinct cell types and tissues. The crosstalk between these tumor suppressor pathways in several types of tissue prompted us to identify further the effect of combined Rb/p53 deficiency in the developing kidney.

L. Role of Rb in kidney development and function

The expression levels of the various CDKs and cyclins are reported to change dynamically in a temporal pattern during renal development (S. K. Park et al. 1997). Moreover, studies have linked deregulated expressions and activities of cell cycle-regulatory proteins with kidney cell-specific diseases such as acute kidney injury (AKI), glomerulophathies and chronic kidney failure (reviewed in (Canaud and Bonventre 2015; Thomasova and Anders 2015)). For instance, the expression levels of cyclin D1 and D3, as well as CDK2 and CDK4 were found to be upregulated during renal tubule regeneration following post-ischemic renal injury. In addition, mesangial cell proliferation in Thyl glomerulonephritis mouse models was associated with increased expressions and activities of CDK2 and p21 (Shankland 1997; Thomasova and Anders 2015; Canaud and Bonventre 2015; Shankland et al. 1997). The Rb protein is a critical determinant of cell proliferation and hypothesized to be controlling glomerular hypertrophy in the developing kidney. A study reports the changes in the phosphorylation status of Rb during mesangial and tubular cell proliferation in normal and pathological conditions (Shankland 1997). Yet, direct evidence about the specific role of the Rb pathway and its downstream targets during renal development and in kidney disease is still lacking.

M. Rationale, Hypothesis and Specific aims

We generated tamoxifen-inducible Nestin-CreERT2-YFP p53-/-; $Rb^{fl/fl}$ mice (mixed FVBN x C57BL6 x129/Sv background) by crossing p53 null mice (p53-/-) and Rb

floxed/floxed ($Rb^{fl/fl}$). The Rb floxed allele has exon 19 flanked by two LoxP sites (Marino et al., 2000). Nes-CreERT2/Rosa26R-YFP/YFP mice (designed by Lagace et al., 2007) carry the inducible Nes-CreERT2 cassette, which includes the Nestin promoter combined with exons 1-3 of the Nestin gene, and controls the expression of the Cre recombinase enzyme fused with ERT2 (a mutated estrogen receptor). Upon tamoxifen administration, Cre translocates to the nucleus and excises genes that are flanked by two LoxP sites. Preliminary data was generated from two Nestin-CreERT2; p53-/-; $Rb^{fl/fl}$ mice that were treated at E18.5 and sacrificed at P40. These two animals exhibited reduced body size and weight overall, a 2-fold dramatic decrease in kidney size, and elevated levels of creatinine by 5-6 folds as compared to wild type and non-treated Nestin-CreERT2; p53-/-; $Rb^{fl/fl}$ mice, suggesting the presence of severe failure in kidney function in the absence of both Rb and p53. Of note, p53-/- mice did not exhibit any major renal defects at least in the mixed genetic background used here.

Given the main role of Rb in cell cycle progression, its other regulatory roles during progenitor cell development in diverse tissues, and the preliminary data described above in p53null; Rb-cKO mice, we **hypothesized** that Rb plays an essential role in kidney development and may be required for the control of nephron progenitor proliferation and/or survival in immature nephrons. We also propose the presence of potential crosstalk between the Rb and p53 pathways that coordinates one or more of these developmental processes during nephrogenesis in order to achieve proper kidney development.

We set **three aims** in this project. The first aim is to investigate a potential role for Rb in regulating renal development, particularly nephrogenesis by using tamoxifen-inducible Nestin-CreERT2-YFP/YFP mice, and, $Rb^{fl/fl}$ mice, to induce conditional

deletion of Rb targeted to Nestin-positive cells and their progeny in the developing kidney. Then, we will perform histological analyses and gene expression studies to examine the renal phenotype in Nestin-CreERT2- $Rb^{fl/fl}$ mutants (referred to as Rb-/-) in comparison with $Rb^{fl/+}$ (referred to as Rb+/-) and wild-type control (referred to as Rb+/+) mice at distinct developmental stages. The second aim is to investigate the combined roles of Rb and p53 in regulating renal development and nephrogenesis by inducing a conditional deletion of p53, and, combined deletions of Rb and p53, then to characterize the renal phenotypes in these animals. The third aim is to identify potential changes in gene expression affecting Rb and/or p53 target genes involved in kidney development by quantitative Real-time (qRT-PCR); this will help uncover the molecular mechanisms underlying the roles of both pathways during renal development.

Embryonic/Adult	Stem/Progenitor Cell Types	Effect(s) of Rb deletion	Reference(s)
Embryonic	Retinal Progenitors	Ectopic proliferation; Failed cell-cycle exit in progenitors and elevated cell death of specific differentiated cell populations	(MacPherson et al. 2004; Danian Chen et al. 2004; J. Zhang et al. 2004)
	Developing Neural Progenitors	Ectopic proliferation of cortical neuroblasts, cerebellar granule cells and immature olfactory sensory neurons (OSNs); increased apoptosis of cerebellar granule progenitors and mature OSNs; blocked neuroblast migration and differentiation in the cortex	(Ferguson et al. 2002; M. G. Andrusiak et al. 2011; Ghanem et al. 2012; Jaafar et al. 2016)
	Spermatogonial Stem Cells	Defective self-renewal and rapid depletion of the spermatogonial stem cell pool	(Hu, de Rooij, and Page 2013)
	Bone Progenitors	Cell cycle exit defects and impaired osteoblast differentiation	(Flowers, Xu, and Moran 2013)
	Intestinal Progenitors	Increased proliferation; Delayed cell cycle exit; Increased Hedgehog signaling; Aberrant differentiation	(Yang and Hinds 2007)
	Pluripotent Stem Cells	Reprogramming of differentiated cells to iPSCs	(Kareta et al. 2015)
Adult	Muscle Progenitors	Cell cycle re-entry; Expansion of myoblast pool; Increased progenitor cell death; Reduced differentiation	(Hosoyama et al. 2011)
	Skin stem cells	Increased Proliferation; Aberrant differentiation	(Ruiz et al. 2004)
	Adult Neural Stem and Progenitor Cells	Enhanced progenitor proliferation; No effect on neuroblast migration or differentiation; loss of long-term survival of adult-born neurons; No effect on stem cell self-renewal	(Vandenbosch et al. 2016; Naser et al. 2016)
	Hematopoietic Stem Cells	Impaired cell cycle exit under stress conditions	(Daria et al. 2008)

Table 1 Summary of the major phenotypes resulting from Rb inactivation in various cell/tissue types during development and in the adult.

Embryonic/ Adult	Cell Types	Effect(s) of combined deletions of Rb and p53	References
Adult	Mesenchymal stem cells	Expansion of $Sca1^+$ (stem cell marker) multipotent mesenchymal progenitors, blocked terminal differentiation, osteosarcoma development	(Berman et al. 2008; Choi et al. 2010)
	Adult Cerebellar granule progenitors	Ectopic and prolonged proliferation, delayed differentiation and apoptotic response resulting in genomic instability	(Shakhova et al. 2006)
	Adult neural progenitors	Increased proliferation and no effect on differentiation *in vitro*	(Halaby N, Saliba A and Ghanem N, et al. in preparation)
	Mammary progenitors	Development of aggressive EMT-type tumors from bipotent progenitors	(Z. Jiang et al. 2010)
	Prostate stem/progenitor cells	Growth of tumor lesions of luminal and neuroendocrine origins consisting of atypical cells expressing the proliferation marker Ki67 and Sca1	(Zhou, Flesken-Nikitin, and Nikitin 2007)
	Renin-expressing cells	Development of pancreatic neuroendocrine carcinoma	(Glenn et al. 2014)
Embryonic and adult	Liver hepatocytes	Elevated levels of PCNA, down-regulation in the expression of genes related to metabolic pathways, Increased DNA ploidy (large nuclei)	(McClendon et al. 2011)

Table 2 Summary of the major phenotypic defects resulting from the combined deletions of Rb and p53 in various cell types/tissues during development and in the adult.

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CHAPTER 2

II. STUDY OF THE ROLE OF RB AND, THE COMBINED ROLES OF RB AND P53 PATHWAYS, DURING KIDNEY DEVELOPMENT

A. Abstract

Deficit in mature nephrons results in chronic kidney disease (CKD), a major health problem worldwide. Hence, generation of an accurate number of functional nephrons is a critical developmental process that necessitates a refined balance between nephron progenitor cells (NPCs) self-renewal/proliferation and terminal differentiation/maturation. The Retinoblastoma protein, Rb, and the p53 pathways play key roles in the control of cell proliferation, senescence and apoptosis in many organs. Previous studies have uncovered a requirement for p53 in the regulation of nepron stem cell self-renewal and their terminal differentiation. However, the role of Rb during kidney development remains unknown. Here, we have crossed tamoxifen-inducible Nestin-CreER^{T2}-YFP-YFP mice with single and/or double floxed/floxed ($^{fl/fl}$) Rb and/or p53 mice, to induce single or double conditional deletion(s) in these genes that are targeted to Nestin-positive cells and their progeny. As a result, we report for the first time a specific role for Rb in the control of NPCs' proliferation during development, and importantly, a critical requirement for Rb in survival of newborn nephrons around birth. Hence, we show that loss of Rb leads to enhanced nephrogenesis associated with increased NPCs' proliferation (but not stem cells), and subsequent differentiation; however, this was counterbalanced with severe cell survival defects around birth. Furthermore, consistent with previous literature, we have identified multiple renal defects in p53$^{fl/fl}$ treated embryos including renal hypoplasia and pronounced differentiation defects leading to nephron deficit. Unexpectedly, combined deletions of both genes did not rescue the

survival defects observed in the absence of Rb; rather, it exacerbated them and gave rise to a more severe hypoplasia with almost complete loss of immature glomeruli at birth. These findings are the first to highlight the role of Rb during kidney development, and the existence of distinct as well as synergistic functions attributed to the Rb and p53 pathways that are indispensable for proper kidney development.

B. Introduction

The mammalian kidneys are fully derived from the intermediate mesoderm (IM). The dorsal IM known as the nephric duct (ND) secretes signals that induce the commitment of the ventral IM at the level of the hind limb to form the metanephric mesenchyme (MM). Reciprocal signals emanating from the MM stimulate an outgrowth from the ND called the ureteric bud (UB) which invades the MM and initiates nephrogenesis (Grobstein 1955; Little and McMahon 2012). Once IM cells commit to the nephron lineage, they activate the expression of the homeobox transcription factor, Six2 that primarily maintains NPC self-renewal throughout kidney development until termination of nephrogenesis (Mugford, Sipilä, McMahon, et al. 2008). In the developing kidney, self-renewing (Six2+;Cited1+) nephron progenitor cells (NPCs) are condensed closest to the UB tip and form the cap mesenchyme (CM) (S. Boyle et al. 2008; Kobayashi et al. 2008), from which all epithelial segments of the nephron arise via a mesenchyme-to-epithelial transition (MET) (Costantini and Kopan 2010). While Cited1 deletion is shown not to cause any renal defects (S. Boyle et al. 2008), Six2 deletion causes premature CM depletion and ectopic epithelialization (Self et al. 2006a). Within each UB-CM niche, a subset of the (Six2+;Cited1+) population becomes (Six2+;Cited1-;Wnt4+) and forms pre-tubular aggregates (PA), which generate epithelial structures known as renal vesicles (RV). RV undergo elongation and segmentation to give rise to comma-shaped bodies (CSBs) then S-shaped bodies (SSBs). Finally, the SSBs further elongate and differentiate into functional nephrons (Bard et al. 2001). The most proximal component of the nephron that filters the blood is the glomerulus; it occupies the external cortex of the adult kidney and consists of a capillary tuft surrounded by interdigitating foot processes known as podocytes (Quaggin and Kreidberg 2008). In

addition to the glomerulus, the mature nephron consists of a tubular epithelium that is essential for the modification of the glomerular filtrate. This tubular epithelium is comprised of the proximal tubules, the loop of Henle running through the medullary region of the kidney, the connecting segment and the distal tubules connected to the collecting duct system through which urine drains for disposal (Bulger and Hebert 1988). Nephrogenesis is critically regulated by niche signals in the nephrogenic zone to ensure proper nephron endowment. Iterative rounds of epithelialization generate nephrons throughout the developmental period in the mammalian kidney. Hence, part of CM is maintained (by self-renewal) and expanded to ensure enough NPCs are available to generate all the nephrons of the mature kidney over the entire period of development from E11.5 until P4 in mice, and between 15 weeks and 35 weeks of gestation in humans (Little 2015). The generation of the right number of mature nephrons is critical given that reduced number of nephrons resulting from defective kidney development is associated with chronic kidney disease (CKD) in humans (Boubred et al. 2013).

The Retinoblastoma, Rb, and p53 pathways are master regulators of cell division and senescence in many organs. The role of p53 in kidney development is well studied (Saifudeen, Dipp, and El-Dahr 2002; Godley et al. 1996; S. S. El-Dahr et al. 2014; Saifudeen et al. 2009; 2012; Y. Li et al. 2015). Hence, p53 was shown to be required for terminal differentiation of renal epithelial cells during development (Saifudeen, Dipp, and El-Dahr 2002). p53 germline deletion is associated with an incompletely penetrant renal phenotype that is dependent on the mice genetic background, and is most frequently manifested by hypoplasia, nephron deficit and formation of double ureters (Saifudeen et al. 2009; Y. Li et al. 2015). Similarly, $Six2^{p53-/-}$ (a specific deletion of p53 in NPCs) led to the formation of smaller kidneys, nephron deficit, and dispersed cap

mesenchymal cells as early as E13.5. In addition, $Six2^{p53-/-}$ embryos exhibit progressive depletion of the (Six2+;Cited1+) population coupled to a prolonged cell cycle, suggesting a decrease in their self-renewal capacity but without any obvious survival defects or increase in apoptosis (Y. Li et al. 2015). Subsequent transcriptome analyses performed on $Six2^{p53-/-}$ mice revealed the presence of altered expressions in important metabolic genes, therefore attributing the above nephron progenitor phenotype (compromised self-renewal) to reduced metabolic fitness (Y. Li et al. 2015).

Rb controls cell cycle progression at the G1-S phase restriction point through direct inhibition of E2f transcriptional activity (Sherr 1994; Hamel, Gallie, and Phillips 1992). Hypo-phosphorylated/active Rb remains associated with E2F1-3 transcription factors (activator E2Fs), thereby favoring degradation of cyclin E and blocking S phase entry (Serrano, Hannon, and Beach 1993; Kitagawa et al. 1996). Rb is inactivated when hyper-phosphorylated by complexes made of cyclins; cyclin-dependent kinases (CDKs) (Genovese et al. 2006). Hence, CDK4/6-cyclin D complexes bind and phosphorylate Rb, then additional phosphorylation by the CDK2/cyclin E complex renders Rb inactive. The INK4 and CIP/KIP families of CDK inhibitors including p16 and p21, respectively carry additional cell cycle regulation. In fact, damage-induced p53 activation leads to inhibition of cyclin dependent kinase 2 (Cdk2)/cyclin E complex by its downstream effector p21. In turn, this p53-mediated Cdk inhibition prevents Rb phosphorylation, thereby arresting the cell cycle (Sherr and McCormick 2002). It is reported that there is a tight temporal regulation in the expression levels of CDKs and cyclins throughout renal development. Hence, the expression levels of the various CDKs and cyclins change dynamically in a temporal pattern during renal development (Park et al. 1997). Moreover, studies have correlated deregulated expressions and

activities of cell cycle-regulatory proteins with kidney cell-specific diseases such as acute kidney injury (AKI), glomerulophathies and chronic kidney failure (reviewed in (Canaud and Bonventre 2015; Thomasova and Anders 2015)). For instance, the expression levels of cyclin D1 and D3, as well as CDK2 and CDK4 are found upregulated during renal tubule regeneration following post-ischemic renal injury. In addition, mesangial cell proliferation in Thyl glomerulonephritis mouse models is associated with increased expressions and activities of CDK2 and p21 (Shankland 1997; Thomasova and Anders 2015; Canaud and Bonventre 2015; Shankland et al. 1997). Besides the few studies above, the role of cell cycle proteins during renal development remains largely unknown. Moreover, Rb is a critical determinant of cell proliferation and hypothesized to be controlling glomerular hypertrophy in the developing kidney. One study have reported a change in Rb phosphorylation status during mesangial and tubular cell proliferation under normal and pathological conditions (Shankland 1997). Yet, direct evidence about specific role(s) of the Rb/E2F pathway and its downstream target genes during renal development and kidney disease is still lacking. Also, it is unknown whether there is crosstalk between the Rb and p53 pathways during kidney development as reported in various other tissues during development (Berman et al. 2008; Choi et al. 2010; Z. Jiang et al. 2010; Zhou, Flesken-Nikitin, and Nikitin 2007; Glenn et al. 2014; McClendon et al. 2011; Shakhova et al. 2006).

Here, we have investigated for the first time the role played by Rb in kidney development by inducing its conditional deletion in Nestin-positive progeny including NPCs. Our results uncovered a specific role for Rb in the control of NPCs proliferation. Importantly, Rb is required for the survival of immature nephrons and proper kidney function in a p53-independent manner. The combined deletions of both Rb and p53

exacerbated the developmental defects leading to severe kidney hypoplasia and massive loss in newborn nephrons.

C. Materials and methods

1. Mice and tissue preparation

All animals procedures including animal handling, maintenance and treatments were performed according to protocols approved by the "the Institutional Animal Care and Use Committee"-IACUC at AUB. Nestin-CreERT2/Rosa26R-YFP/YFP mice (designed by Lagace et al., 2007) were mated with p53 -/- (Jackson Laboratory; FVB.129-Trp53^{tm1Brn}), and with Rb floxed/floxed (fl/fl) and/or p53 floxed/floxed mice to generate conditional Rb and/or p53 deletions(s). Rb fl/fl females were bred and maintained on a mixed FVBN- C57/Bl6 genetic background, while the p53 fl/fl females carried the Sv/126 background. The Rb floxed allele has exon 19 flanked by two LoxP sites (Cre-specific sites) (Marino et al., 2000). P53 floxed mice have LoxP sites flanking exons 2-10 (The Jackson Laboratory, B6.129P2-*Trp53^{tm1Brn}*/J). The inducible Nestin-CreERT2 cassette includes the Nestin promoter combined with exons 1-3 of the nestin gene, which controls the expression of the Cre recombinase enzyme fused with ERT2 (mutated estrogen receptors). Tamoxifen, an estrogen analog, binds to ERT2 allowing Cre translocation to the nucleus where it excises the genes flanked by two LoxP sites including the Rb and/or p53 floxed alleles, and the stop codon of the YFP reporter gene (used to assess recombination efficiency as internal control).

Pregnant females were given single tamoxifen treatments by oral gavage (at E10.5 or E13.5) and according to body weight (180 mg/kg; Tamoxifen Citrate; abcam cat# ab120656, prepared at 44.8 mg/ml in 60% sunflower oil and 40% DMSO). E0.5 corresponds to the day when a vaginal plug was observed. Moreover, pregnant females received a single intraperitoneal BrdU injection 2h prior to sacrifice. All animals were anesthetized with a mixture of 1.5µl/g Ketamine and 0.25µl/g Xylasine followed by

cervical dislocation. Embryos were harvested at E15.5, E17.5 or P0. Kidneys were dissected out and fixed in 4% PFA (paraformaldehyde) at 4°C overnight. The following day, kidneys were washed in 1xPBS and dehydrated in a sucrose gradient prepared in 1x PBS as follows: 12% sucrose for 1 day, 18% for 1 day, then 22% sucrose for 3 consecutive days. Kidneys were embedded and well oriented in molds containing OCT media (Surgipath FSC 22 3801480), then frozen at -35°C with cold isopentane (Sigma Aldrich M32631) on dry ice. Frozen sagittal sections of 8-10µm thickness were collected on superfrost slides (Thermofisher Scientific) using the cryostat at a controlled temperature of -21°C. Finally, sections were stored at -80°C, and used within a one-month period (maximum) for optimal section quality and best results.

