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CELLULAR AND MOLECULAR REGULATION OF RENAL PROGENITOR POPULATIONS

THE ROLES OF RECEPTOR TYROSINE KINASE
SIGNALING IN NEPHRON AND COLLECTING DUCT
PROGENITOR MAINTENANCE AND DIFFERENTIATION

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ACADEMIC DISSERTATION

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“Nature uses only the longest threads to weave her patterns, so each small piece of her fabric reveals the organization of the entire tapestry.”

– Richard P. Feynman

ABSTRACT

Background

Disruptions in MAPK/ERK signaling have been implicated in the development of various renal diseases, including renal cancer and nephrotic syndrome as well as the developmentally derived childhood renal cancer, Wilms' tumor. Moreover, our lab, among others, has shown that MAPK/ERK is a crucial regulator of normal kidney development, where its functions are required both for ureteric bud branching morphogenesis as well as for nephrogenesis and progenitor expansion. Thus, understanding the role of MAPK/ERK signaling in kidney development is crucial for advancing our understanding of the mechanisms underlying renal diseases and for the development of new therapeutic strategies, including stem cell and organoid-based therapies.

Kidney development begins when signals originating from the metanephric mesenchyme induce the initial ureteric bud outgrowth from the Wolffian duct and drive subsequent branching morphogenesis. The ureteric bud is comprised of the collecting duct progenitors, and their maintenance depends on the metanephric mesenchyme-derived signals. In turn, signals from the ureteric bud induce the metanephric mesenchyme, comprised of the nephron progenitor cells, to differentiate and begin the process of nephron formation. Due to the reciprocal interactions, the extent of ureteric bud branching dictates not only the size and shape of the organ but also the final nephron number. Nephron number additionally depends on the molecular regulation and maintenance of the nephron progenitors. As the kidney is a non-regenerating organ, nephron endowment is ultimately defined during the fetal period, and low nephron number predisposes renal and related cardiovascular issues throughout life.

Results

We performed RNA-sequencing on ureteric bud and nephron progenitor cell populations isolated from mice with tissue-specific deletion of MAPK/ERK to identify transcriptional targets downstream of the cascade in renal progenitors. Our results indicate high tissue-specificity for MAPK/ERK, with only a small portion of genes differentially expressed in both cell populations. Of the genes common to both tissues, histone-related genes were over-represented, indicating that MAPK/ERK's effects on cell cycle and stem cell maintenance could be mediated through epigenetic mechanisms. In the MAPK/ERK-deficient ureteric bud, there was a dramatic loss of progenitor-specific gene expression with a concomitant increase in differentiation-associated genes, confirming a role for MAPK/ERK in the maintenance of progenitor status in ureteric bud cells. In the nephron progenitors lacking

MAPK/ERK, there was a dysregulation of genes related to metabolism, which we functionally validated.

We next performed metabolomics profiling to identify metabolite-level targets in MAPK/ERK-inhibited nephron progenitor cells. Utilizing a systems biology approach, we used the identified metabolite targets to support the *in vivo* transcriptional changes identified in MAPK/ERK-deficient nephron progenitors. The major pathways affected at both the transcript and metabolite levels include mitochondria, ribosome and amino acid metabolism, and diminished pyruvate and proline metabolism. *Ex vivo* kidney cultures demonstrated a dosage-specific pyruvate function in the regulation of the shape of the UB tip, which acts as the extrinsic niche for nephron progenitors. Inactivation of two proline synthesis enzymes, pyrroline-5-carboxylate reductases 1 and 2 (*Pycr1/2*) was used as a model of disrupted proline metabolism, which exhibited accelerated differentiation and premature exhaustion of nephron progenitors without an increase in apoptosis.

Finally, we demonstrated that an increase in metanephric mesenchyme-derived GDNF, previously known for its function in ureteric bud morphogenesis, essentially determines how long nephron progenitors are maintained in the maturing kidney. Our results show that GDNF prolongs the nephron progenitor lifespan and nephrogenesis beyond its typical cessation, despite its negative effect on overall kidney size. We show that the mechanism by which excess GDNF prolongs the nephrogenic program is through its increased secreted target genes and by enhancing WNT signaling. Besides upregulating its known targets in embryonic kidneys, excess GDNF also upregulates *Wnt9b* and *Axin2* leading to slower nephron progenitor proliferation. Postnatal decline of GDNF normalizes the ureteric bud, producing an environment that permits continued nephrogenesis. The effect of excess GDNF is therefore bi-phasic in nephron progenitors, which are capable of responding to GDNF dosage during the fetal and postnatal periods.