Bromodeoxyuridine (BrdU) treatment

10 mg/ml BrdU (Sigma B5002-250MG) solution was prepared using 0.9% NaCl and 1N NaOH and administered to mice according to body weight (50mg/kg). Prior to BrdU immunostaining, tissue sections were first incubated in 1N HCl for 20 minutes at 37°C (DNA denaturation) followed by neutralization in 0.1M sodium borate (pH=8.5) (Fisher scientific S-249) for 10 min.

2. Genotyping

DNA extraction was performed using Phenol-chloroform-isoamyl alcohol mixture (SIGMA cat. #77617) as per standard phenol-chloroform extraction protocol. Genomic DNA screening was done by PCR using the following primers (Sigma-Aldrich): Nes-Cre transgene Cre-forward 5’ ATT TGC CTG CAT TAC CGG TC 3’, Cre- reverse: 5’ ATC AAC GTT TTC TTT TCG G 3’, Rb flox-forward 5’ GCA TTT AAT TGT CCC

CTA ATC C 3', Rb flox-reverse 5' CTC ATG GAC TAG GTT AAG TTG TGG 3', YFP-forward 5'AAA GTC GCT CTG AGT TGT TAT 3', 5' GCG AAG AGT TTG TCC TCA ACC 3', and YFP-reverse 5' GGA GCG GGA GAA ATG GAT ATG 3', p53 flox-forward 5'CAC AAA AAC AGG TTA AAC CCA 3' and p53 flox-reverse 5'AGC ACA TAG GAG GCA GAG AC 3'. Nes-Cre+/-; Rb fl/fl;YFP/YFP (or Rb-/-) kidneys were compared with Nes-Cre+/+; Rbfl/fl; YFP/YFP (wild-type) or Nes-Cre+/-; Rbfl/fl; YFP/YFP (Rb+/-) kidneys from the same litters.

3. Immunohistochemistry

Frozen sections removed from -80°C and air dried for at least 30 minutes. Then, they were fixed in 4% PFA for 5 minutes, washed in 3x5minutes with 1 xPBS, and blocked for 1-2h in blocking solution (1% BSA (amresco 0332-25G), 0.3% Triton X, 5% donkey serum and 0.1M PBS). Sections were incubated with primary antibody(ies) (prepared according to recommended dilutions in blocking solution) overnight at room temperature. The next day, sections were washed 3x10minutes with 1xPBS, then incubated with fluorochrome-conjugated secondary antibodies diluted in blocking solution for 1h at room temperature. The primary antibodies used are: rat anti-BrdU (1:500; Accurate), chicken anti-GFP (1:1000; ab13970), rabbit anti-Six2 [(1:300; Proteintech 11562-1-AP; with antigen retrieval using10mM sodium citrate solution (Fisher scientific BP327-500)], chicken anti-Nestin (1:300; ab134017), rabbit anti-ki67 (1:500, Cell Marque SP6), mouse anti-Bax-3 (1:300; Abcam), rabbit anti-cleaved Caspase 3 (1:500; Cell Signaling D175), mouse anti-WT1 (1:300; sc-7385), mouse anti-nephrin (1:500; sc-376522). The following secondary antibodies (Jackson Immunoresearch) were used: Alexa Fluor donkey anti-mouse 596, donkey anti-rabbit

Cy3 Alexa Fluor, donkey anti-rat Cy5, Alexa Fluor donkey anti-rabbit 488, Alexa fluor donkey anti-chicken 488, and donkey anti-chicken Cy3. Sections were counterstained with Hoechst (1:50,000; Invitrogen H21486), then washed with 1x PBS for 3x5minutes before mounting in 3:1 (PBS:Glycerol) solution.

4. Imaging and cell counting

Fluorescent and bight-field images were visualized and acquired using the upright Leica microscope (DM6B). Representative images were taken from kidney sections at matching levels, and positive cells were counted using the Image J software and reported as absolute numbers and/or ratios when relevant. Images of double/triple positive cells were overlapped using Adobe Photoshop CS5. All counts were performed on three consecutive sagittal kidney sections at medial level, and reported as absolute numbers and/or ratios. AC-3+ cells and Bax3+ tubules were counted in the whole kidney at medial level. BrdU+, Ki67+, Six2+ and BrdU+/Six2+ single and double positive cells were counted in the whole nephrogenic zone region. BrdU+ cells were also counted in the whole medulla region. AC-3+ glomeruli and AC-3+ SSBs were counted in the whole kidney, and reported as absolute numbers. For statistical analysis, independent t-tests was applied, and all results were obtained in triplicates at least (n=3 per genotype).

5. *In situ* hybridization

Anti-sense riboprobes were synthesized using digoxigenin (DIG)-labeled UTP. Hybridization was performed overnight at 65°C. Signal detection was performed using an AP-conjugated anti- DIG antibody (1:1500; Roche cat.# 11093274910) followed by

incubation in staining solution made of 4-nitro blue tetrazolium chloride (NBT) and 5-bromo-4- chloro-3-indolyl-phosphate (BCIP), at room temperature for 4h-12h depending on probe intensity. Mouse anti-sense riboprobes were synthesized from cDNA amplified by PCR with the following primers (all reverse primers included the T7 polymerase sequence):

Primers used for riboprobe synthesis	5'--------3'	**Product Size**
Cre-For	5'-GCAGAACGAAAACGCTGGTT-3'	411
Cre-Rev-T7	5'-**TAATACGACTCACTATAG**TTGCCCCCGTTTCACTATCC-3'	
Wnt4-For1	5'-GGAGACGTGCGAGAAACTCAAA-3'	192
Wnt4-Rev1-T7	5′-**TAATACGACTCACTATAG**TGTGTCACCACCTTCCCAAAGAC-3′	
Wnt4-For2	5′-CGCTAAAGGAGAAGTTTGACGGTG-3′	111
Wnt4-Rev2-T7	5′-**TAATACGACTCACTATAG**GGTCCTCATCTGTATGTGGCTTG-3′	
Slc34a1-For1	5′-CTTCTTCTTCAACATCTCGGGCATC-3′	556
Slc34a1-Rev1-T7	5′ **TAATACGACTCACTATAG**TCTGTCTTTCTACTGTGGGCATTG-3′	
Slc34a1-For2	5′-CGCTGGTGTTTGGCATTTCC-3′	580
Slc34a1-Rev2-T7	5′-**TAATACGACTCACTATAG**GCACTAATGGTCACACAGGCTCAG-3′	
podx1-For1	5′-GCCAAGCAACCCTACACCATTC-3′	359
podx1-Rev1-T7	5′-**TAATACGACTCACTATAG**TCGCTGTGCTCGGTGAAGAATC-3′	
podx1-For2	5′-AATGGTCTGTGATGGTCACGGG-3′	394
podx1-Rev2-T7	5′-**TAATACGACTCACTATAG**GCCTCATTCTGCTGGACATTCC-3′	

6. Western Blot analysis

Protein lysates were prepared from whole embryonic kidneys in Universal Lysis Buffer, RIPA lysis buffer (abcam cat.# 156034), protease inhibitor (Roche cat.# 04 693 124 001) and 20% SDS. Proteins were quantified using the DC protein assay reagents (Bio-rad reagent A500-0113, reagent B 500-0114, reagent S 500-0115). Electrophoresis was performed on 8% Tris-Chloride gels, and proteins transferred to PVDF membranes (Bio-Rad 162-0177) for signal detection. The antibodies used are total RB (BD-Pharmingen, San Diego, CA, Cat#554136, 1:500) and Alpha-tubulin (abcam

cat.#52866, 1:2000). The secondary antibodies used are HRP goat anti-mouse (cs-2005, Santa Cruz 1:2500) and HRP goat anti-rabbit (sc2004, Santa Cruz, 1:2500).

7. Hematoxylin-Eosin staining

Sections were dried for 30-40min, then fixed in 4% PFA for 5min, and rehydrated in 95% and 75% ethanol for 3min each. Sections were transferred to ddH_2O for 5min, then placed in Harris hematoxylin solution (Sigma HHS16) for 1 min. This was followed by washing under running tap water for 3min or until excess stain is removed. Counterstaining was done in Eosin solution (Polysciences, cat.# 09859) for 45 sec, followed by dehydration in 75%, 95% and 100% ethanol solutions for 3min each. Finally, sections were transferred to xylene for 2min, and mounted with permanent mounting media (KLEERMOUNT xylene solution).

8. Quantitative Real Time-PCR analysis (RT-PCR)

Total RNA was extracted from dissected nephrogenic zones in control and mutant kidneys using the RNeasy Plus Mini Kit (Qiagen cat.#74134). This was followed by cDNA synthesis using QuantiTect Reverse Transcription Kit (Qiagen cat.# 205311). Standard curves were generated and optimized for maximal coefficient efficiency. Quantitative RT-PCR was performed using SYBR green (iTaq™ Universal SYBR® Green Supermix from Biorad). For each gene tested, 2-3 independent RT-PCR experiments were carried to confirm the results obtained (using duplicates for each sample and n=3 samples per genotype). The double delta Ct method and the relative standard curve method were both used to analyze the relative changes in gene expression from real-time quantitative PCR experiments. β-actin was used as internal control to

normalize gene expression. The RT-PCR primers (BioRad) used are summarized in the table below:

Primers used for real time (qRT-PCR)	5'--------3'	**Annealing T°C**
Slc12a3- Forward	CCTTTGATACCCAGAGCCATAATG	59
Slc12a3- Reverse	AATGAATGCAGGTCAGCCAGG	
Bmp7- Forward	ACGGACAGGGCTTCTCCTAC	60
Bmp7- Reverse	ATGGTGGTATCGAGGGTGGAA	
Six2- Forward	GCAACTTCCGCGAGCTCTAC	60
Six2- Reverse	CCTTGAGCCACAACTGCTG	
Cited1- Forward	GTCGAGGCCTGCACTTGATG	60
Cited1- Reverse	CCAAGGTTGGAGTAGGCCAGAG	
Wnt4- Forward	GGAGACGTGCGAGAAACTCAAA	61
Wnt4- Reverse	TGTGTCACCACCTTCCCAAAGAC	
Podxl- Forward	GCCAAGCAACCCTACACCATTC	62
Podxl- Reverse	TCGCTGTGCTCGGTGAAGAATC	
β-actin Forward	CATCCGTAAAGACCTCTATGCCAAC	60
β-actin Reverse	ATGGAGCCACCGATCCACA	

9. Cell sorting

Embryonic kidneys from 5-6 animals carrying the same genotype were dissected and pooled, then mechanically dissociated into small pieces. After mild enzymatic digestion using a mixture of Papain (final concentration:20IU/ml, Sigma p3125-1U/ul) and 0.5mM EDTA (final concentration:1.2mM, GIBCO-15574-038), cells were purified with Percoll (GE-healthcare 17-0891-01), fixed with 1%PFA and co-stained with (Six2; YFP) antibodies before being processed for cell sorting (BD FACS Aria SORP cell sorter). For more detail on the sorting procedure, please refer to the protocol described in the next manuscript entitled “Isolation of Nephron Progenitor Cells from Embryonic Kidneys of Nes-Cre ERT2/Rosa26-YFP Mice using Indirect FACS staining”.

D. Results

Nestin-Cre driven deletion of Rb during kidney development

Using Nestin-CreER^{T2}-YFP; Rb$^{fl/fl}$; p53-/- mice, we found that conditional deletion of Rb in p53-/- null mice between E13.5 and P21 results in embryonic lethality around birth with almost full penetrance of phenotype (**Supplementary Table 1)**. However, induced deletion of Rb at P30 or later using the same model did not affect survival in p53-null mice (up to 4 months following loss of Rb). Notably, two tamoxifen-treated mice at E18.5 that exceptionally survived until P40 (probably due to incomplete Cre recombination) were sacrificed, and displayed reduced body size and weight overall, a 2-fold decrease in kidney size, and elevated levels of creatinine by 5-6 folds compared to vehicle-treated p53-null mice and wild type mice (**Supplementary Figure 1 and Suppl. Table 2**)**.** Closer histological examination of the kidneys in these mutant animals revealed severe renal hypoplasia and the presence of thinner and dilated tubules as well as expanded interstitial space compared to wild type controls and p53-/- mice (**Supplementary Figure 2**). These developmental defects suggested the presence of abnormal renal development and severe kidney failure associated with the loss of Rb in p53-null mice carrying a mixed FVBN x C57/Bl6 x Sv/126 genetic background.

To specifically investigate a potential role for Rb in the developing kidney, we generated Nestin-CreERT2-YFP; Rb$^{fl/fl}$ embryos by crossing Nestin-CreERT2-YFP/YFP mice and Rb$^{fl/fl}$ mice, and induced Rb deletion by single tamoxifen treatments administered to pregnant females during mid-gestation (Lagace et al. 2007; Marino et al. 2003). Crosses were designed to obtain mixed litters of Rb$^{fl/fl}$ and Rb$^{fl/+}$ embryos carrying the Nestin-CreERT2 cassette thereafter referred to as Rb-/- (Rb conditional knock-out KO) and Rb+/- (Rb heterozygotes littermate controls), or lacking the Nestin-

Cre cassette and called Rb+/+ (wild type littermate controls). Nestin is an intermediate filament protein widely expressed and used as marker of neural stem and progenitor cell but also shown to be expressed in the developing and adult kidneys (Sun et al. 2014; Dhaliwal and Lagace 2011; Dubois et al. 2006; J. Chen et al. 2006). Studies have indeed shown that, in Nestin-Cre1 embryos, Nestin is expressed throughout the MM compartment (but not UB structures) including Cited1+ CM progenitors and (CD31+; Flk1+) endothelial cells of the immature glomeruli starting at E11.5 (J. Chen et al. 2006; Dubois et al. 2006). Our results obtained with the Nestin-CreERT2-YFP line are consistent with the above studies and showed Nestin-driven Cre mRNA expression inside the renal cortex including CM cells, SSB and immature glomeruli at E15.5 (2 days following tamoxifen treatment) (**Figure 1A-A'**). Accordingly, we detected overlapping protein expressions of Nestin and GFP in the cap mesenchyme-derived nephrogenic structures at E17.5 post-tamoxifen treatment at E13.5 (**Figure 1B-D'**). Moreover, we detected a significant reduction in Rb protein level by western blot analysis performed on E15.5 protein lysates that are derived from embryonic kidneys treated at E10.5 (**Figure 1E**). To further verify that Cre recombination was successful in targeting the NPCs pool, we labeled and sorted by FACS (Six2+;GFP+) double positive cells derived from embryonic kidneys at E18.5 (treated at E10.5) and identified a clear and distinct population of Six2+ NPCs co-expressing GFP at this age (**Figure 1F; population P4**). These results indicate that, using a Nestin-CreERT2 line, we have successfully deleted Rb in most progeny belonging to the CM (including nephrogenic structures) and endothelial lineages during development.

Diverse morphological defects in Rb mutant kidneys at birth

To characterize the kidney phenotype following conditional loss of Rb, we first examined renal morphology by hematoxylin and eosin staining performed on sagittal kidney sections derived from embryos treated at E13.5 and sacrificed at P0. Compared with Rb+/- littermate controls, Rb-/- embryos had a similar kidney size (data not shown), however, they showed diverse histological defects including loss of clear interface between the stroma and the CM, thickening of the nephrogenic zone (NZ), and deterioration/detachment of the tubular epithelium (**Figure 2: compare B-C with B'-C'**). In addition, unlike in Rb+/- where the diverse nephrogenic structures can be clearly identified inside the NZ including pre-tubular aggregates (PA), CSB and SSB, the Rb-/- NZ showed higher cellular density with a predominantly large number of PA at this age suggesting the presence of renal hyperplasia. Of note, immature glomeruli were pushed deeper into the outer and/or inner medulla in the absence of Rb instead of being localized to the outer medulla that is lining the cortical region as seen in Rb+/- controls; this is likely a secondary effect associated with the thickening of the cortical region (**Figures 2A-A', 3A-A' and Suppl. Figure 3A-A'**). Besides this observation, we did not detect any major developmental defects inside the medulla region including the UB structures.

Increased and ectopic nephron progenitor proliferation in the absence of Rb

To gain further insight on the renal phenotype in the absence of Rb, we induced an Rb deletion at E10.5, which is consistent with the onset of nephrogenesis (Davidson 2008). 5 days later (at E15.5), we detected increased and ectopic cell proliferation in the nephrogenic zone and the medulla, that was manifested by extensive BrdU incorporation in both regions (**Figure 3B-B'**). Thus, we scored 2.51-fold and 1.85-fold

increase in the numbers of BrdU-positive cells found throughout the entire nephrogenic zone and the medulla in Rb-/- kidneys compared with Rb+/+ control littermates at E15.5, respectively (**Graph 1a**). These results are consistent with our previous H and E data showing renal hyperplasia and thickening inside the NZ. We next examined whether the excessively proliferating Rb-/- cells are nephron progenitors by co-staining for BrdU and Six2. Quantification of the numbers of single Six2+ and (Six2+; BrdU+) double-labeled cells indeed revealed the presence of around 2- and 1.77-folds increase in cell counts inside the CM in Rb mutant kidneys compared with wild type controls, respectively (**Figure 3C-D'; yellow arrowheads in C', and, Graph 1a**). Of note, we did not find any significant difference in the ratio of (Six2+; BrdU+) cells over the total number of Six2+ cells in Rb-/- kidneys compared with Rb+/+ control, indicating the presence of a proportional increase in proliferation inside the progenitor pool (**Graph 1b**). Notably, almost all (BrdU+; Six2+) double-labeled cells are localized at the lateral edges of the CM, thus indicating that they are either primed NPCs and ready to initiate epithelialization or at the pre-tubular aggregates' stage (**Figure 3D'; white arrows)**. The increase in NPC proliferation in Rb-/- kidneys was further confirmed by Ki67 immunostaining, which showed higher expression by 1.92-and 2.6-folds throughout the nephrogenic zone and the medulla compared with controls, respectively (**Figure 4A-B' and Graph 2**). Altogether, the above data indicate that Rb negatively controls renal progenitor proliferation during nephrogenesis, and this is consistent with its reported role in other tissues including the developing and adult brain as well as the olfactory epithelium (Ghanem et al. 2012; Naser et al. 2016; Jaafar et al. 2016; Vandenbosch et al. 2016).

Enhanced nephrogenesis in the Rb mutant kidney

Given the increase in NPCs proliferation described above, we next assessed whether progenitor differentiation is affected in the absence of Rb by examining potential change(s) in the levels of key differentiation genes that mark distinct stages of nephrogenesis. Hence, we co-stained for Nestin and WT-1 (Wilm's tumor 1), whose expression is upregulated during early stage of mesenchyme condensation and found high in SSB and immature glomeruli at later stages (Hartwig et al. 2010; Berry et al. 2015). Our results showed upregulated WT-1 expression in differentiating nephrogenic structures as well as in immature glomeruli at P0 following loss of Rb at E13.5. Hence, the numbers of double positive (Nestin+; WT1+) proximal SSB and glomeruli were found increased by 2.63- and 1.72-folds inside the whole kidney in the absence of Rb, respectively (**Figure 4C-D', Graph 3**). Furthermore, using *in situ* hybridization and DIG-labeled anti-sense riboprobes, we assessed the transcript levels of the early differentiation gene Wnt4, known to be an essential inducer of nephron differentiation including MET, and, of mid-to-late stages differentiation genes including the proximal tubules' marker, Slc34a3 and the immature podocytes' marker, Podxl . Consistent with our earlier findings, we detected enhanced mRNAs expressions of Slc34a3 and Podxl in the Rb-/- kidneys compared with Rb+/+ control littermates at E15.5. Interestingly, the mRNA expression of Wnt4, an inducer of NPC commitment by mesenchymal to epithelial transition, is reduced in the absence of Rb. This result is consistent with the upregulated expression of Six2, which was shown to negatively regulate the transcription of Wnt4 and promote NPCs self-renewal/maintenance by preventing commitment to a nephrogenic fate (Jingyue Xu et al. 2014; J. S. Park et al. 2012) (**Figure 5A-C'; black arrows in A'-C'**).