Conclusions

My thesis work found that MAPK/ERK maintains ureteric bud cells as undifferentiated and regulates renal progenitor cell maintenance through effects on chromatin accessibility and energy metabolism. MAPK/ERK-dependent nephron progenitor metabolism functionally contributes to progenitor preservation by controlling pyruvate availability and proline metabolism in developing kidneys. Furthermore, my data shows that monitoring the degree of signaling activity is a crucial way by which GDNF and possibly other molecules contribute to the specification of the lifespan of nephron progenitors. These studies provide some previously missing information about the regulation of renal development, which could contribute to the improvement of diagnostic and/or therapeutic strategies for renal diseases.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are included here as appendices. The original articles have been reprinted with permission from the publishers. In the text, original publications are referred to by their Roman numerals (I-III).

- I **Kristen Kurtzeborn***, Hyuk Nam Kwon*, Vladislav Iaroshenko, Imrul Faisal, Martin Ambrož, Xing Jin, Talha Qureshi, Jussi Kupari, Anneliis Ihermann-Hella, Juho Väänänen, Henna Tynysmaa, Iva Boušová, Sunghyouk Park, and Satu Kuure (2022). “Comparative whole-genome transcriptome analysis in renal cell populations reveals high tissue specificity of MAPK/ERK targets in embryonic kidney.” *BMC Biology* 20(1):112.

- II Hyuk Nam Kwon*, **Kristen Kurtzeborn***, Vladislav Iaroshenko, Xing Jin, Abigail Loh, Nathalie Escande-Beillard, Bruno Reversade, Sunghyouk Park, and Satu Kuure (2022). “Omics profiling identifies the regulatory functions of the MAPK/ERK pathway in nephron progenitor metabolism.” *Development* 149(19).

- III Hao Li, **Kristen Kurtzeborn**, Jussi Kupari, Yujuan Gui, Edward Siefker, Benson Lu, Kärt Mätlik, Soophie Olfat, Ana R Montaña-Rodríguez, Sung-Ho Huh, Franklin Costantini, Jaan-Olle Andressoo, and Satu Kuure (2021). “Postnatal prolongation of mammalian nephrogenesis by excess fetal GDNF.” *Development* 148(10).

*Kristen Kurtzeborn and Hyuk Nam Kwon contributed equally to these publications.

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ABBREVIATIONS

3PG	3-phosphoglycerate
α -KG	alpha-ketoglutarate
acetyl-CoA	acetyl coenzyme-A
ADAM10	A disintegrin and metalloproteinase domain-containing protein 10
Akt	protein kinase B
AMPK	adenosine monophosphate-activated protein kinase
ATP	adenosine triphosphate
<i>Axin2</i>	axis inhibition protein 2
BAK	B cell leukemia/lymphoma 2-antagonist/killer 1
BAX	B cell leukemia/lymphoma 2-associated X protein
BMP4/7	bone morphogenetic protein (isoforms 4 and 7)
CAKUT	congenital anomalies of the kidney and urinary tract
<i>Ccnd1</i>	cyclin D1
CITED1	Cbp/p300-interacting transactivator with Glu/Asp-rich carboxy-terminal domain 1
<i>Crlf1</i>	cytokine receptor-like factor 1
DE(G)	differentially expressed (gene)
DHAP	dihydroxyacetone-phosphate
dko	double knockout
DNA	deoxyribonucleic acid
DNMT1	DNA methyltransferase 1
DUSP6	dual specificity phosphatase 6
ECM	extracellular matrix
ELISA	enzyme-linked immunosorbent assay
<i>EphA4</i>	Eph receptor A4
ERK1/2/5	extracellular signal-regulated kinase (isoforms 1, 2, and 5)
ESC	embryonic stem cell
<i>Ets1</i>	E26 transformation-specific proto-oncogene 1
ETV4/5	E26 transformation-specific factor (isoforms 4, 5)
EYA1	eyes absent homolog 1
FACS	fluorescence-activated cell sorting
FGF(8/9 /20)	fibroblast growth factor (isoforms 8, 9, and 20)
FGFR1/2	fibroblast growth factor receptor (isoforms 1 and 2)
FOXD1	forkhead box D1
FOXI1	forkhead box I1
FRET	Förster resonance energy transfer
GA3P	glyceraldehyde-3-phosphate
GATA3	GATA binding protein 3
GDNF	glial cell line-derived neurotrophic factor