To further correlate the developmental defects observed above with potential changes in gene expression, we compared the expression levels of Cited-1, Six2, Wnt4, Bmp7, Slc12a3 and Podx1 by quantitative real-time PCR using cDNA derived from dissected and dissociated NZ cells (derived from cortex and part of the outer medulla) in both genotypes (by excluding the inner medulla region and UB structures). Interestingly, our results showed 4.5-folds increase in Six2 transcript expression but no significant change in Cited1 expression in the Rb-/- NZ compared with littermate controls (**Graph 4**). This data confirms that Rb specifically controls proliferation of the nephron progenitor cells or NPCs population (Cited1-;Six2+), but not the self-renewing stem cell population (Cited1+; Six2+), in striking similarity with its role during development in other tissues such as the developing and adult brain. Consistent with the increase in Six2 expression, we detected a 2.7-folds decrease in Wnt4 transcript expression in the absence of Rb. In addition, we detected a 2.8-folds increase in the transcript expression of Bmp7, which was shown to be required for NPC proliferation (**Graph 4**) (Blank et al. 2009). Importantly, we observed 2.3-folds increase in the transcript level of the distal tubule marker, Slc12a3, which is consistent with the upregulated expressions of Slc34a3 and Podx1 described earlier (**Graph 4 and Figure 5B-C')**. Unexpectedly, we did not detect an increase in Podx1 mRNA level; this is likely because the majority of glomeruli are abnormally localized deeper in the medulla (close to the inner medulla) in Rb-/- kidneys, a region that was excluded from the dissected tissues used in this experiment. Taken together, our data demonstrate that Rb controls the rate of nephrogenesis during development and its loss leads to enhanced nephrogenesis.

Rb is required for nephron survival during late development

Despite the enhanced nephrogenesis associated with loss of Rb, we did not detect a significant change in kidney size in Rb-/- embryos compared with littermate controls throughout development nor at birth. Given the critical role played by Rb in cell survival during maturation in various embryonic and adult tissues e.g. neuronal survival, we sought to determine whether it is needed for cell survival during nephrogenesis. Immunostaining against the apoptotic marker Bax3, and, Nephrin, a marker of glomerular podocytes and late SSBs (Ruotsalainen et al. 2000; Putaala 2001), showed massive cell death affecting several renal tubules and glomeruli in Rb mutant kidneys compared with Rb+/+ controls at birth (**Figure 6; arrows in B'-D' and Suppl. Figure 4; arrows in C')**. Similar results were obtained following co-staining with Bax3 and active-caspase 3 (AC-3), a second apoptotic marker, at E17.5 (**Suppl. Figure 3A-D'**). Cell counts performed at the latter age confirmed these survival defects, thus showing 2.74- and 1.79-folds increase in the numbers of Bax3+ tubules and AC-3+ cells in the absence of Rb, respectively (**Graph 5**).

To further elucidate whether apoptotic cell death in the absence of Rb could be mediated by p53, we generated Nestin-CreERT2-YFP Rb;p53 double floxed mice and assessed survival by co-staining with AC-3 and Nestin at E15.5 (5 days after tamoxifen treatment). Compared with Rb-/- kidneys, we unexpectedly detected a more severe apoptosis coupled to massive reduction in the numbers of immature glomeruli in double mutant kidneys at this age (**Suppl. Fig. 4C-C''**). The above findings indicate that both Rb and p53 are needed for survival in the developing nephrogenic structures and that loss of p53 could not rescue the survival phenotype observed in the absence of Rb.

Moreover, this increased cell death likely offsets any potential increase in kidney size associated with enhanced nephrogenesis in Rb-cKO embryos.

Combined deletions of Rb and p53 leads to additive phenotypic defects and exacerbates the nephron survival defect

Given that the existing crosstalk between the Rb and p53 pathways in the control of cell proliferation, senescence and survival in various tissues during development, we sought to characterize further the renal phenotype in Nestin-CreERT2-Rbfl/fl; p53fl/fl or double mutant mice in comparison with p53 single mutant and wild-type controls. We found that inducible deletion of p53 at E10.5 results in a remarkable renal hypoplasia (**Figure 7A-A' and Supplementary Figure 4A, A'**), in addition to an enlargement of Bowman's capsule and the formation of irregularly shaped glomeruli at E15.5 (**Figure 7B, B'; white arrows in B' inset**). Of note, we detected a slight but not significant decrease in cell proliferation as assessed by the total numbers of BrdU+ cells and (BrdU+;Six2+) double positive cells inside the kidney at this age (**Figure 7C-D' and Graph 6**). Moreover, the proliferative ratio of (BrdU+; Six2+) over the total number of Six2+ cells is similar in p53-cKO and wild type controls **(Graph 1b).** These results suggest that the increase in apoptosis is the likely cause of renal hypoplasia rather than the presence of a proliferation defect in p53-/- kidneys.

(**Supplementary Figure 4C, C'**).

On the other hand, double mutant embryos displayed exacerbated defects when compared with Rb and p53 single mutant mice. These defects include more severe reduction in kidney size (**Figure 7A-A"'**), paucity of immature glomeruli (**Figure 7B-B'"**) and increased cell death affecting distinct renal structures along the nephrogenic lineage at E15.5 (embryos treated at E10.5, **Supplementary Figure 4C-D''**). Of note,

the severity of the phenotype in double mutant mice commonly led to embryonic lethality few days before birth, which prevented further characterization of the phenotype at later ages in development. To overcome this, we induced combined deletions of both genes at E13.5 and closely assessed the renal phenotype 4 days later. Compared with wild type littermate controls, E17.5 double mutant embryos showed decreased proliferation inside the cortical region as assessed by BrdU labeling and co-staining for (Brdu;Six2) (**Fig. 7D-D'', Suppl. Fig. 5D-D' and graph 6**). Of note, the proliferative ratio was not affected in the double mutants when compared to all other genotypes **(Graph 1b**). Moreover, they displayed severe nephron differentiation and maturation defects including paucity of differentiating renal structures, low count of immature glomeruli with irregular shape, and expansion of the mesangial matrix as revealed by the sharp decrease in the expressions of Nestin, NeuN, and Nephrin **(Suppl. Fig. 5B-C' and E-E')**. Put together, these findings indicate that the Rb and p53 pathways seem to control distinct functions during kidney development such as NPCs' proliferation by Rb versus differentiation/maturation by p53. However, they also show crosstalk in the control of other processes such as cell survival, given that single and combined deletion(s) in these genes affect renal survival to various extents.

E. Discussion

We report here for the first time a requirement for the cell cycle protein, Rb, during kidney development. We show that Rb specifically controls NPC proliferation but does not seem to regulate renal stem cell self-renewal or nephron differentiation. However, it is indispensable for the survival of immature nephron around birth. Therefore, loss of Rb around mid-gestation leads to enhanced nephrogenesis throughout development, and this is counterbalanced by increased apoptosis in the nephrogenic progeny during late embryonic stages **(Figure 8)**. Moreover, we uncover some of the interactions between the Rb and p53 pathways during kidney development and report the presence of distinct as well as shared functions between the two pathways.

The role of p53 is well studied during kidney development; previous studies have shown that it plays a central role in NPC self-renewal, nephron differentiation and UB branching in the developing kidney (Godley et al. 1996; Y. Li et al. 2015; Saifudeen, Dipp, and El-Dahr 2002; Saifudeen et al. 2012; 2009; S. S. El-Dahr et al. 2014; S. El-Dahr, Hilliard, and Saifudeen 2017). Besides this, the roles of various cell cycle proteins and cell cycle machinery during kidney development are poorly characterized and limited to few studies only. In fact, it was reported that the expression levels of cyclins and cyclin-dependent kinases change in a temporal manner during kidney development (Park et al. 1997). Moreover, Rb hyperphosphorylation was associated with mesangial cell proliferation (Shankland et al. 1997) while the non-classical cell cycle regulator, Cdk5, was shown to be expressed *de novo* in differentiated podocytes at E18 (Griffin et al. 2004).

In contrast, a substantial body of literature investigated the changes in gene expression of several cell cycle proteins and their regulatory roles in the adult kidney under

homeostatic conditions as well as in renal injury and disease (Thomasova et al. 2015, Marshall and Shankland, 2006). Hence, it was shown that the expressions of Cyclin D, CdK2 and Cdk4/6 are upregulated after renal tubule injury (Park et al. 1997). Similarly, higher levels of Cyclins D and E, Cdk2 and Cdk4 were associated with mesangial cell proliferation in Thy1 glomerulonephritis rat models. In other diseases or following injury, cell cycle regulators were shown to play opposing roles whereby they either contribute to a defective podocyte phenotype or play a protective role depending on the context. For instance, in collapsing focal segmental glomerulosclerosis (FSGS), altered expression of CDK inhibitors (CDKI) and cyclins help bypass the cell cycle checkpoints, thus leading to podocyte loss by mitotic catastrophe. In contrast, in adriamycin- induced podocyte injury, high expression of p21 plays a protective pro-survival role in podocytes (Marshall et al. 2010). Notably, pharmacological inhibition of Cdk4/6 in ischaemia-reperfusion mouse model induces transient cell cycle arrest to allow repair of DNA damage, thereby ameliorating tubular cell death (DiRocco et al. 2014). On the other hand, under homeostatic conditions, high levels CDKI including p21, p27 and p57 are necessary for maintaining a post-mitotic/quiescent state in mature podocytes, thus preventing aberrant cell cycle activation, which may lead to podocyte detachment, loss of function, and eventually apoptosis (Kriz and Lemley 2015; Thomasova and Anders 2015; Liapis, Romagnani, and Anders 2013). Given the novel role of Rb reported here and the interplay between Rb, Cdks and Cdkis during cell cycle regulation, future studies should aim at identifying the molecular interactions between Rb and these key cell cycle regulators in the developing kidney.

Nestin-CreERT2-YFP targeted deletion of Rb during kidney development

This study is the first to induce successfully a targeted deletion of Rb using Nestin-CreERT2-YFP; $Rb^{fl/fl}$ mouse model in the developing kidney (Lagace et al., 2007; Morano et al. 2000). Consistent with previous studies, we found that Nestin expression starts around E10.5, which coincides with the onset of nephrogenesis (Dubois et al. 2006). Moreover, we show that Nestin primarily targets Cre recombination (Rb deletion) in the metanephric mesenchymal lineage including all nephrogenic structures (excluding UB structures), and becomes confined to immature glomeruli during late developmental stages (Figure 1, 3 and 4; (Bertelli et al. 2007a; J. Chen et al. 2006). Using this model, we successfully deleted Rb in the nephrogenic zone (cortex region) including Six2+ NPCs, differentiating nephrogenic structures and podocytes (Figures 1 and 3), and efficiently isolated a pure population of (Six2+;GFP+) double positive cells from Rb mutant embryonic kidneys using indirect FACS staining. These cells could be used to conduct cell cycle analysis e.g. by propidium iodide staining, and molecular studies e.g. assess changes in gene expression by qRT-PCR. Furthermore, we successfully generated primary cultures of mixed renal progenitors that were derived by mild enzymatic digestion from the cortical region in Rb mutant and wild type embryonic kidneys using a previously described protocol (Brown et al. 2011). Interestingly, these cultures were highly enriched in Rb-null NPCs expressing Cited-1 and/or Six2, and were used to conduct complementary studies of the role of Rb *in vitro* (Hammoura I, Jaafar C and Ghanem N; unpublished data). Despite the fact that Nestin expression is also reported in some endothelial cells, the Nestin-CreERT2 is a suitable model to study the role of Rb (and other developmental genes) in the MM progeny both *in vivo* and *in vitro.*

Rb primarily controls nephron progenitor proliferation during development

We demonstrate here that Rb negatively regulates NPC proliferation without affecting (at least directly) their subsequent differentiation. Our findings also indicate that loss of Rb does not affect the self-renewing (Cited-1+; Six2+) stem cell population, which is refractory to differentiation signals and localized most apically in the cap mesenchyme. In contrast, loss of Rb leads to increased and ectopic proliferation of the (Cited-1-; Six2+;Wnt4-) population, which marks the early NPCs, and eventually give rise to the (Cited-1-; Six2+;Wnt4+) late progenitors that initiate differentiation and undergo epithelialization to RV. Several observations strongly support the above conclusions for several reasons. First, our qRT-PCR studies showed that Cited-1 expression is not changed whereas there is 4.5-fold increase in Six2 expression that is paralleled by a 2.7 decrease in Wnt4 expression (Wnt4 being a direct target of Six2) in the Rb mutant nephrogenic zone (Graph 4, see next paragraph). Second, our histological studies revealed that around 90% of (BrdU+; Six2+) double positive cells are localized to the lateral regions of the cap, normally occupied by clusters of (Cited-1-; Six2+) cells including early and late/committed progenitors as well as pre-tubular aggregates. Third, we detected a significant increase in the numbers of Six2+ and BrdU+ cell populations in Rb-/- cap mesenchyme cultures compared with Rb+/+ cultures (unpublished data; Hammoura I, Jaafar C and Ghanem N). Of note, we were not able to confirm the above conclusions by triple immunostaining for Cited-1, Six2 and BrdU because the Cited-1 available antibody works well *in vitro* only. In the future, we could sort the (Cited-1+; GFP+) population to overcome this, and co-stain for BrdU, Six2 and/or Wnt4 to confirm our findings. Finally, the role of Rb in the control of NPCs proliferation is

consistent with its role in the developing and adult brain as well as the olfactory epithelium where it specifically regulates progenitor proliferation (Jaafar et al. 2016; Vandenbosch et al. 2016; Ghanem et al. 2012; Naser et al. 2016).

While the exact role of Cited-1, a key marker of the renal stem cell population, is not yet known in the developing kidney (Boyle et al. 2007), Six2 is described as the gatekeeper given its central role in maintaining the proliferative state of NPCs pool throughout the whole period of nephrogenesis (Self et al. 2006). With each round of UB branching, only a fraction of NPCs commits to nephron formation (Short et al. 2014). Studies have uncovered some of the molecular mechanisms that regulate the balance between expansion of the NPC pool and their commitment. In fact, it was shown that Six2 induces its own transcriptional expression as well as the expressions of other proliferation genes including GDNF and Osr1. In addition, Six2 interacts with Osr1 and forms a repressor complex with TCF and the Groucho-related repressor proteins that inhibits the transcription of Wnt4 and other pro-differentiation genes (Park el. 2012, Xu et al. 2014). Our data is consistent with these studies given that loss of Rb leads to upregulation in Six2 expression and downregulation in Wnt4 (Graph 4). On the other hand, following activation of Wnt9b/β-catenin signaling, β-catenin competes with Groucho and directly converts the above repressor complex (Six2/TCF/Groucho) to an activator one, which in turn upregulates Wnt4 expression (Xu et al. 2014). Interestingly, previous studies have shown the existence of interaction/crosstalk between the Rb-E2f1 and Wnt/β-catenin canonical pathways. For example, E2f1 was shown to inhibit Wnt/β-catenin signaling (TCF/LEF dependent transcription) in human colorectal tumors. In this context, E2f1 directly activates ICAT (an inhibitor of β-catenin), thus leading to downregulation of β-catenin target genes such as cMyc and CyclinD1 (Wu et al. 2011;

Morris et al. 2008). Moreover, the Rb regulatory function(s) are primarily mediated through its direct interaction with and repression of the E2f family of transcription factors, typically E2F1, 2 and 3 (activator E2Fs) during development (Burkhart and Sage 2008; Indovina et al. 2015). Future work should examine whether similar interactions exist between the Rb/E2F pathway and the canonical Wnt/β-catenin signaling in the developing kidney, and whether one or more key renal regulatory genes such as Six2 could be direct targets of the Rb-E2F pathway.

Other Rb family members and cell cycle proteins could contribute to the regulation of self-renewal of the stem cell population in the developing kidney as reported in other tissues. For instance, p107 was shown to negatively control self-renewal of neural stem cell in the adult brain via inhibition of the Notch signaling pathway (Vanderluit et al. 2004; Chapouton et al., 2010), and could play a similar role during renal development. Moreover, p53 was shown to positively regulate self-renewal in Cited1+ cells through maintenance of their metabolic fitness. Actually, Liu et al showed that Six2-Cre driven p53 deletion resulted in renal hypoplasia that was linked to decreased self-renewal ability of (Cited1+;Six2+) stem cell pool. In fact, deregulated gene expression in key metabolic pathways, particularly glycolysis, led to pre-mature depletion of the pool (Jiao Liu et al. 2015). The same study showed that stem cells and early NPCs exhibit elevated level of PI3K-Akt signaling, which potentiates the rate of glycolysis and therefore, maintenance of the stem cell pool. In contrast, late/committed NPCs have reduced PI3k-Akt signaling and decreased glycolytic flux, thus stimulating their exit from the stem cell pool and nephrogenic commitment (Liu et al. 2015).

Rb is dispensable for nephron differentiation during development

Previous studies have shown that Rb is required for neuroblast differentiation in a context-dependent and spatial-dependent manner. For instance, Rb is needed for proper neuroblast differentiation in the dorsal cortex and the subventricular zone (SVZ) during brain development (Matthew G Andrusiak et al. 2012; Ghanem et al. 2012; Ferguson et al. 2002). In contrast, it is not needed for olfactory sensory neuroblasts' differentiation in the developing olfactory epithelium (Jaafar et al. 2016), or terminal differentiation and migration of adult-born neuroblasts in the adult SVZ/olfactory bulb and the hippocampus (Naser et al. 2016 and Vandenbosch et al. 2016). Our results suggest that Rb is also dispensable for NPCs differentiation and does not seem to play a direct role in this process. In fact, compared with Rb littermate controls, Rb-/- embryos had enhanced nephrogenesis that is manifested by: 1) the increased expression of several differentiation markers including Slc12a3, Slc34a3 and Podxl (Graph 4 and Figure 5), and, 2) a higher number of differentiating nephrogenic structures including SSB's and immature glomeruli detected between E15.5 and P0 (Figures 5 and 6). While these results cannot rule out a possible role for Rb in nephron differentiation, they are likely to be secondary effects associated with deregulated NPC proliferation in the absence of Rb. Future experiments should exclude any obvious cell cycle exit defect and thus, confirm these observations. Alternatively, other pocket proteins such as p107 could be playing could compensatory role(s) to ensure proper cell cycle exit and nephron differentiation. Previous studies have indeed reported compensatory roles among pocket proteins; for instance, dual deletion of both Rb and p107 induced an increase in the proliferative index in differentiating cortical neurons as compared to Rb deletion alone (Oshikawa et al. 2013). Moreover, p107 plays a protective role against the development

of Retinoblastoma in retinal interneurons in absence of both Rb and p103 (Ajioka et al. 2007).

Rb is required for immature nephron survival during late development

Numerous studies highlighted the critical anti-apoptotic role of Rb in various embryonic and adult tissues, where Rb was shown to be indispensable for the maintenance of a post-mitotic state via preventing cell cycle entry and/or inducing senescence (Knudsen et al. 2000; Payton et al. 2006; De Leon, Sherry, and Krucher 2008; Akakura et al. 2010; Alexander, Yang, and Hinds 2003; Duminuco et al. 2015; Chicas et al. 2010; Collard et al. 2012; Hilgendorf et al. 2013). Our data fall in line with this body of literature; in fact, we report massive apoptosis in tubule-epithelia of the immature nephron in the Rb-/- kidney compared with littermate controls at late developmental stages (Figure 6 and Suppl. Figure 3). We also show that loss of p53 does not rescue the survival defects observed in Rb-/- kidneys; on the contrary, it exacerbates them (Suppl. Figure 4). Notably, p53 single mutants also showed severe renal hypoplasia and cell death in the developing kidney (Suppl. Figure 3). Altogether, this data indicate that the both Rb and p53 maintain renal cell survival via distinct and/or common mechanism(s), which warrant further investigation. These findings are consistent with our previous studies showing increased apoptotic cell death and deterioration of the OE during late development (Jaafar et al. 2016), and severely compromises long-term survival of adult-born neurons during adult neurogenesis in the absence of Rb (Naser et al. 2016).

The pro-survival role of Rb is mostly attributed to its repression of E2f1 transactivation. Hence, the massive apoptosis observed in Rb-/- mouse embryos can be rescued by loss of E2f1 (Tsai et al. 1998; Saavedra et al. 2002). In fact, E2f1 can trigger apoptosis in a p53-dependent manner (Hershko et al. 2005; Fogal et al. 2005; D. Chen et al. 2005);

hence, it upregulates the expression of ASPP1/2, which binds to p53 and directly activate the transcription of the pro-apoptotic gene Bax (Fogal et al. 2005). In parallel, E2f1 can also downregulate the expression of anti-apoptotic genes such as BCL2 and MCL1 (Eischen et al. 2001; Croxton et al. 2002). Alternatively, elevated expression of E2f1 can also activate the expressions of pro-apoptotic genes in cells lacking p53 (Polager and Ginsberg 2008). This p53-independent E2f1 function is mediated by p73, which can transactivate common targets and pro-apoptotic genes such as APAF1 and PUMA (Stiewe and Putzer 2000; Lissy et al. 2000; Crighton et al. 2007). Future studies should examine the mechanism(s) mediating nephron apoptosis in the absence of Rb, particularly the dynamic role of E2F1 (with dual function as oncogene and tumor suppressor gene) in this context.

Functional interplay between the Rb and p53 pathways during kidney development

The phenotype of p53-/- embryonic kidney reported in our study is consistent with the findings of previous studies that have examined the role of p53 during kidney development. We and others have found that p53-/- embryos display renal hypoplasia (reduction in kidney size), reduction in the number of immature nephron and irregularly shaped glomeruli (Figure 7) (Saifudeen et al. 2009; Yuwen Li et al. 2015). These defects are likely due to metabolic deficit negatively affecting stem cell self-renewal and/or decreased nephron differentiation in the absence of p53 as reported earlier (Saifudeen et al. 2012; Y. Li et al. 2015). In contrast, unlike the findings reported by Li et al., our data does not support the presence of a proliferative defect in the stem cell pool in the absence of p53 as reflected by the similar numbers of total BrdU+ cells and (BrdU+;Six2+) cells as well as the comparable proliferative ratios inside the NZ

between p53+/+ and p53-/- embryos (Figure 7 and Graph 6 and Graph 1b, Liu et al. 2015). On the other hand, our data clearly indicate the presence of increased apoptosis in the p53-/- kidney (both inside the cortex and medulla), which can strongly explain the observed renal hypoplasia phenotype (Suppl. Figure 4). Such an obvious survival defect was surprisingly not reported in Six2-Crep53$^{fl/fl}$ mice or at least overlooked (Liu et al. 2015). Of particular interest to our study is the observed phenotype in Rb-p53 double mutant embryos showing exacerbated survival defects (with more reduction in kidney size) that could be due to either additive or synergistic effects following combined loss of both genes (Suppl. Figure 4). In addition, the double mutants exhibited severe proliferation and differentiation defects (Figure 7 and suppl. Figure 5), that could be attributed to the distinct roles played by Rb and p53 in the control of renal progenitor proliferation (by Rb) versus control of stem cell proliferation/differentiation (by p53). These distinctive functions of Rb and p53 in the control of stem vs progenitor cell development are found conserved in other tissues such as the adult brain (Naser et al. 2016; Gil-Perotin et al. 2006).

Taken together, our data strongly highlight the presence of different roles attributed to Rb and p53 during kidney development as well as the existence of crosstalk between both pathways, particularly in the context of cell survival. Ongoing work in our laboratory is aimed at performing RNA-sequencing studies using total RNA/cDNA extracted from the dissected nephrogenic zones in Rb+/+, Rb-/-, p53-/- and Rb-/-; p53-/- embryos. The purpose of this experiment is to decipher the molecular mechanisms and target genes that are mediating the distinct versus redundant functions of both genes during kidney development. The anticipated gene expression profiles will also have

direct implications on the potential role(s) for these tumor suppressor pathways during kidney development and disease in humans.

FIGURES

Figure 1

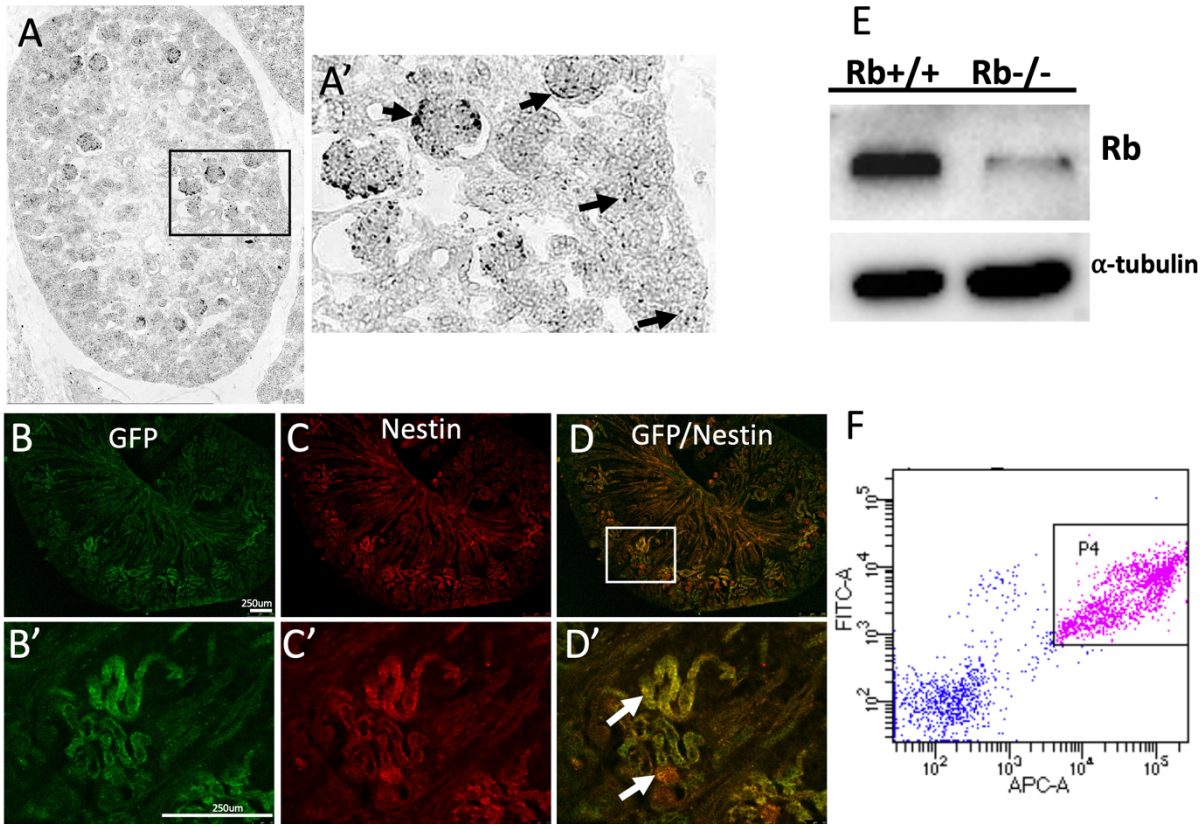


Figure 1: Proof of Rb deletion by Nestin-Cre recombination in the developing kidney. (B-D') Double immunostaining with antibodies against Nestin and GFP performed on sagittal kidneys sections in Nestin-CreERT2-YFP;Rbfl/fl embryos at E17.5 (treated at E13.5). (B'-D') higher magnification images of the region shown in boxes in (B-D). Note the overlapping expression patterns of Nestin and GFP in differentiating nephrogenic structures in D' (white arrows). (A-A') *In situ* hybridization showing Cre transcript expression in the renal cortex and immature glomeruli in Rb-/- kidneys treated at E13.5 and sacrificed 2 days later (black arrows in A'). (E) Western blot analysis showing significant reduction in Rb protein level at E15.5 in Rb-/- embryonic kidneys compared with wild type littermates (treated at E10.5). (F) FACS-sorting of (Six2+;GFP+) double positive cells derived from E18.5 embryonic kidneys in Rb-/- embryos (treated at E10.5) and showing a double positive (FITC+;APC+) population in P4. Scale bar =250um.

Figure 2

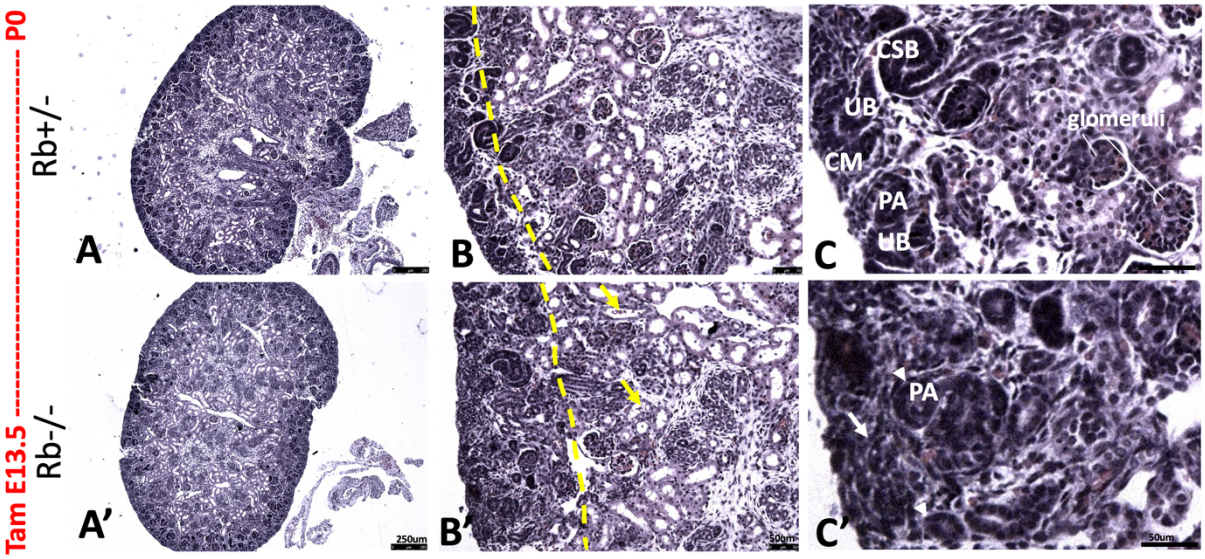


Figure 2: Diverse morphological defects in Rb mutant kidneys at birth. Hematoxylin and Eosin staining performed on P0 sagittal kidney sections derived from E13.5.5 treated Nestin-CreERT2-YFP;$Rb^{fl/fl}$ mice and $Rb^{fl/+}$ heterozygote littermate controls. Compared with Rb+/- littermate controls, Rb-/- embryos show increased thickness of the nephrogenic zone (delineated by the dashed yellow line), loss of clear interface between CM and UB (white arrow in C'), high number of pre-tubular aggregates (white arrowheads in C') and detachment of tubular epithelium (yellow arrows in B'). Scale bar (in A, A') =250um. Scale bar (in B-C') =50um.

Figure 3

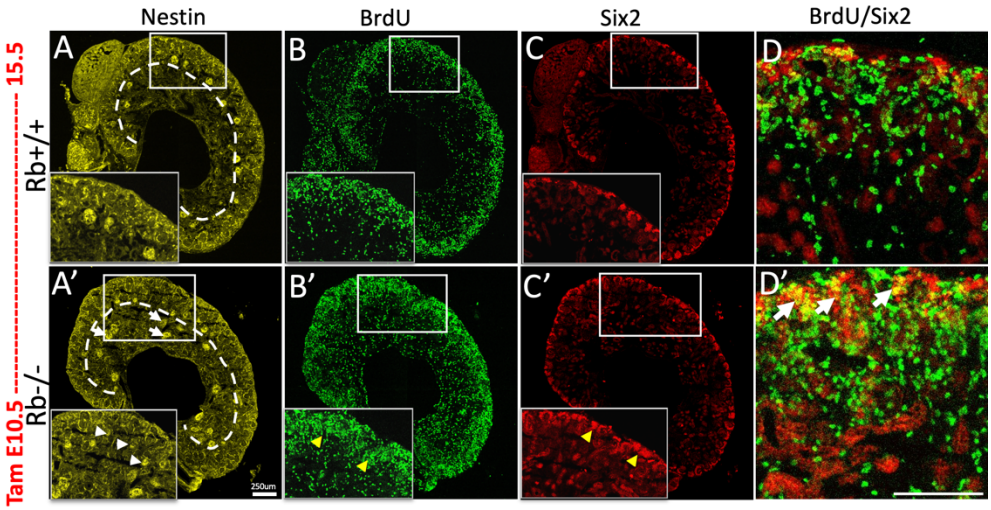
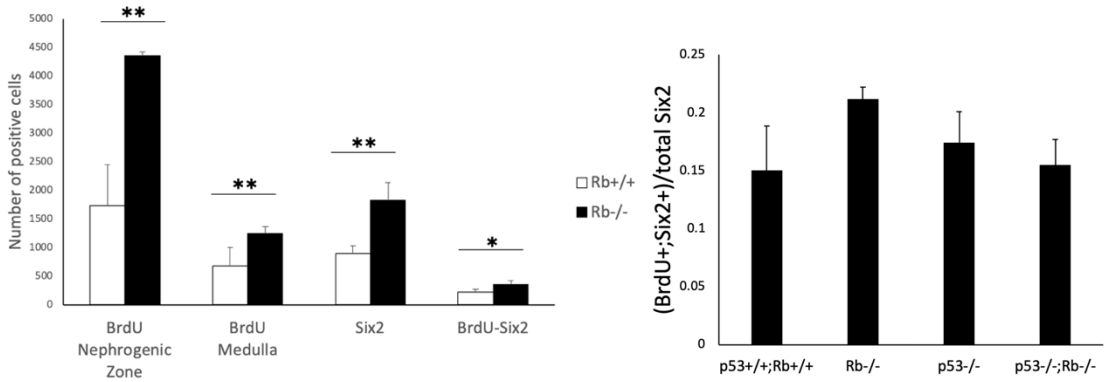


Figure 3 Loss of Rb leads to enhanced nephron progenitor proliferation during development. (A-D') Triple immunostaining performed on sagittal kidney sections using antibodies against Nestin (yellow), Six2 (red) and BrdU (green) in Rb+/+ (A-D) and Rb-/- (A'-D') embryos at E15.5 (treated at E10.5). (D-D') are higher magnification pictures showing the overlap in BrdU and Six2 expressions. Compared with Rb+/+ littermate controls, the Rb-/- embryos have an increase in the number of Nestin-expressing SSB (white arrowheads in A'-inset-), displaced Nestin-positive glomeruli pushed towards the inner medulla (white arrows in A'), upregulated Six2 and BrdU expressions (yellow arrowheads in insets in B' and C'). Note that most (BrdU+;Six2+) co-labeled cells are localized at the lateral edges of the cap mesenchyme, indicating that they are primed NPCs or pre-tubular aggregates (white arrows in D'). White dashed lines in A and A' delineates the nephrogenic zone. Scale bar =250um

Graph 1 a) b)



a) Quantification of single and double positive cells expressing BrdU and/or Six2 in Rb-/- versus Rb+/+ embryos. There is 2.51 and 1.85-folds increase in BrdU+ cells in the nephrogenic zone and the whole medulla at E15.5, respectively. Around 2 and 1.77-folds increase was also detected in Six2+ and (BrdU+;Six2+) double positive cells in the nephrogenic zone in the absence of Rb, respectively. Cell counts were done on three consecutive sagittal sections at medial level and the average of the absolute number of positive cells across the whole nephrogenic zone or medulla region is reported here. Error bars represent SD of measurements from n=3 and asterisks indicate a statistically significant difference between genotypes using t-tests, (*) represents $p<0.05$ and (**) represents $p<0.01$

b) Graph showing no difference in the ratios of double positive (BrdU+;Six2+) cells over the total number of Six2+ cells in Rb-/-, p53-/-, p53-/-;Rb-/- embryos compared with wild type embryos at E15.5. Error bars represent SD of measurements from n=3.

Figure 4

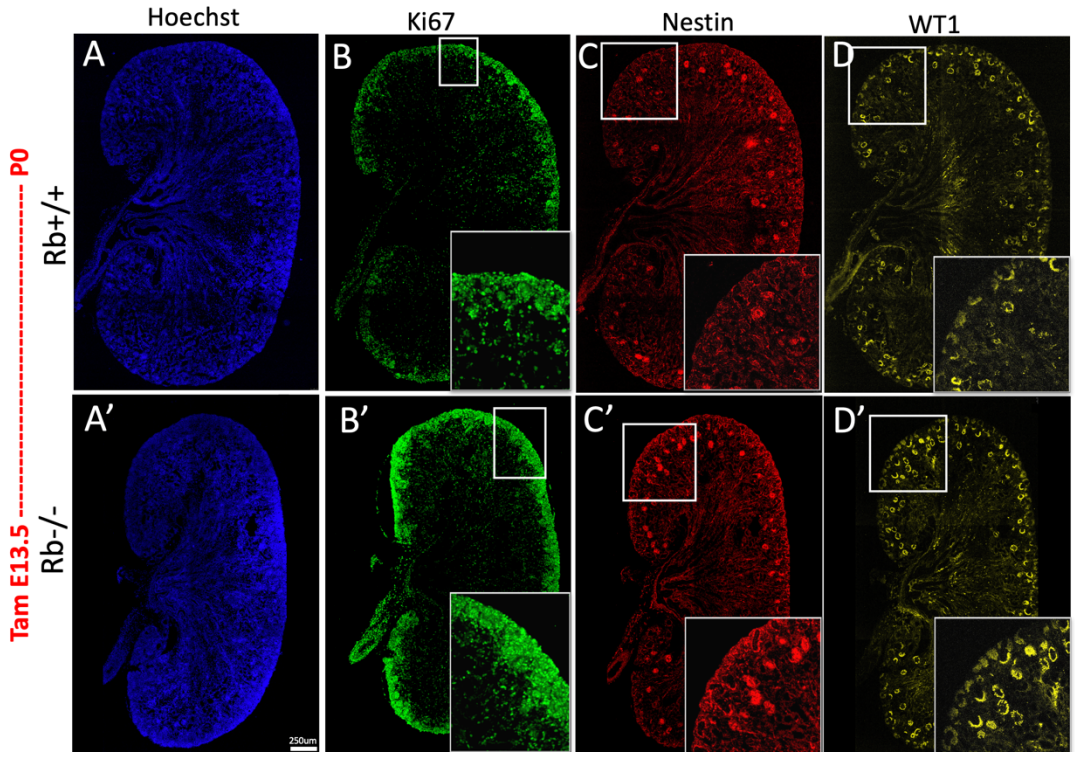
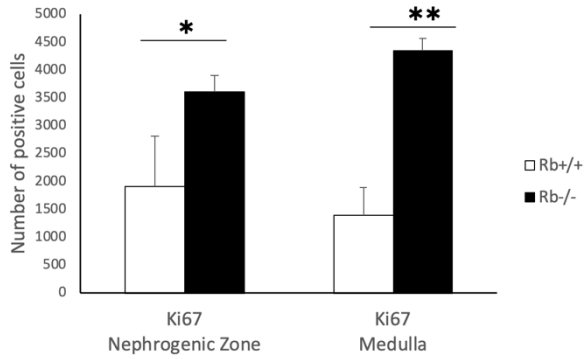
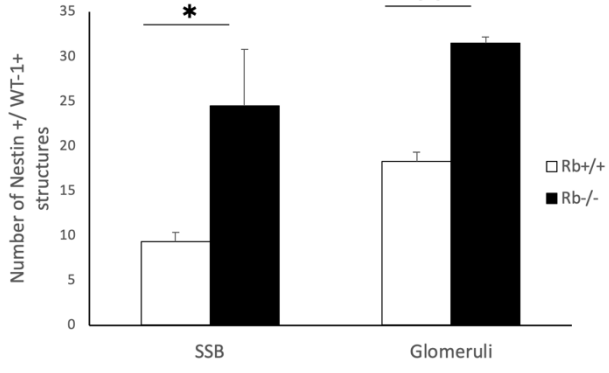


Figure 4 Enhanced nephrogenesis in the Rb mutant kidney. (A-D') Triple immunostaining performed on sagittal kidney sections at P0 using anti-Ki67 (green), anti-Nestin (red) and anti-WT1 (yellow) in Rb-/- embryos (A'-D') and Rb+/+ wild type littermates (A-D). Note, in the absence of Rb, the increased cell proliferation inside the cortical region as shown by Ki67 staining, and the increased number of glomeruli stained with Nestin (C'; inset) and WT1 (D'; inset) in comparison with controls (B) and (C), respectively. Scale bar = 250um.