GFP	green fluorescent protein
GFRa1	GDNF family receptor alpha-1
GLUT	glucose transporter
GO	gene ontology
GTP	guanosine triphosphate
H3K9me2	histone H3 lysine 9 dimethylation
H3K27me3	histone H3 lysine 9 trimethylation
HGF	hepatocyte growth factor
HIF	hypoxia-inducible factor
<i>Hoxc8</i>	homeobox C8
<i>Hoxd11</i>	homeobox D11
<i>Hoxb7</i>	homeobox B7
hPSC	human pluripotent stem cell
IGF2	insulin-like growth factor 2
IL-1 β	interleukin 1 beta
iPSC	induced pluripotent stem cell
ITGA8	integrin alpha 8
<i>Jag1</i>	jagged canonical Notch ligand 1
JNK	c-Jun N-terminal kinase
LC-MS	liquid chromatography-mass spectrometry
<i>Lef1</i>	lymphoid enhancer-binding factor-1
<i>Let-7</i>	lethal-7 microRNA precursor
LHX1	LIM homeobox 1 (previously LIM1)
LIM1	LIM homeobox 1 (renamed to LHX1)
<i>Lin28</i>	lin-28
MAPK	mitogen-activated protein kinase
MCT	monocarboxylate transporter
MEK1/2	dual specificity mitogen-activated protein kinase kinase (isoforms 1 and 2)
<i>Meox2</i>	mesenchyme homeobox 2
MM	metanephric mesenchyme
MPC	mitochondrial pyruvate carrier
mTOR	mammalian target of rapamycin
MYC	master regulator of cell cycle entry and proliferative metabolism proto-oncogene
NAD ⁺	oxidized nicotinamide adenine dinucleotide
NADH	H ⁺ reduced nicotinamide adenine dinucleotide
<i>NF1</i>	neurofibromatosis type 1
NOTCH	neurogenic locus
NP	nephron progenitor
OAA	oxaloacetate
OSR1	odd-skipped related transcription factor 1
OXPHOS	oxidative phosphorylation
p38	mitogen-activated protein kinase 14
p53	transformation related protein 53

p63	transformation related protein 63
PAX2/8	paired box (isoforms 2 and 8)
PCR	polymerase chain reaction
PGC-1 α	peroxisome proliferator-activated receptor gamma coactivator-1alpha
PI3K	phosphatidylinositol 3-kinase
PKC	protein kinase C
PKM2	pyruvate kinase isozyme M2
PLC γ	phospholipase C gamma
PPP	pentose phosphate pathway
PTA	pre-tubular aggregate
PYCR1/2/L	pyrroline-5-carboxylate reductase 1/2
RAF	rapidly accelerated fibrosarcoma proto-oncogene serine/threonine-protein kinase
RAS	rat sarcoma virus
RET	rearranged during transfection proto-oncogene
RNA-Seq	ribonucleic acid sequencing
ROBO2	roundabout guidance receptor 2
RTK	receptor tyrosine kinase
RV	renal vesicle
SALL1	spalt like transcription factor 1
<i>Shh</i>	sonic hedgehog
SIX1/2/4	sine oculis-related homeobox (isoforms 1, 2, and 4)
Smad	suppressor of mothers against decapentaplegic
<i>Spred2</i>	sprouty related EVH1 domain containing 2
SPRY	sprouty receptor tyrosin kinase signaling antagonist
SSB	S-shaped body
succ-CoA	succinyl coenzyme-A
TCA	tricarboxylic acid
TCF	T-cell factor
TNF- α	Tumor necrosis factor alpha
TSC1	TSC complex subunit 1 (aka hamartin)
UB	ureteric bud
UTR	untranslated region
VPC	vascular endothelial progenitor cell
WD	Wolffian (nephric) duct
WNT(4/9b/11)	wingless-type MMTV integration site family (members 4, 9b, and 11)
<i>Wt1</i>	Wilms tumor 1 transcription factor

Conventional murine gene/protein nomenclature is used throughout this document. Gene names are italicized with the first letter capitalized. Protein names are not italicized and are fully capitalized. Example: *Gene* and PROTEIN.