Graph 2



Graph 3

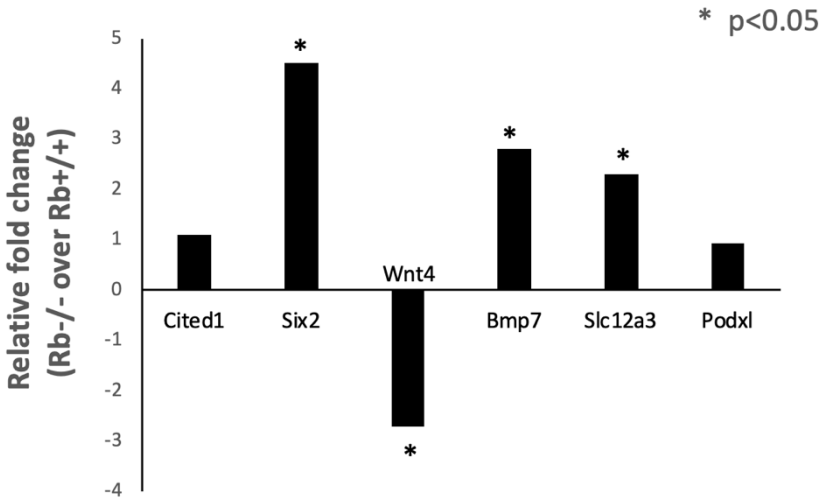


Graph 2 Quantification of the total number of Ki67+ cells showing 1.92 and 2.6 folds increase in cell counts in the nephrogenic zone and the medulla in Rb mutant embryos compared to

controls at P0, respectively (treatment done at E13.5). Counts were done on three consecutive sagittal sections at medial level and the average of the absolute number of positive cells across the whole nephrogenic zone or medulla region is reported here. Error bars represent SD of measurements from n=3 and asterisks indicate a statistically significant difference between genotypes using t-test, (*) represents $p<0.05$ and (**) represents $p<0.01$

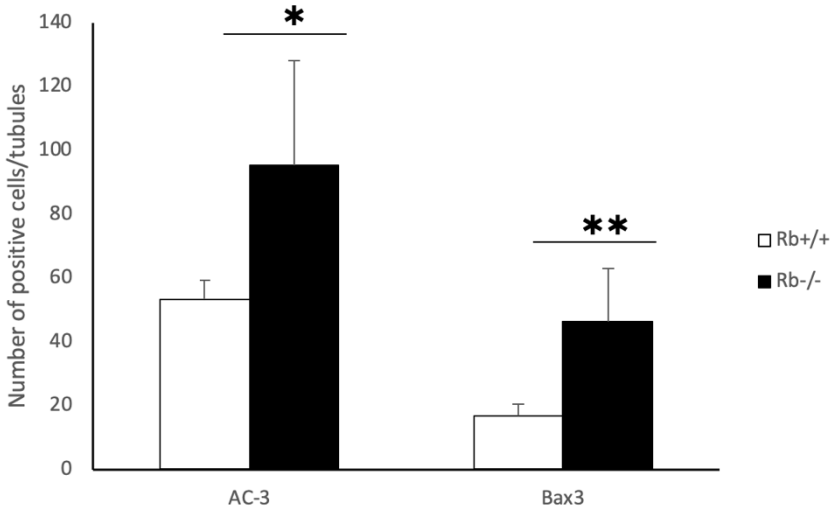
Graph 3 Quantification of the numbers of (Nestin+; WT1+) S-shaped bodies (SSB) and (Nestin+; WT1+) glomeruli in the whole kidney showing 2.63 and 1.72 folds increase in Rb-/- versus Rb+/+ at P0, respectively (treatment done at E13.5). Counts were done on three consecutive sagittal sections at medial level and the average of the absolute number of positive cells across the whole kidney section is reported here. Error bars represent SD of measurements from n=3 and asterisks indicate a statistically significant difference between genotypes using t-test, (*) represents $p<0.05$ and (**) represents $p<0.01$

Graph 4



Changes in gene expression performed by qRT-PCR using cDNA extracted from dissected and dissociated nephrogenic zones in Rb mutant and control kidneys at P0 (treated at E13.5, refer to the white dashed line in figure 3 delineating the dissected region of the NZ). Results showed 4.5, 2.7 and 2.3-folds increase in the expressions of Six2, Bmp7 and Slc12a3 in the absence of Rb, respectively. There is a 2.7 decrease in Wnt4 expression and no significant change in Cited 1 or Podx1 expressions between genotypes. Error bars represent SD of measurements from n=4 and asterisks indicate a statistically significant difference between genotypes using t-test, (*) represents $p<0.05$ and (**) represents $p<0.01$

Graph 5



Quantification of AC-3+ cells and Bax3+ tubules detected in the whole kidney in Rb-/- versus Rb+/+ at E17.5 (4 days post-Rb deletion). Note the significant increase by 1.79 and 2.74 folds in AC3+ cells and Bax3+ tubules, respectively. Cell counts were done on three consecutive sagittal sections at medial level and the average of the absolute number of positive cells/tubules across the whole kidney section is reported here. Error bars represent SD of measurements from n=3 and asterisks indicate a statistically significant difference between genotypes using t-test, (*) represents $p<0.05$ and (**) represents $p<0.01$.

Figure 5

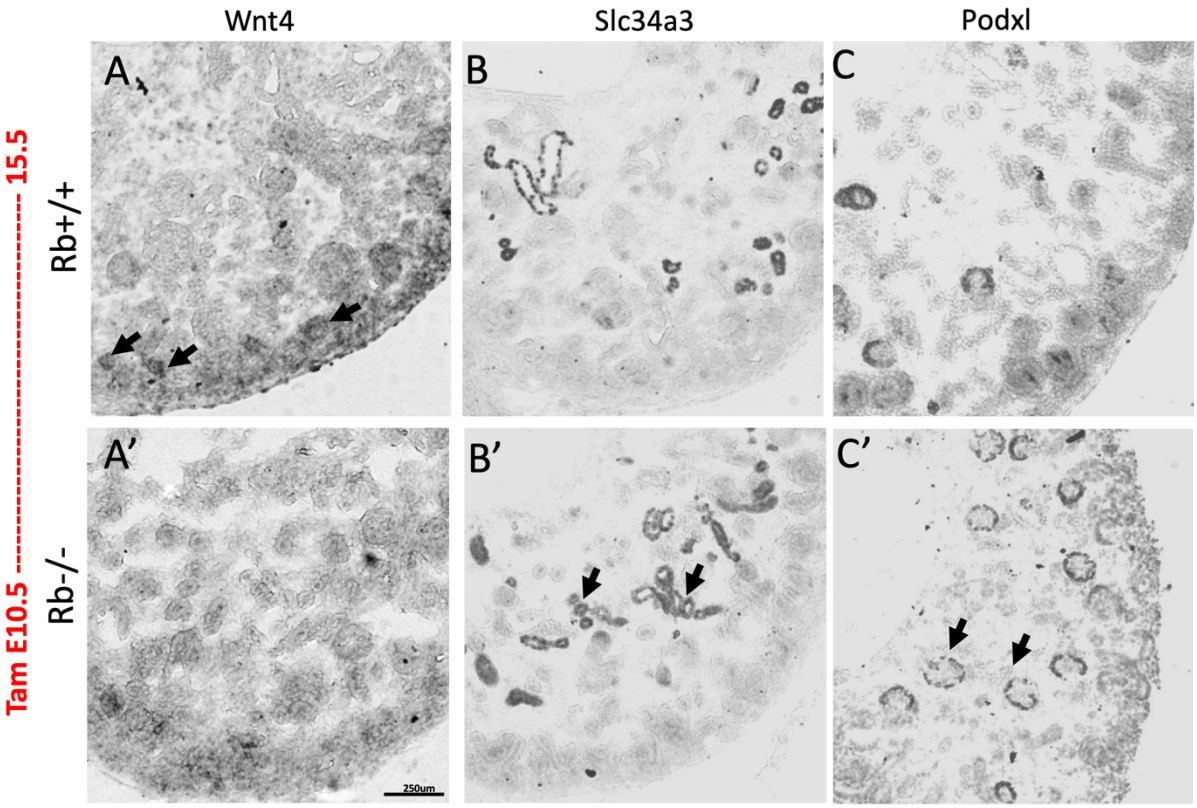


Figure 5: Enhanced nephron differentiation in the mutant Rb kidney. (A-C') mRNA expressions of three differentiation genes marking distinct stages of kidney development was assessed by *in situ hybridization* in Rb-/- versus Rb+/- at E15.5: the mesenchymal to epithelial induction marker, Wnt4 (A, A'), the proximal tubule marker, Slc34a (B, B') and the podocyte marker, Podxl (C, C'). Note the downregulation in the transcript expression of Wnt4 (black arrows in A) and upregulation in the expressions of Slc34a3 and Podxl (black arrows in B' and C'). Scale bar = 250um.

Figure 6

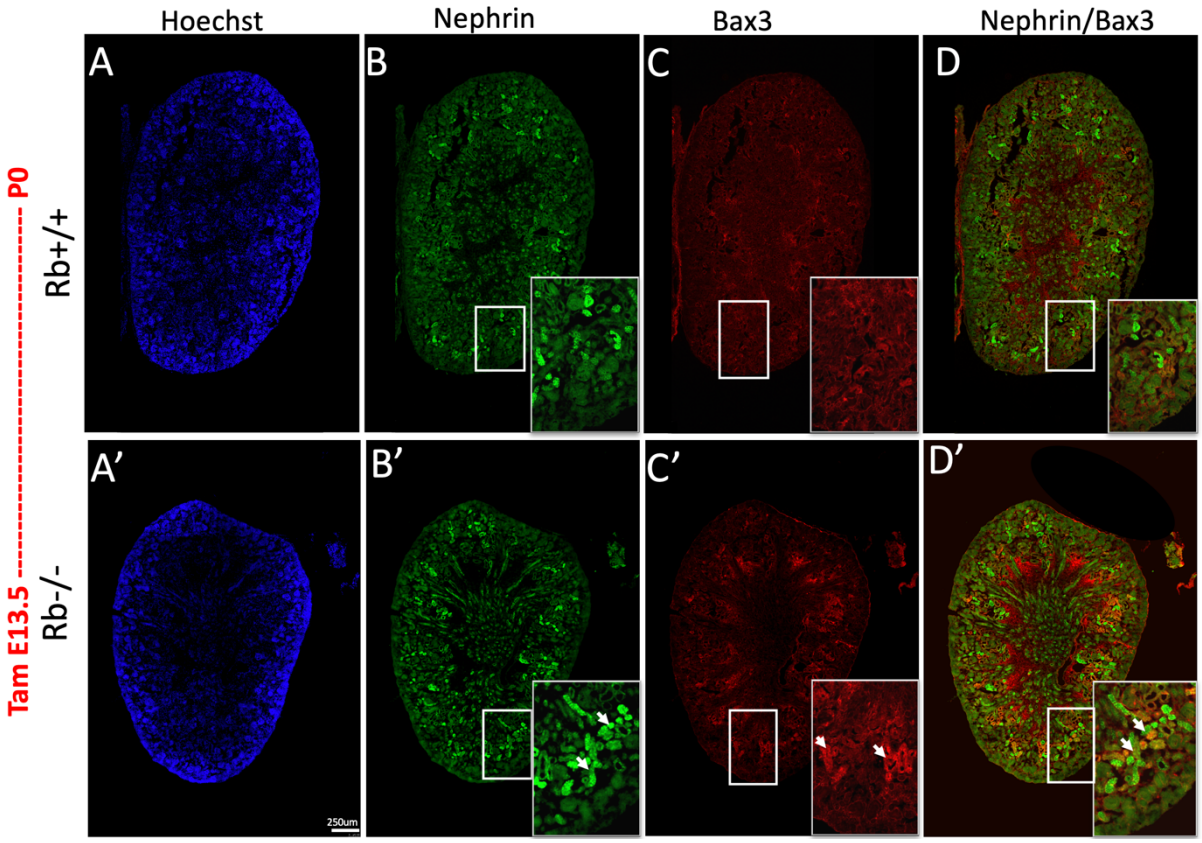


Figure 6: Increased apoptosis in Rb mutant kidneys at birth. (A, A') Hoechst staining, (B, B') Immunostaining showing upregulated expression of the nephron differentiation marker, Nephrin, (white arrows in insets in B'-D') as well as increase in renal tubules undergoing apoptosis by Bax3 staining (white arrows in inset in C'). (D, D') panels showing overlapping patterns of expression of both markers in the absence of Rb. Scale bar=250um.

Figure 7

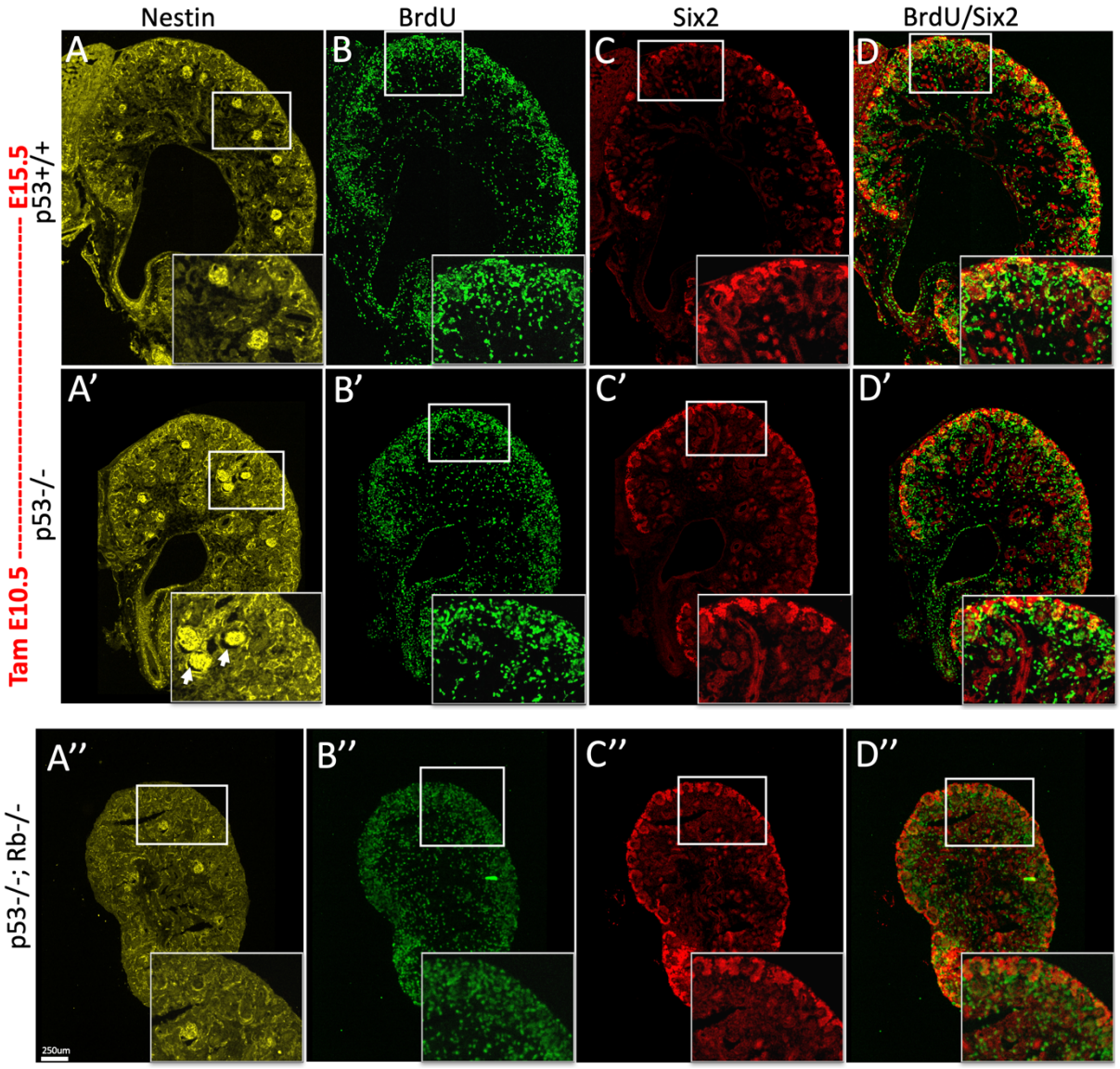
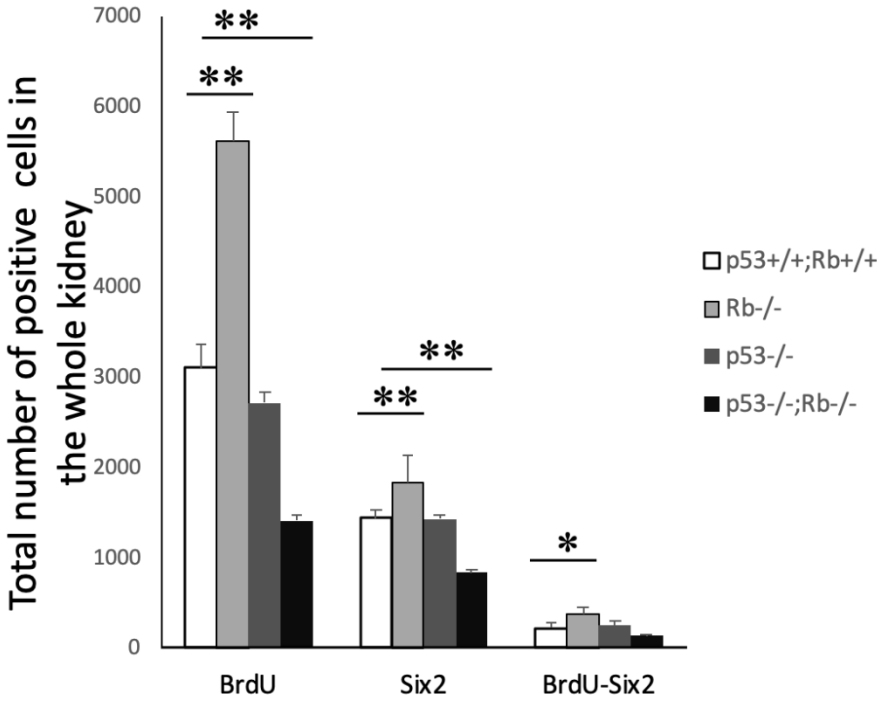


Figure 7 p53 deletion leads to multiple renal defects and exacerbated defects when combined to the loss of Rb in the developing kidney. (A-D'') Triple immunostaining performed on sagittal kidney sections using anti-BrdU (green), anti-Nestin (yellow) and anti-Six2 (red) in p53+/+ (A-D), p53-/- (A'-D'; Nestin-CreERT2-YFP;p53$^{fl/fl}$) and Rb;p53 double mutant embryos (A"-D"; Nestin-CreERT2-YFP; Rbfl/fl;p53fl/fl) at E15.5 (2 days after tamoxifen treatment). Compared with p53 littermate controls, p53 mutant kidneys exhibit significant reduction in kidney size, irregularly shaped glomeruli (compare B' and B) and nephron maturation defects manifested by enlargement of Bowman's capsule (white arrows in inset in A'). Of note, there was no significant change in cell proliferation in the absence of p53 as assessed by BrdU incorporation (compare B' with B and refer to graph 6 below); this was also true for Six2 staining (C-C''). Moreover, compared with p53 mutant embryos, p53 and Rb double mutant embryos showed a more severe reduction in size and a pronounced decrease in the number of immature nephrons (compare A-A''). Scale bar = 250um

Graph 6



Quantification of single and double positive cells expressing BrdU and/or Six2 in Rb-/-, p53-/-, p53-/-;Rb-/-versus wildtype embryos. We found no significant change in all cell counts shown here in p53-/- embryos compared with wild type. However, there is a significant decrease by 2.19, 1.72 and 1.67 folds in the numbers of BrdU+ cells, Six2+ cells, and (BrdU+; Six2+) double positive cells in the nephrogenic zone in double mutant animals compared with wild type controls, respectively.

Cell counts were done on three consecutive sagittal sections at medial level and the average of the absolute number of positive cells across the whole kidney section is reported Error bars represent SD of measurements from n=3 and asterisks indicate a statistically significant difference between genotypes using t-test, (*) represents $p<0.05$ and (**) represents $p<0.01$

Figure 8

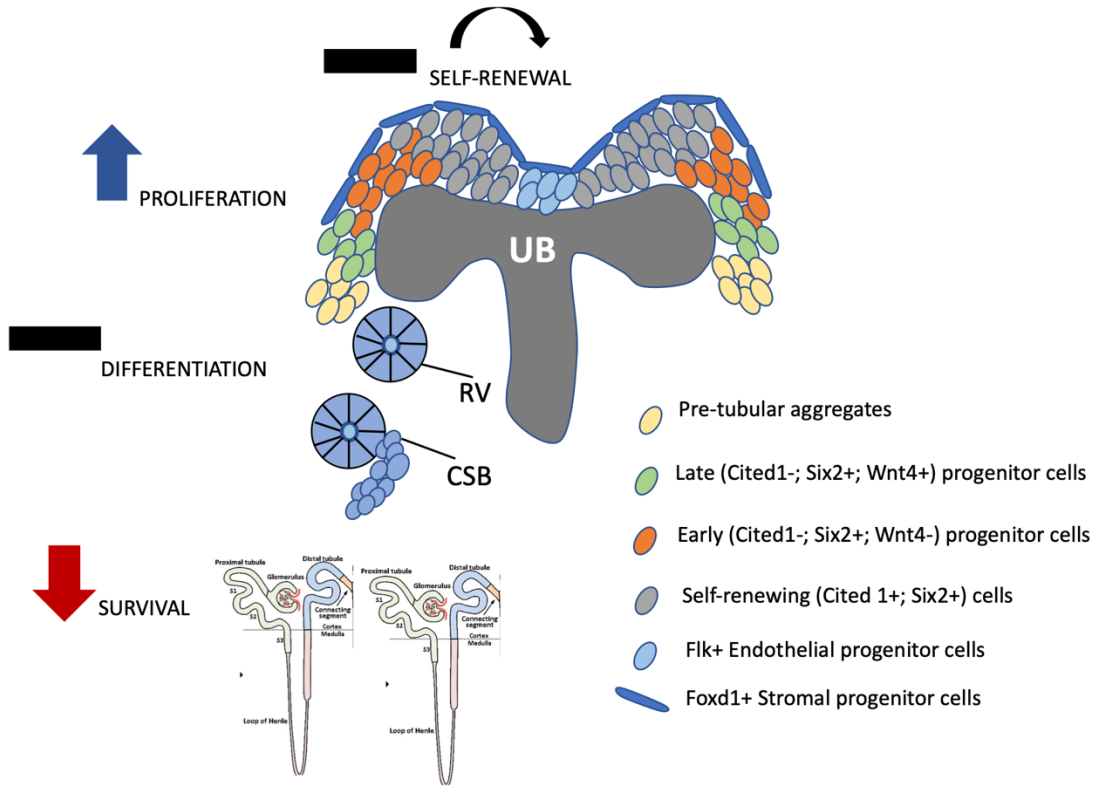


Figure 8 Schematic diagram illustrating the role of Rb during nephrogenesis. Rb specifically controls proliferation of (Cited1-; Six2+; Wnt4-) -early progenitor population- (but not the self-renewing (Cited1+; Six2+) cell population), without affecting subsequent differentiation of RV into CSB or formation of nascent nephrons. Rb is also required for nephron survival. **In the absence of Rb**, we report decreased cell survival (Red arrow), increase in progenitor proliferation Blue arrow; and no major change in self-renewal or differentiation **(solid black line).** UB: Ureteric bud. RV: Renal vesicle. CSB: Comma-shaped body.

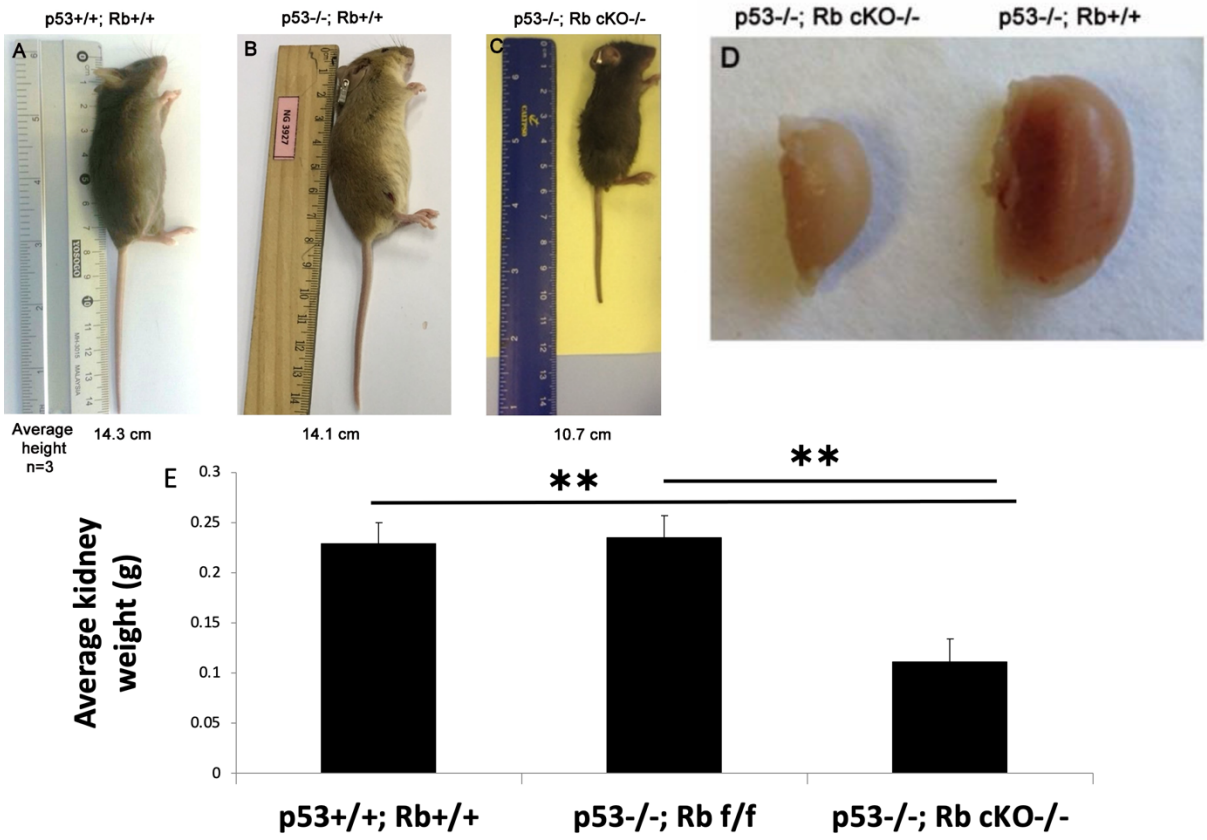
Table 1

Cell type/nephrogenic structures/ nephron components	Effect(s) of Rb deletion	Effect(s) of p53 deletion
Self-renewing (Cited 1+; Six2+) cells	No apparent effect on self-renewal	Reduced self-renewal
Early (Cited1-; Six2+; Wnt4-) progenitor cells	Increased and ectopic progenitor proliferation	No significant effect on progenitor proliferation
S-shaped bodies (SSB)	Increased number of SSB	Decreased number of SSB
Proximal/Distal tubules	Increased production of tubules; Detachment and degeneration of tubular epithelia (cell death)	Decreased number of tubules Increased tubular cell death
Immature glomeruli	Increased number of glomeruli, normal morphology Glomerular cell death	Decreased number of glomeruli, irregularly shaped glomeruli, expansion of Bowman's capsule

Table 1 Summary of the phenotypes observed following loss of Rb or p53 during kidney development.

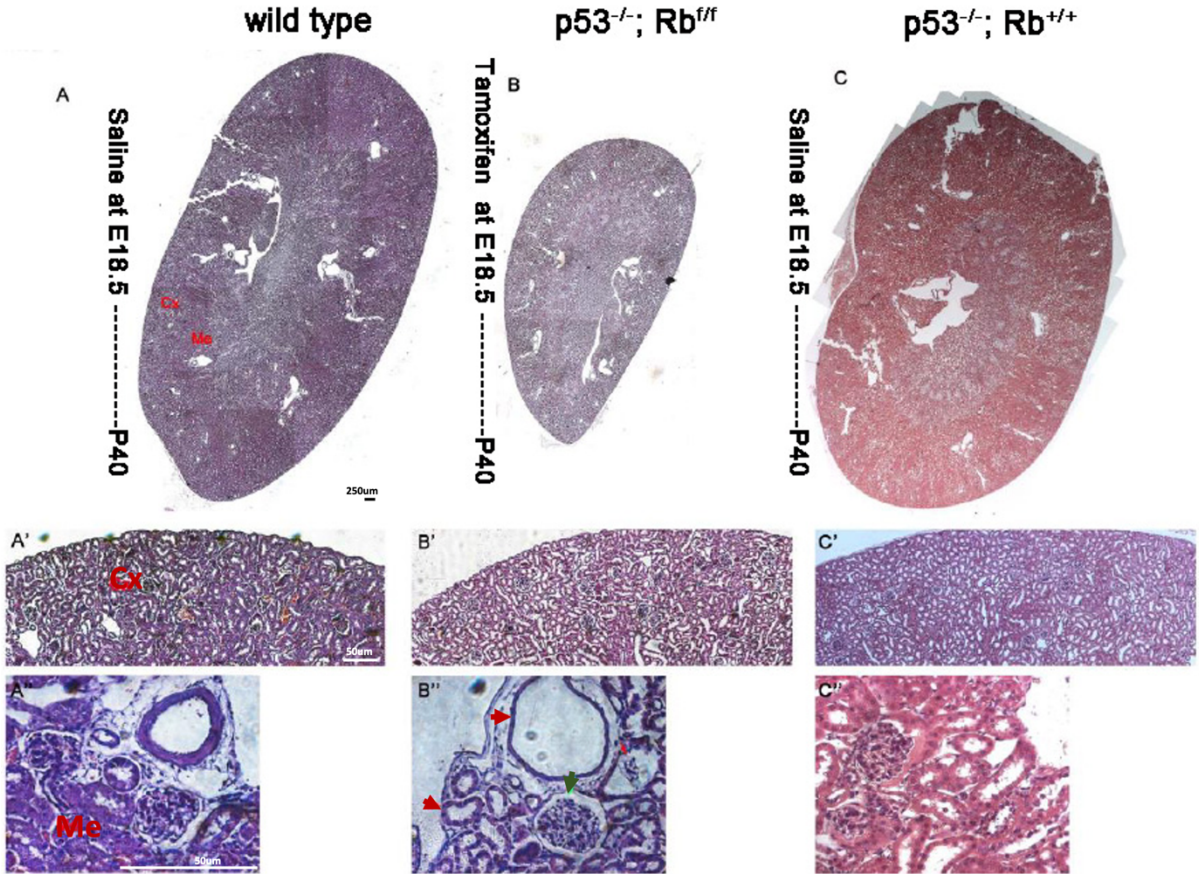
SUPPLEMENTARY FIGURES

Suppl. Figures 1 and 2



Supplementary Figure 1: p53-/-; RbcKO-/- mice show reduced body size and abnormal kidney development. (A, C) Images of P40 (A) wild type, (B) p53-/- and (C) p53-/-; RbcKO-/- animals showing a significant reduction in body size in the latter genotype compared with the other two genotypes (n ranging from 2 to 3 per genotype). (D) Images showing significant reduction in the size of the embryonic kidney in p53-/-; RbcKO-/- embryos compared with p53-/- embryos. (E) Average kidney weight per genotype, t-test, **p<0.01.

Suppl. Figure 2



Supplementary Figure 2: Renal developmental defects in p53-/-; RbcKO-/- mice. (A-C'') Hematoxylin and eosin staining performed on kidney sagittal sections and showing renal hypoplasia, dilation and thinning of tubules (red arrows in B'') and expansion of the mesangial matrix and interstitial space in glomeruli (green arrow in B'') in p53-/-; RbcKO-/- mice (tamoxifen-treated at E18.5 and sacrificed at P40) compared with wt and p53-/- mice. Cx; cortex, Me: medulla

Suppl. Table 1

Age at treatment	Litter size (n)	Survival rate (n)	Age at time of death / (n)	Age at sacrifice / (n)
E13.5	5	0/5	P2 (5/5)	NA
E15.5	3	0/3	P2 (2/3) P60 (1/3)	NA
E18.5	5	3/5	P2 (1/5) P40 (1/5)	P40 (n=3)
P3	9	0/9	P7 (9/9)	NA
P8	8	0/8	P15 (8/8)	NA
P21	6	0/6	P25 (6/6)	NA
P30	4	3/4	P60 (1/4)	P60 (1/4) P90 (2/4)
P60	10	9/10	P120 (1/10)	P90 (1/10) P180 (8/10)

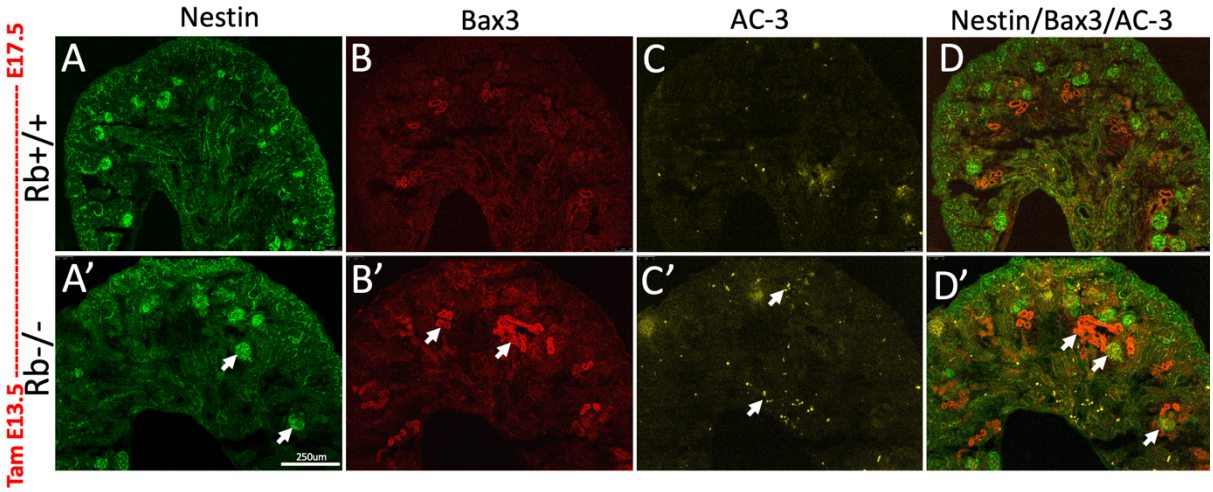
Suppl. Table 1: Summary of the survival rates of Nestin-CreER^{T2}-YFP;Rb$^{fl/fl}$;p53-/- mice treated with tamoxifen at various developmental and adult ages. Note that all animals treated between E13.5 and P21 died before one month of age with the exception of three mice treated at E18.5 that survived until P40, and sacrificed at P40 for phenotypic analysis.

Suppl. Table 2

Genotype	Mouse	Creatinine levels (micromol/L) (using enzymatic assay)	Average per genotype (micromol/L)
p53-/-; Rb f/f Nestin-CreERT2; YFP/YFP (not treated with tamoxifen)	Single mutant 1	5.4	7.86±2.25
	Single mutant 2	9.8	
	Single mutant 3	8.4	
p53-/-; Rb cKO -/- Nestin-CreERT2; YFP/YFP (treated with tamoxifen)	Double Mutant 1	34.9	44.05±12.94 *
	Double Mutant 2	53.2	
			* p<0.01

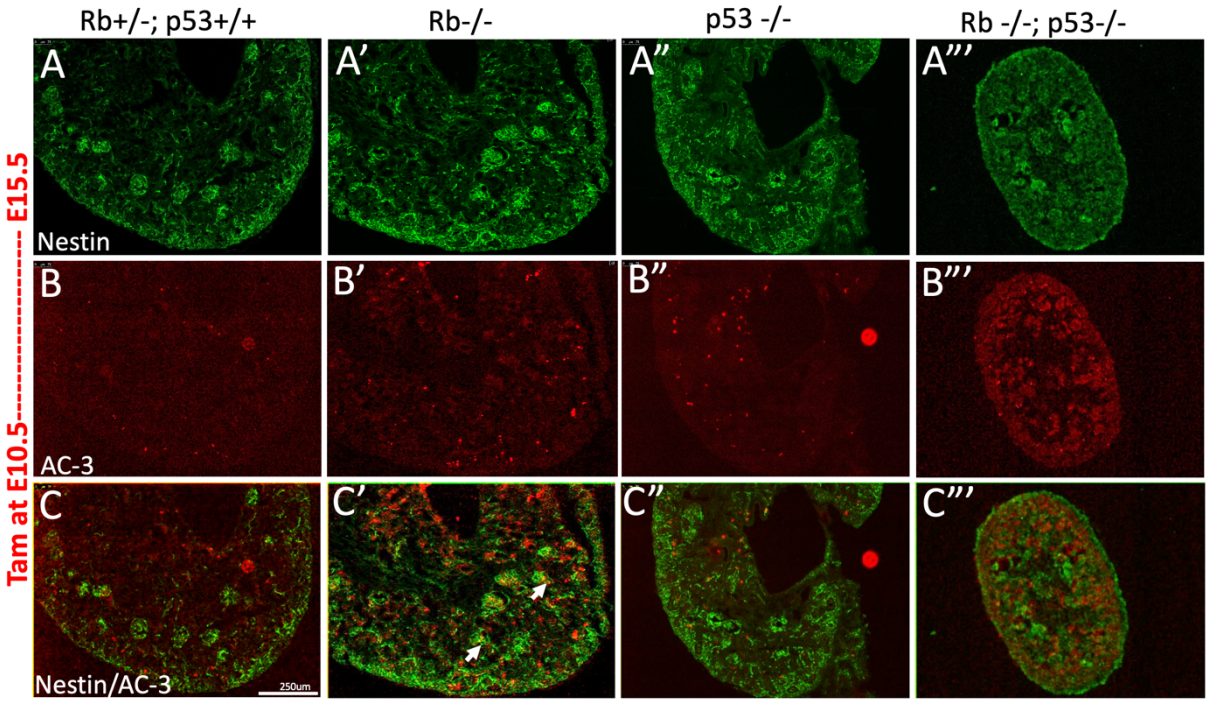
Suppl. Table 2 Blood creatinine levels reveal **s**evere kidney failure in **tamoxifen treated** Nestin-CreER^{T2}-YFP;Rb$^{fl/fl}$;p53-/- mice in comparison with p53-/- untreated animals of the same age.