1 INTRODUCTION

The increasing prevalence of kidney diseases arises from metabolic diseases in adults and congenital anomalies in children (Kovesdy, 2022). Congenital anomalies of the kidney and urinary tract (CAKUT) are among the most common birth defects, often leading to lifelong issues such as chronic kidney disease and even end-stage renal disease. However, the genetic and molecular causes of congenital renal anomalies remain poorly understood (Kohl et al., 2021). Current treatments, dialysis and transplantation, are insufficient, and the development of much needed new diagnostic and therapeutic options, including stem cell- and organoid-based therapies, is hindered by a lack of sufficient data. Current differentiation protocols for stem cell and organoid applications are based solely on the knowledge obtained from developmental biology studies in the mouse, and more recently in humans (Khoshdel-Rad et al., 2022). My project delivers some of the missing information and provides insight into possible underlying mechanisms behind congenital renal defects.

The kidneys perform many functions in the body. Not only do they filter and purify the blood, but they also maintain the body's fluid and salt balance, release hormones that are involved in blood pressure regulation, produce an active form of vitamin D, and have a role in the production of red blood cells. The adult kidney (Figure 1) consists of the outer cortex, the medulla in the middle, and the renal pelvis in the concave hilum of the kidney, which is where the nerves, blood vessels, and ureters connect to the kidney. The filtering units of the kidney are the nephrons. The nephron is made up of the renal corpuscle, the tubules, and the associated capillary network. The renal corpuscle consists of the capillary network called the glomerulus, surrounded by Bowman's capsule. Emerging from the glomerulus is the renal tubule, comprised of the proximal convoluted tubule, the loop of Henle, and the distal convoluted tubule. Each nephron is connected by its distal tubule to a collecting duct that extends into the medulla, eventually emptying into the ureter. Each of these structures is composed of specialized cells which allow the kidney to function properly, and it has been proposed that the adult kidney contains as many as 73 distinct cell populations (Balzer et al., 2022; Schumacher et al., 2021).

Like many organs, the embryonic kidney utilizes branching morphogenesis and inductive tissue interactions to drive growth, patterning, and differentiation, aspects that are defective in CAKUT (Saxen, 1987). In developing kidneys, ureteric bud (UB) branching not only defines the organization of the collecting duct system and the organ's ultimate size and shape but also determines the final nephron number and thus the health of the adult organ (Cebrian et al., 2014). Due to the nature of inductive tissue interactions, UB branching maintains nephron progenitors (NP) and induces their differentiation. Reciprocally, NPs sustain branching in the UB tips, which

differentiate into the collecting duct network and ureter. The understanding of the molecular and cellular regulation of renal progenitor maintenance, differentiation, and niche characteristics is only about to emerge.