Suppl. Figure 3



Supplementary Figure 3: Survival defects in the developing kidney in Rb-/- embryos at E17.5. (A-C') Triple immunostaining with anti-Nestin (A, A'), anti-Bax (B, B') and anti-AC3 (C, C') showing ectopically displaced Nestin+ glomeruli in the inner medulla (A'; white arrows), as well as an increase in the number of Bax3+ tubules (B'; white arrows) and AC3+ cells (C'; white arrows) in Rb-/- embryos compared with littermate controls. Scale bar =250um

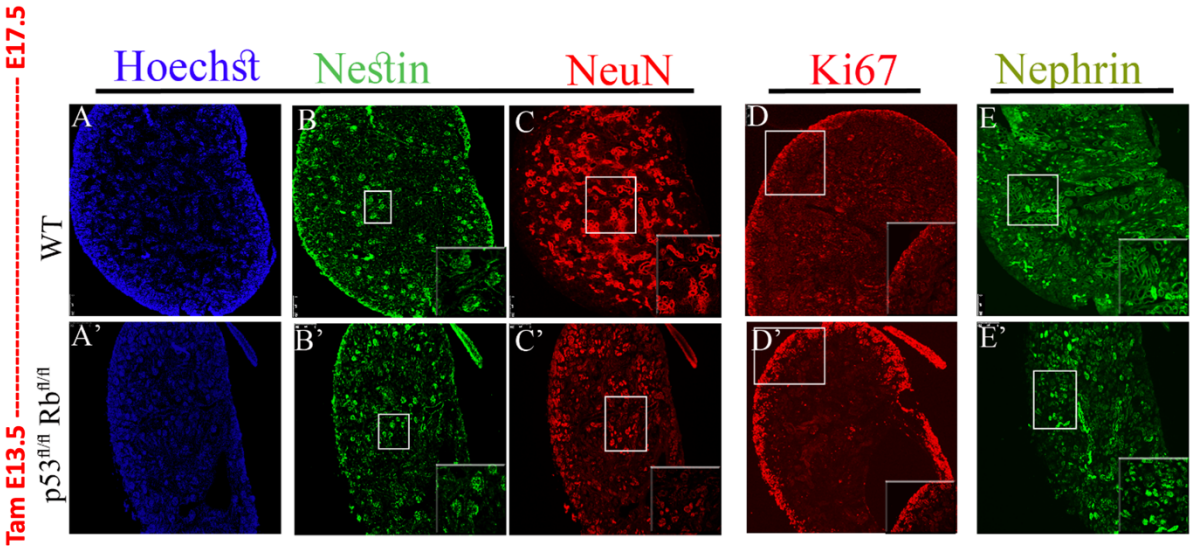
Suppl. Figure 4



Supplementary Figure 4: Severe survival defects in the developing kidney in the absence of both Rb and p53. (A-C"') Immunostaining with anti-Nestin (A-A"') and anti-AC-3 (B-B"') showing severe nephron maturation defects coupled to massive apoptosis with almost complete loss of Nestin+ glomerular structures in p53-/- embryos (A") and Rb;p53 double mutant embryos

(A'") compared with Rb+/-; p53+/+ controls (A). Note that cell death is affecting double labelled Nestin+/AC-3+ glomeruli (C'; white arrows) in Rb-/- embryos. Scale bar =250um

Suppl. Figure 5



Supplementary Figure 5: Severe renal proliferation and differentiation defects in the developing kidney in Rb;p53 double mutant embryos at E17.5. (A-E') Immunostaining with antibodies against Nestin (B-B'), NeuN (C-C' late differentiation marker), Ki67 (D-D') and anti-nephrin (E-E') showing ectopic proliferation inside the cortical region (D'), severe differentiation and maturation defects (C' and E'), displaced glomeruli with irregular shape (B') in Rb;p53 double mutant embryos compared with WT at E17.5.

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F. Isolation of Nephron Progenitor Cells from Embryonic Kidneys of Nestin-Cre ERT2/Rosa26-YFP Mice using Indirect FACS staining

Abstract

Utilization of inducible Nestin-Cre ERT2 transgenic mouse models has proved to be highly efficient in studying adult neurogenesis in the brain in the past decades. However, the suitability of using inducible Nestin-Cre ERT2 transgenic mice to study renal development is not assessed to date. A study by Dubois et al. 2006 reported Nestin expression in the developing and adult kidneys by examining Cre-mediated recombination in Nestin-Cre1 transgenic mice. Using the Nestin-CreERT2/YFP line designed by Lagace et al., we show here Nestin expression in the developing kidney including the Cap Mesenchyme (CM), the deep cortex and the developing glomeruli starting mid-gestation until birth (Lagace et al. 2007). The CM consists of the Six2+ nephron progenitor population that gives rise to all epithelial components of the nephron. In this study, we describe an efficient methodology to sort (Six2+;GFP+) double positive cells present in the Nestin-expressing population in the embryonic kidneys in transgenic Nestin-Cre ERT2/Rosa26-YFP mice using indirect FACS staining (Lagace et al. 2007). We show that the GFP+ population cannot be sorted from these embryonic kidneys without proper fixation and staining prior to performing FACS. Our approach highlights the need for cell fixation before staining in order to preserve cell integrity. This is in contrast with the successful isolation of unstained and unfixed GFP+ cells from the adult brain in the same Nestin-Cre ERT2/Rosa26-YFP line by relying on the endogenous YFP signal.

1. Introduction

Over the previous two decades, studies using transgenic mouse models provided significant insight onto key developmental pathways underlying kidney formation and nephrogenesis. Significantly, research into renal morphogenesis in the mouse is anticipated to contribute to our understanding of kidney disease in humans. The Cap Mesenchyme (CM) is a region of the metanephric mesenchyme consisting of a Six2+ progenitor population condensed around the ureteric bud (UB) tips and contributes to the genesis of the epithelial components of the developing nephron by associating with the UB and stromal elements. The homoeobox gene, *Six2*, was first revealed to play a role in repressing the epithelialization signals that promote nephrogenesis in order to maintain self-renewal of progenitor cell population during kidney development in mice (Self et al. 2006b). Later, Six2 was identified as a key molecular marker of the multipotent nephron progenitor population that generate all nephron structures throughout renal organogenesis (Kobayashi et al. 2008). Most studies addressing developmental and regulatory pathways governing nephron progenitor cells (NPC) proliferation, differentiation and survival during nephrogenesis relied on using Six2-Cre transgenic models (e.g. generated by Kobayashi et al.) to achieve targeted conditional deletion of genes specifically in the progenitor population (Kobayashi et al. 2008). Nestin is an intermediate filament protein widely known as a neural stem and progenitor cell marker, and, was later shown to be also expressed in renal progenitors. A study by Dubois et al. 2006 examined Cre-mediated recombination in the Nestin-Cre1 transgenic line, in which the rat Nestin promoter and an enhancer element (intron 2 of the Nestin gene) drives the expression of the Cre recombinase enzyme. The study reported for the first time Nestin expression in the developing kidney (Dubois et al. 2006). Further evidence provided by cell lineage studies using the same Nestin-Cre1line revealed

specific distribution of Nestin expression throughout the CM compartment excluding UB structures in mice. Hence, Nestin expression was detected in Cited1+ CM progenitors starting at E11.5 as well as CD31+ and Flk1+ endothelial cells of the immature glomeruli during development, and, becomes restricted to Nephrin+ podocytes and associated with CDK5 in mature glomeruli in the adult kidney (J. Chen et al. 2006; Bertelli et al. 2007b). In humans, Nestin was detected in different cells of the developing human kidney including renal vesicles, podocyte layer as well as endothelial cells of S-shaped bodies and mesangial progenitors (Bertelli et al. 2007b). Interestingly, one study also reported the presence of a reservoir of Nestin+ mesenchymal stem cells (MSCs) in the postnatal kidneys. These Nestin+ cells were shown to have a reparative potential when injected into acute ischemic kidney injury murine models (M. H. Jiang et al. 2015). Moreover, it is proposed that under damage conditions the induced increase in Nestin expression stimulates mesangial cell proliferation (Daniel et al. 2008) and promotes proximal tubular cell migration during the repair process (Wen et al. 2012). In this study, we used the Nestin-CreERT2-YFP transgenic line designed by Lagace et al. to isolate YFP+ and (YFP+; Six2+) renal cells during development. The inducible Nestin-CreERT2 cassette includes the Nestin promoter combined with exons 1-3 of the nestin gene (Lagace et al. 2007), and controls the expression of the Cre recombinase enzyme fused with ERT2 (a mutated estrogen receptor). Tamoxifen, an estrogen receptor modulator, binds to ERT2 allowing Cre translocation to the nucleus where it excises the stop codon flanked by two LoxP sites allowing the expression of the YFP reporter gene that is used to assess recombination efficiency. Using this line, we show localized Nestin expression in the CM, differentiating nephronic structures and developing glomeruli; our results are consistent

with the literature described above **(Fig. 1)**. Our laboratory has successfully sorted GFP+ cells from the adult olfactory bulbs in Nestin-Cre ERT2/Rosa26-YFP (without cell fixation or immunostaining) **(Fig. 2 and Naser et al. 2016).** In this study, we demonstrate the utility of the inducible Nestin-Cre ERT2/Rosa26-YFP transgenic line to isolate (YFP+; Six2+) NPCs from the Nestin-expressing population in the developing kidney, as well as YFP+ cells derived from differentiating nephronic structures and immature glomeruli. We optimized a protocol to successfully fix, stain and sort the double positive (Six2; GFP) cell population using indirect FACS staining. **(Fig. 3B and 4B)**. Notably, we were not able to sort unfixed and unstained GFP+ cells from the embryonic kidneys in this line by relying on endogenous YFP fluorescence (unlike in the adult olfactory bulbs) **(Fig. 3A and 4A)**.

Hence, we show that fixation followed by staining is required in order to preserve cell integrity before staining. Using this optimized protocol, we show that the majority of GFP+ cells can be collected intact in the parent population, and used for downstream applications e.g. in gene expression studies.

2. Materials

2.1 Preparation of solutions and buffers

1. Phosphate Buffer Saline (PBS): To prepare 1 L of 10x PBS, dissolve 80g of NaCl, 14.4g of Na_2HPO_4, 2g of KCl, and 2.4g of KH_2PO_4 in 1L of deionized distilled water (ddH_2O). Autoclave solution and adjust the pH to 7.4 by adding 3-4 drops of 12N HCl. To prepare 1 L of 1xPBS, dilute 100mL of 10x PBS in 900mL of ddH_2O, then readjust the pH to 7.4.

2. Paraformaldehyde (PFA): To prepare 100ml of 1%PFA, weigh 1g of PFA (HIMEDIA, RM 3660), dissolve them in 80ml ddH_2O, add 25ul 10N NaOH and heat below 65°C while stirring till the PFA dissolves. Filter the solution through a filter paper then add 10ml 10x PBS. Chill at 4°C before use. For long-term storage, store aliquotes at -20°C.
3. Blocking Solution: To prepare 1mL, dissolve 0.01g of bovine serum albumin (BSA), 50µL of donkey serum (final concentration, 5%), and 30µL of 10% Triton-X (final concentration, 0.3%) in ~920µL of 1x PBS.

2.2 Staining

1. Primary Antibodies: combination of chicken anti-GFP (1:1000, ab13970) with rabbit anti-Six2 (1:500, Proteintech 11562-1-AP) prepared in blocking solution.
2. Secondary fluorochrome conjugated antibodies: Donkey anti-chicken 488 Alexa Fluor (1:400, Jackson Immunoresearch) and Donkey anti-rabbit Cy5 Alexa Fluor (1:400, Jackson Immunoresearch) prepared in blocking solution.

2.3 Digestion

1. Digestion Medium: To prepare 1ml, we add 978ul DMEM:F12 (Sigma D8437 with HPES, NaHCO3, pyridoxine and L-glutamine), add 0.5mM EDTA (final concentration:1.2mM, GIBCO-15574-038), add 20ul Papain (final concentration:20IU/ml, Sigma p3125-1U/ul).
2. 10%FBS: To prepare 2ml, add 200ul of heat inactivated-FBS (Sigma-F9665) to 1.8ml DMEM:F12.

3. Percoll Medium: To prepare 5ml, add 1.1ml Percoll (GE-healthcare 17-0891-01) to 3.9ml DMEM:F12.
4. Collection Medium: To prepare 2ml, add 1ml FBS to 1ml DMEM:F12.

3. Methods

3.1 Animals and Tissue treatment

All animals were maintained, and all animal procedures were performed according to protocols approved by the "the Institutional Animal Care and Use Committee"-IACUC. Nestin-CreERT2/Rosa26R-YFP/YFP mice were mated with Rb/p53 floxed/floxed mice (designed by Lagace et al., 2007). Pregnant females were treated according to weight (180 mg/kg) with Tamoxifen Citrate (abcam, ab120656, prepared at 44.8 mg/ml in 60% sunflower oil and 40% DMSO) by oral gavage at -Embryonic day- E10.5 (E0.5 corresponding to the day a vaginal plug was observed). Animals were anesthetized with a mixture of 1.5µl/g Ketamine and 0.25µl/g Xylasine followed by cervical dislocation-. Embryos were then harvested at E18.5 **(Note 1)**. Pups were collected in 1x PBS solution then decapitated **(Note 1)**. Collected kidneys are placed in 1x PBS solution in a petri dish.

3.2 Digestion of kidney tissue

1. Remove 1xPBS from the petri dish carefully leaving the kidneys behind, then chop the kidney tissues using a clean blade until they become a soft paste.

2. Add 1mL of digestion medium as prepared in **2.3.1** to the paste then transfer the medium and tissue paste to a 2ml tube **(Note 2)**. Use 1ml digestion media for each six E18.5 kidneys **(Note 3)**.
3. Incubate the sample with digestion media at 37ºC for 30 minutes on shaker **(Note 4)**.
4. Meanwhile, prepare 10%FBS as described in **2.3.2** for nourishing the cells and neutralizing the effect of the enzyme. Use 2ml 10%FBS for each 1ml digestion media.
5. After the incubation, triturate the digest 40 times **(Note 5)**, it will become a homogeneous solution then transfer it to a 50mL tube.
6. Add 10%FBS to the digest and mix gently by pipetting 6 times.
7. Centrifuge the sample for 5 min at 2000 rpm at 4°C. If pellet still floating and supernatant is still cloudy, centrifuge for another 5 minutes.
8. Remove supernatant by pouring it slowly without disturbing the pellet **(Note 6)**.
9. Add 1mL of percoll media prepared as in **2.3.3** to pellet then triturate 40 times. Use 1ml of percoll media for each six E18.5 kidneys.
10. Complement with percoll media up to 5mL. Then mix by pipetting 6 times.
11. Centrifuge sample for 15 minutes at 2000rpm at 4°C.
12. Remove supernatant then resuspend pellet in 5ml 1xPBS and mix gently by pipetting.
13. Pass the cell suspension through a 40µm cell strainer (Falcon, 352340) **(Note 7)**.
14. Centrifuge sample for 5 minutes at 2000rpm at 4°C.

15. Remove supernatant then resuspend pellet in 500µL 1xPBS.
16. Count cells using the hemocytometer.
17. Proceed to FACS to sort unstained cells or proceed for staining as in the steps of section **3.3** below. The unstained samples are used for gating the negative population (negative control) (**Fig. 3A and 4A**). We collect the unfixed unstained cell sample in this step and transfer the cell suspension to15ml tube suitable for the BD FACS Aria SORP cell sorter ready for sorting. The fixed unstained cell sample is collected after step **3.3.3** below.

3.3 Indirect FACS Staining

In our procedure, we split the volume in step 15 into two halves (half of the cells (250ul) to be fixed with PFA. We complement the volume to 500ul 1xPBS and then proceed as in step 1 below. The second half (250ul) kept without PFA fixation- for this part we complement the volume to 2ml 1xPBS then procced from starting step 4 below.

1. Fixation: add to the 500ul cell suspension fresh and chilled PFA solution (final concentration:1% PFA), incubate for 10 minutes at room temperature. Dilute with 1xPBS by adding 3ml 1xPBS.
2. Centrifuge the sample for 5minutes at 1000rpm at 4ºC.
3. Remove supernatant then add 2ml 1xPBS to wash the cells.
4. Centrifuge the sample for 5minutes at 1000rpm at 4ºC.
5. Remove supernatant and add 900ul blocking solution. Incubate cells for 30min at 4ºC.
6. Meanwhile prepare primary antibody according to recommended dilutions as described in **2.2.1 (Note 8)**.

7. Incubate with primary antibody for 15minutes on mutator at 4ºC.
8. Centrifuge the sample for 5minutes at 1000rpm at 4ºC.
9. Remove supernatant then add 2ml 1xPBS to wash away non-bound primary antibody.
10. Centrifuge the sample for 5minutes at 1000rpm at 4ºC.
11. Meanwhile, dilute secondary antibody in blocking as described in **2.2.2.**
12. Incubate with 1ml secondary for 15minutes in the dark on mutator at 4ºC.
13. Centrifuge the sample for 5minutes at 1000rpm at 4ºC.
14. Remove supernatant then add 2ml 1xPBS to wash away non-bound secondary antibody.
15. Centrifuge the sample for 5minutes at 1000rpm at 4ºC.
16. Remove supernatant and resuspend pellet in 500µl 1xPBS
17. Count the cells on hemocytometer
18. Proceed to sorting using the BD FACS Aria SORP cell sorter **(Note 9).**

4. Figures:

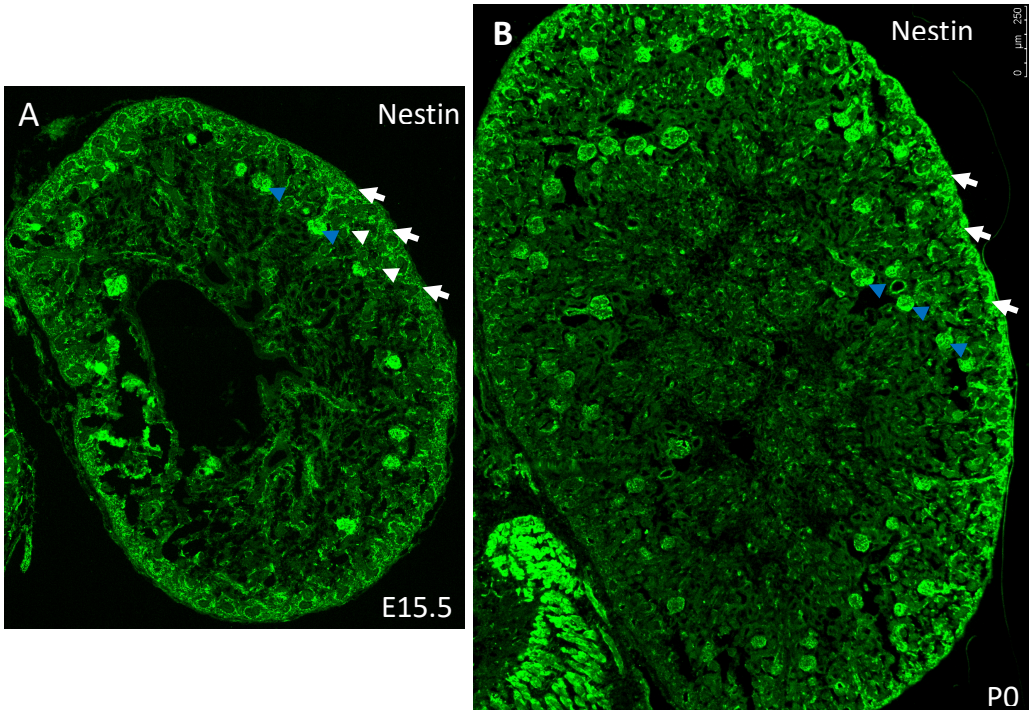

Figure 1 Histological assessment of Nestin expression pattern in the developing kidney of Nestin-Cre ERT2/Rosa26-YFP mice at E15.5 and P0. Immunofluorescent staining using anti-Nestin antibodies on sagittal embryonic kidney sections collected at E15.5 (A) and at P0 (B) demonstrate localization of Nestin expression to the Cap Mesenchyme (white arrows in A and B), in differentiating renal structures in the deep cortex such as S-shaped bodies (white arrowheads in A) and in immature glomeruli (purple arrowheads in A and B).