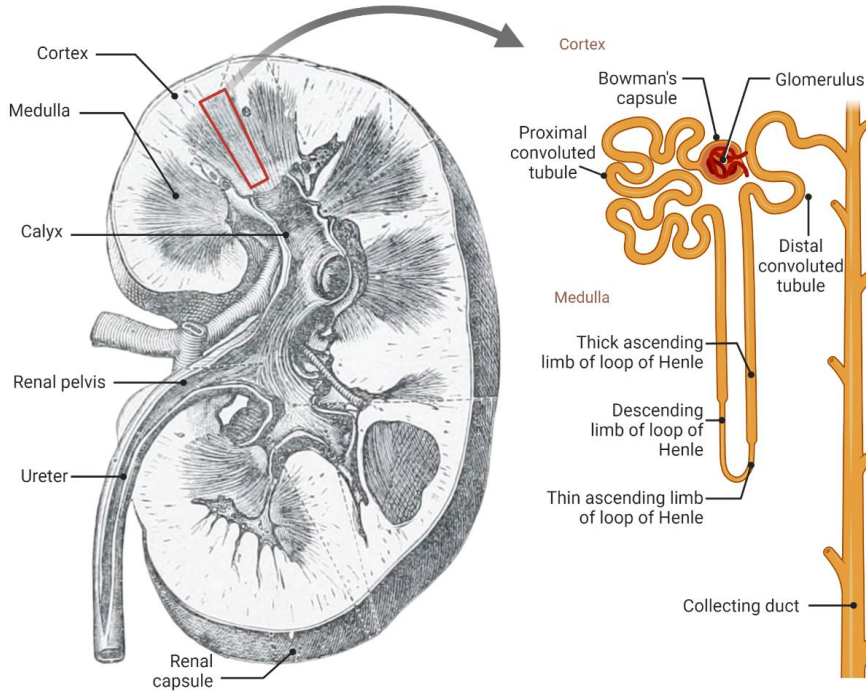


Figure 1 Anatomy of the adult, vertebrate kidney and nephron. Kidney image modified from *An Atlas of Human Anatomy for Students and Physicians* (1903). The image is public domain under the Creative Commons 1.0 universal license. Images labeled and adapted from the “Kidney Anatomy” template at Biorender.com (2023; <http://app.biorender.com/biorender-templates>).

On the molecular level, branching morphogenesis of the UB is largely guided by receptor tyrosine kinase (RTK) signaling downstream of glial cell line-derived neurotrophic factor (GDNF)/RET/GFRa1 as well as by fibroblast growth factors (FGFs) (Sainio et al., 1997; Walker et al., 2016). For further information on signaling during kidney development, there are several reviews in the literature (Khoshdel Rad et al., 2020; Krause et al., 2015; Kurtzeborn et al., 2018; Kurtzeborn et al., 2019). The initial outgrowth of the UB occurs when GDNF, expressed by the metanephric mesenchyme, interacts with its receptor RET and co-receptor GFRa1 in the Wolffian duct (WD), giving rise to the initial outgrowth of UB from the WD (Costantini, 2010). The UB then continues to branch for several generations. Among the many factors regulating UB branching, MAPK/ERK signaling downstream of GDNF/RET is

an important branching regulator, and our lab has demonstrated its requirement for UB tip cell regulation and novel branch formation (Ihermann-Hella et al., 2014).

My studies have contributed to the characterization of the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway, acting downstream of GDNF, as a crucial regulator of kidney size and nephron number. MAPK-deficient kidneys exhibit severe hypodysplasia due to abnormalities in cellular adhesion and cell cycle progression (Ihermann-Hella et al., 2018; Ihermann-Hella et al., 2014). In this thesis, I examine: 1) the transcriptional and metabolic targets regulated by MAPK/ERK signaling and their roles in renal progenitor cell maintenance and differentiation; 2) how the MAPK/ERK cascade regulates different aspects of metabolism during NP maintenance; and 3) how excess fetal GDNF affects NPs, and how the activity levels of GDNF specify NP lifespan. In these studies, I utilized a combination of mouse genetic manipulations, ex vivo kidney cultures, immuno- and histological staining, RNA sequencing (RNA-Seq), and metabolomics analyses to resolve the key molecular mediators of MAPK/ERK functions in renal progenitor populations and examine their functional requirements for normal kidney development. Thus, my research provides some currently missing information about the cellular and molecular mechanisms controlling progenitor maintenance and differentiation as well as guiding kidney growth, information that will prove useful for the continued development of novel treatment options for kidney disease and CAKUT.

2 REVIEW OF THE LITERATURE

2.1 OVERVIEW OF KIDNEY DEVELOPMENT

Kidney morphogenesis is a complex process that gives rise to functional, non-regenerating organs. During mammalian embryonic development, the kidney forms in three spatially and temporally distinct stages, all derived from the intermediate mesoderm (Taguchi et al., 2014): the transient pronephros and mesonephros, and finally the metanephros, which becomes the adult form of the kidney. Here, I focus on metanephric kidney development, which begins at embryonic day 10.5 (E10.5) in mice and during weeks 4-5 of gestation in humans when the metanephric blastema or metanephric mesenchyme (MM) induces outgrowth of the epithelial ureteric bud (UB) from the common nephric duct (or “Wolffian” duct) (Saxen, 1987). An overview of early kidney development can be seen in Figure 2.