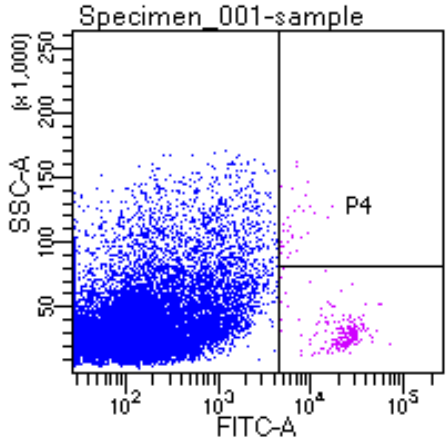

Figure 2 FACS-sorting of GFP+ cells from Nestin-Cre ERT2/Rosa26-YFP adult olfactory bulbs. Live cells from adult GFP+ olfactory bulbs emitted FITC+ signal without the need for staining shown in the P4 population.

A B

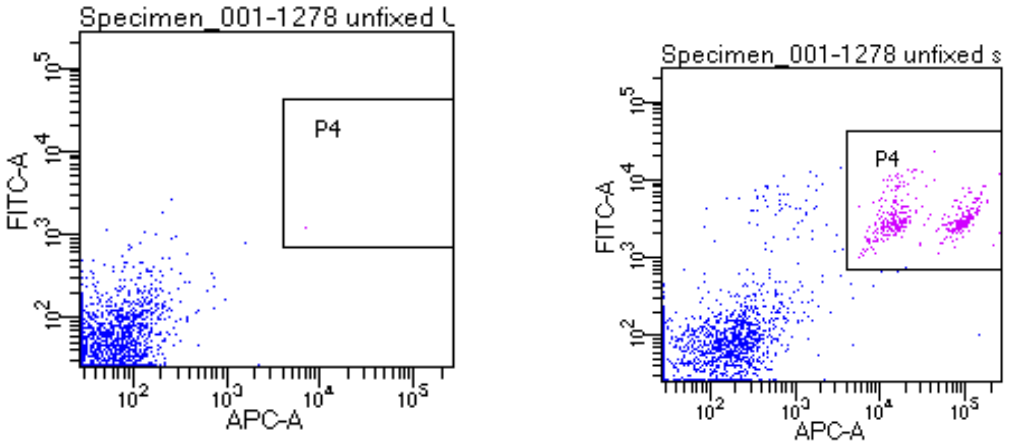

Figure 3. FACS-sorting of unfixed (Six2+;GFP+) double positive cells from E18.5 embryonic kidneys in Nestin-Cre-ERT2/Rosa26-YFP mice treated at E10.5. (**A**) Panel showing the profile for unfixed and unstained cells; FITC and APC signal is absent in these cells as shown in P4. (**B**) Panel displaying the profile for unfixed and stained cells, gated based on high APC and medium to high FITC signal as shown in the P4 population (Six2+;GFP+). Note the presence of a population with low Six2 expression comprised of progenitor cells that are transitioning to pre-tubular aggregates, and, a high Six2+ population characteristic of proliferating progenitor cells.

A B

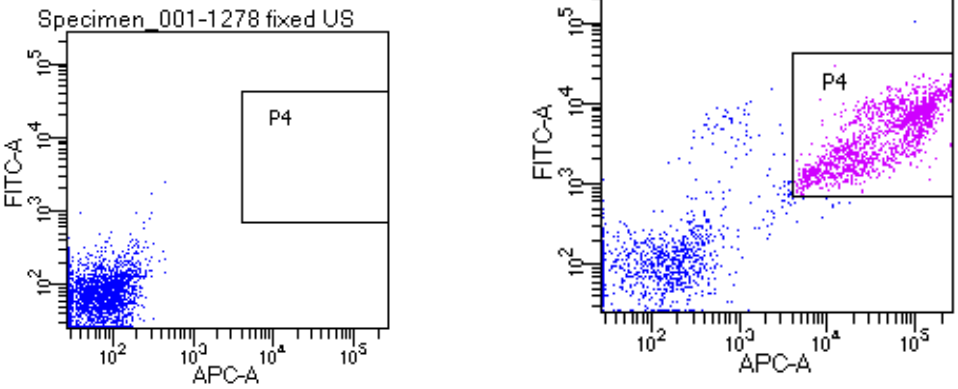

Figure 4. FACS-sorting of fixed (Six2+; GFP+) double positive cells from E18.5 embryonic kidneys in Nestin-Cre-ERT2/Rosa26-YFP mice treated at E10.5. (**A**) Panel showing the profile for fixed and unstained cells; FITC and APC signal is absent as shown in P4. (**B)** Panel displaying the profile for fixed and stained cells, gated based on high APC and medium to high FITC signal as shown in the P4 population (Six2+;GFP+). Fixation preserved the integrity of cells during the staining procedure and yielded a higher percentage of sorted cells in the parent population compared to unfixed stained cells.

5. Notes

1. Remove the uterus containing the embryos out of the pregnant female and place it in a petri dish containing 1xPBS. Remove each embryo from its sac and decapitate. Transfer decapitated embryos to a clean dish. Place the embryo on its back and hold the shoulders using one forceps, using the other forceps, make a fine vertical incision on the abdominal wall of the embryo to expose the internal organs. Grab the tissue at the top of the liver and drag the internal organs from the liver down to the urinary organs out of the embryo. Once the urinary system is dragged out, carefully locate the kidneys attached to the back of the organs, gently detach the pair of kidneys (still connected) from the rest of the organs. Carefully using forceps, hold the tissue separating the two kidneys and using the other forceps hold the adrenal gland and gently remove it, while removing the adrenal gland it will drag the renal capsule (transparent thin membrane) with it peeling it off the kidney, make sure to gently drag the capsule while peeling it without applying too much pressure to avoid splitting the kidney in half. Repeat the same procedure with the other kidney.
2. Use round bottomed 2ml tubes to provide large surface area of contact for optimal enzymatic digestion.
3. To minimize cell loss, rinse the part of the blade that was used to chop the tissue with digestion media.
4. Flick tube before placing it on shaker to ensure the tissue is loose inside the tube (not stuck at the bottom of the tube).
5. While triturating, gently pipette up and down to dissociate pellet. Do not apply too much pressure on the cells, not to kill them.

6. Do not use pipette to remove supernatant, the pressure might cause the pellet to detach off the bottom of the tube.
7. Wet the cell strainer with 1xPBS before adding the cell suspension. Passing the cells through a filter is required to get rid of large clumps of debris or cells, otherwise these will block the nozzle while sorting.
8. Add 1ul chicken anti-GFP (1:1000) and 2ul rabbit anti-Six2 (1:500) to 1ml blocking solution. Do not wash the cells after removing the blocking solution.
9. Technical recommendations to improve the number of cells collected and to preserve cell integrity during the sorting procedure: Nozzle size:100um (the size of the nozzle should preferentially be five times the diameter of the cell), this will reduce the pressure exerted in the cells during sorting. Use 2%FBS/ PBS in the sheath container, this will help nourish the cells during the sorting procedure. Use a viability dye to better assess cell quality before and after sorting.

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CHAPTER 3

III. DISCUSSION

A. On the role of Rb in kidney development: first insights, remaining questions and future directions

Despite being the first tumor suppressor gene to be identified in the early 1980s with critical roles in cell cycle control, survival and senescence, the role of the Retinoblastoma protein, pRb, in mammalian kidney development including humans is still unknown (Sage 2012; Dyson 2016). To our knowledge, there is no report describing the role of Rb in human kidney disease or injury to date. This is the first study to examine and uncover a critical requirement for Rb in kidney development, particularly during nephrogenesis where it specifically controls the rate of nephron progenitor proliferation and importantly, maintains survival in newborn nephrons (**Figure 8 and Table 1**). These functions are noteworthy given that the function of the adult/mature kidney mainly relies on generating the proper number of functional nephrons during development. Moreover, they directly pertain to chronic kidney diseases and renal injury where either loss or damage to mature nephrons is a main contributor among other factors, and cannot be repaired.

To examine the role of Rb, we were the first to use and confirm the suitability of the tamoxifen-inducible Nestin-Cre-ER^{T2}-YFP line originally developed by Lagace et al. for brain research (Lagace et al. 2007). We thus demonstrated the usefulness and high reliability of this line to study gene function during renal development. We particularly reported high Nestin expression in the metanephric mesenchyme lineage throughout development including immature nephrons, and restrictively, in podocytes at late stages (**Figure 1**). Using this line as a Cre recombination method, we optimized a protocol to

efficiently sort nephron progenitor cells (Six2+; GFP+) from embryonic kidneys by indirect FACS staining. These highly purified and recombined cells (or other sorted populations) could be used in several downstream applications to further characterize the cellular and molecular changes following gene deletion e.g. cell cycle analysis, changes in gene expression etc. One other successful application associated with the use of this line is the ability to perform primary cultures of mixed renal progenitor cells; yet, highly recombined and enriched in NPCs following Cre recombination *in vivo* (Hammoura I, Jaafar C and Ghanem N, unpublished data). On the hand, we have faced some challenges and limitations associated with the use of this line including Nestin expression in non-mesenchymal cells e.g. endothelial cells (which complicates phenotypic analysis), embryonic lethality around birth, incomplete/unequal recombination even among littermate embryos, and difficulty in delivery/birth when pregnant females receive tamoxifen treatment early in development e.g. E10.5. While some of these setbacks are linked to the loss of Rb, we could work around them using a slightly lower tamoxifen dosage or by inducing recombination at later developmental stages for example. Moreover, one could use other inducible models to target specific cell populations if needed such as the Six2-CreERT2 line (Kiefer, Robbins, and Rauchman 2012; Ramalingam et al. 2018; Volovelsky et al. 2018; Jiao Liu et al. 2017; Kanda et al. 2014; L. Zhang et al. 2018; Kobayashi et al. 2008; Yuwen Li et al. 2015). Alternatively, we can rely on cell sorting from the Nestin-Cre line as described earlier. This study is considered a proof of principle shedding light on the important role of the Rb pathway during kidney development, which warrants more investigation at many levels including basic research, and in human kidney disease or injury. Several essential questions still need to be addressed: what molecular mechanisms and target genes are

mediating the role(s) of the Rb-E2F pathway during kidney development? Does Rb play similar role(s) in the adult kidney; e.g. is it needed for survival of mature nephrons? What is the clinical relevance of our findings and future results to renal development and disease in humans? The answers to these questions will surely have important implications on human kidney development and function, and possibility, on renal regeneration (which is still a debatable process).

B. Regenerative potential of Nestin-positive cells in the adult kidney following injury or disease

As described above, we have induced an Rb deletion in the developing kidney and optimized a technical approach based on the use of indirect FACS to isolate recombined (Six2+; GFP+) NPCs, all by relying on Nestin as driver of Cre recombination. In a related context, several studies have characterized the abundancy and the distribution of Nestin-positive cells in the healthy adult kidney. Importantly, they also reported a remarkable upregulation in Nestin's expression after acute renal ischemia and therefore, proposed a potential contribution of Nestin-expressing cells to renal repair. For instance, on study showed that, under homeostatic conditions, Nestin-expression in the adult human kidney is mainly localized in the papillary region, vascular components of the medulla and glomeruli, but gradually decline in the outer cortical regions (Patschan et al. 2007). Another study reported that ischemic renal injury induces a transient upregulation in Nestin expression and triggers medullo-cortical rapid migration of Nestin-expressing cells whereby these cells acquire an endothelial phenotypic signature and contribute to angiogenesis (Patschan et al. 2007). Similarly, it was found that the level of Nestin expression correlates well with the degree of tubulointerstitial fibrosis; thus, Nestin is transiently upregulated in tubule and endothelial cells upon renal impairment (Sakairi et al. 2007). Interestingly, de novo Nestin expression is also

observed in mesangial cells that repopulate diseased glomeruli (Daniel et al. 2008). Given this functional plasticity of Nestin+ cells and their migratory potential to damaged regions in the adult kidney make them important target cells where specific gene manipulation(s) can be performed in order to ameliorate renal injury and/or enhance renal regeneration. Such manipulation(s) will likely have clinical relevance given that Nestin expression in the adult human kidney is highly similar to the one in mouse. It is found expressed in the glomerular formation within the mesangium (starting at early stages) as well as in the endothelium and podocytes, after which it only persists in adult podocytes of the mature nephron (Bertelli et al. 2007b).

C. Potential role of Rb in the regulation of cell adhesion during kidney development

Following loss of Rb, we have reported the absence of clear interface between the stroma and the CM in the developing kidney (**Figure 2**). In addition, throughout development, we have frequently detected the presence of high number of abnormally displaced glomeruli that are pushed toward the inner medulla in Rb-/- embryonic kidneys, instead of being normally localized at the border lying between the cortex and outer medulla (**Figure 4 and Supplementary Figure 3**). The above observations could be attributed to the fact that loss of Rb could negatively affect the expression levels of key adhesion genes such as E-cadherins, integrins and/or others; thus, disturbing cell-to-cell and/or cell-to-ECM interactions. In fact, previous studies have shown that Rb acts upstream of and regulates the expression of E-cadherin as well as other MET-related factors including Vimentin, N-cadherin, Slug, Zeb1 and Fibronectin (Arima et al. 2008; 2012; Engel, Cress, and Santiago-Cardona 2014). As such, Rb interacts with AP-2α to regulate cell adhesion in epithelial cells by inducing E-cadherin transcription (Batsché et al. 1998). Reciprocally, in another study, E-cadherin was shown to cause Rb

dephosphorylation, yielding elevated levels of p27 and reduced expression of Cyclin D1 (St. Croix et al. 1998). Therefore, Rb and E-cadherin seem to act in a positive feedback loop in order to maintain epithelial cells' properties. With respect to kidney, cadherins are differentially expressed within the distinct nephron structures throughout development (Dahl et al. 2002). Hence, Cadherin 4 is initially expressed in the proximal region as RV differentiate to become CSB and SSB whereas E-cadherin is mainly expressed in the distal region. Later on, while SSB further elongate to give rise to the capillary loop structure, E-cadherin becomes the predominant cadherin in the proximal and distal tubules (Dahl et al. 2002). Importantly, other studies have also documented a strong link between Rb and cell adhesion proteins, whereby Rb regulates cadherin-dependent and integrin-dependent adhesion in diverse tissues (Engel, Cress, and Santiago-Cardona 2014). Given all of the above, close assessment of the expressions of key adhesion proteins that are known to contribute to normal renal development such as E-cadherin and their interactions with Rb is an ultimate necessity.

D. Strong crosstalk between the Rb and p53 pathways during development and in cancer

The Rb and p53 pathways are considered two major tumor suppressors pathways given their critical roles in the vast majority of tissues during development and in the adult including cell cycle control, survival/apoptosis, senescence and genomic stability. Moreover, numerous studies have underlined strong and intrinsic crosstalk between the two pathways, which was shown to be often complex, and, context-dependent. For instance, the p53 pathway most commonly compensates (at least partially or temporarily) for the deregulated cell cycle in the absence of Rb. Therefore, most human cancers tend to gradually accumulate loss-of-function mutations leading to dual inactivation of both pathways. For example, the synergistic effect of the combined loss

of Rb and p53 in postnatal cerebellar granule cells triggers ectopic and extended proliferation accompanied by delayed differentiation, ultimately leading to the induction of medulloblastoma in mice (Shakhova et al. 2006). Similarly, combined deletion of both genes in mammary progenitors not only contributes to tumorigenesis but also dictates the subtype of tumor formation (luminal-B-like versus basal-like tumors) (Z. Jiang et al. 2010). Remarkably, in the developing liver, loss of p53 alone does not elicit a change in cell proliferation; however, p53 inactivation in Rb-null liver cells enhances significantly cell proliferation compared with loss of Rb alone (McClendon et al. 2011). Moreover, in response to genotoxic insult, combined loss of Rb and p53 in hepatocytes help bypass the cell cycle restriction points, eventually inducing genomic instability and tumor formation (McClendon et al. 2011). Similar consequences for the combined inactivation of the Rb and p53 pathways have been reported in the formation of osteosarcomas and pancreatic (Berman et al. 2008; Glenn et al. 2014).

In parallel to the above studies, developmental studies have started to uncover the identity of the cell type(s) and developmental stage(s) that are controlled by the Rb and p53 pathways. Remarkably, they have reached common conclusions with respect to the distinct versus redundant functions attributed to these genes in various tissues. For instance, our laboratory and others have shown that p53 negatively regulates neural stem cells self-renewal in the adult brain without directly affecting progenitor cell proliferation (Gil-Perotin et al. 2006; Halaby N, Saliba A and Ghanem N et al. in preparation). In contrast, we have demonstrated that Rb specifically controls neural progenitor cell proliferation without being involved in stem cell maintenance in the developing and adult brain (Vandenbosch et al. 2016; Ghanem et al. 2012; Naser et al. 2016). Interestingly, during kidney development, our data and results from others

studies examining the role of p53 are consistent with the findings reported in the brain; thus showing that p53 and Rb regulate stem cell self-renewal and nephron progenitor proliferation as distinct functions, respectively (this study and Li et al. 2015). However, it remains to examine whether similar or distinct molecular mechanisms are acting downstream of the two pathways in different tissues in the future.

E. Can transient manipulation of the Rb-E2f pathway help enhance nephrogenesis for therapeutic purpose following kidney injury or disease in the future?

Nephrogenesis ceases by P4 in mice and by week 36 of gestation in humans. The final number of nephrons in the adult kidney ranges between 200 000 and 2 million in humans, and around 16 000 in mice on average (Short et al. 2014). Balance between progenitor expansion and commitment ensures that proper number of nephrons are formed for normal function in the adult kidney. Therefore, an abnormally low nephron endowment during fetal development is a risk factor associated with hypertension and chronic kidney disease (Luyckx and Brenner 2019). Notably, nephron induction is limited to embryonic development; hence, once NPCs are consumed, de novo nephrogenesis is not possible and the adult kidneys are only capable of repairing damage. This repair occurs through the segment-specific postnatal renal progenitors (residual cells) that persists near terminally differentiated cells and replaces worn out tubule cells, but cannot regenerate new nephrons even in cases of severe injury (Rinkevich et al. 2014). Thus, a better comprehension of the molecular mechanisms and pathways that control nephron generation during development as well as regenerative process(es) that can contribute to nephron formation will offer critical insights for therapeutic interventions in many renal diseases. We found that Rb negatively controls progenitor proliferation; its loss leads to expansion in the progenitor pool and

subsequently, enhanced nephrogenesis (higher nephron counts at P0) without significantly affecting nephron differentiation or maturation (yet with compromised survival). Of note, this enhanced progenitor proliferation is still sustained at P0 in the absence of Rb but not in control mice (**Figure 4**), suggesting that loss of Rb may be associated with delayed cessation of nephrogenesis, or prolonged nephrogenesis owing to the cumulative/higher number of produced NPCs during development. This interesting observation requires further investigation in the future. It can be confirmed by inducing an Rb deletion during late development (e.g. E16.5-E18.5), then assessing the renal phenotype after postnatal day 4 (time when nephrogenesis is normally terminated) to check whether there could be residual Six2+ NPCs around the cap. Furthermore, future work at the basic research level should examine whether transient manipulation of the Rb pathway e.g. transient Rb knockdown could help expand the pool of NPC and their progeny without compromising their fate and function both *in vivo* and *in vitro*.

A common view holds that post-injury renal regeneration in the adult mouse kidney is attributed to the surviving tubule cells that de-differentiate and proliferate again or to repair induced by the proliferation of segment specific renal progenitors (Berger et al. 2014; Kusaba et al. 2014). No evidence exists to date suggesting the presence or contribution of multipotent stem cells to reparative function post-renal injury in mouse or humans. Nevertheless, studies have suggested the involvement of rare multipotent stem cells in renal repair in the adult human kidney; yet, this observation is still highly debatable and awaiting formal evidence (Ronconi et al. 2009; Angelotti et al. 2012). Basic research involving the role of key developmental pathways including the Rb and p53 pathways will surely bring new insights into the broad picture, and hopefully, help

improve regeneration in the adult human kidney by therapeutic interventions such as targeting and expanding what is believed to be ‘a rare stem cell population’.

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