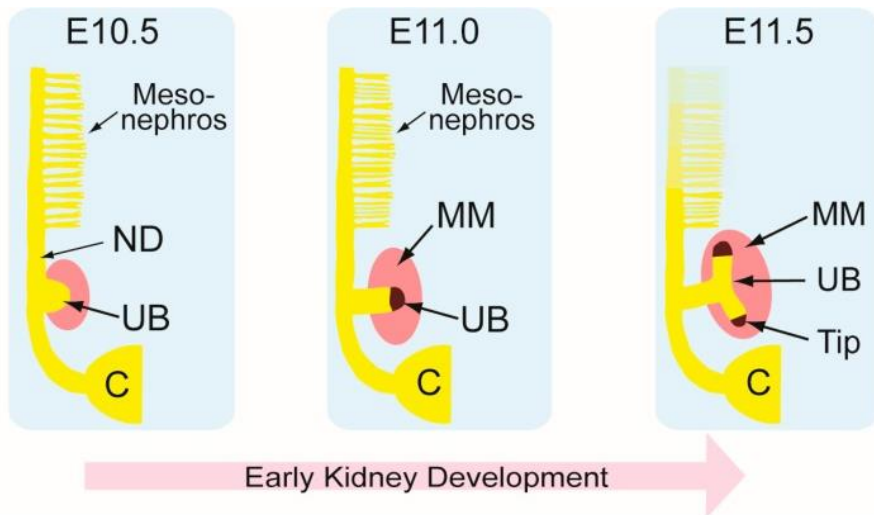


Figure 2 Early murine kidney induction and development. The transient, embryonic mesonephros is predominant at E10.5 and begins to regress as development progresses. The adult kidney, the metanephros, begins to form at E10.5 when signals from the metanephric mesenchyme (MM, pink) induce ureteric bud (UB) outgrowth from the nephric duct (ND) near the cloaca (C). The signals from the MM attract the UB, which becomes divided into the progenitor-containing UB tips (red) and trunk (trunk). Reciprocal inductive interactions between UB and MM initiate UB branching around E11.5. Image adapted and used with permission (Kurtzeborn et al., 2019).

The establishment of the initial MM mass is guided by transcriptional regulators such as EYA1, GATA3, SIX1/4, OSR1, and PAX2/8 (Bouchard et al., 2002; Brophy et al., 2001; Dressler, 2011; James et al., 2006; Kobayashi et al., 2007; Li et al., 2003; Lim et al., 2000; Narlis et al., 2007; Sajithlal et al., 2005; Wang et al., 2005; Xu et al., 2003; Xu et al., 1999). The MM and nephric duct communicate through reciprocal inductive interactions to cause the initial outgrowth of the UB from the nephric duct. These interactions are molecularly mediated mainly through glial cell derived neurotrophic factor (GDNF) and fibroblast growth factor (FGF) signaling as well as by integrins that mediate signaling from the extracellular matrix into the cell (Mathew et al., 2012). MM cells secrete GDNF which binds to the nephric duct through RET tyrosine kinase receptor and its co-receptor GDNF family receptor alpha-1 (GFRa1) (Costantini, 2010; Golden et al., 1999; Hellmich et al., 1996; Pachnis et al., 1993). *Ret* expression is increased in the UB tips in response to retinoic acid signaling from the surrounding stroma (Batourina et al., 2001). Cells with high levels of RET actively move to the area of UB outgrowth in response to GDNF (Chi et al., 2009), a process mediated by the ETS transcription factors ETV4 and ETV5 (Kuure et al., 2010; Lu et al., 2009). The roles of GDNF, RET, and GFRa1 in the process of UB outgrowth have been extensively studied, and it is known that all three pathway components are required for normal UB outgrowth (Cacalano et al., 1998; Moore et al., 1996; Pichel et al., 1996; Sainio et al., 1997; Sanchez et al., 1996; Schuchardt et al., 1994).

Several negative regulators of GDNF/RET signaling are active to prevent ectopic UB formation and improper branching. Bone morphogenetic protein 4 (BMP4) inhibits GDNF/RET in the MM (Michos et al., 2007; Miyazaki et al., 2000), and is blocked by its antagonist Gremlin1 (Church et al., 2017; Michos et al., 2007). Another GDNF/RET inhibitor, Sprouty homolog 1 (SPRY1) also regulates UB growth (Michos et al., 2010; Rozen et al., 2009). Slit homolog 2 (SLIT2) and its receptor roundabout homolog 2 (ROBO2) also act to prevent abnormal UB outgrowths (Grieshammer et al., 2004). Additionally important for UB outgrowth is FGF signaling through receptors FGFR1/2 (Zhao et al., 2004). FGF signaling has a role in the MM where it acts to prevent ectopic UB formation, although the mechanism by which this occurs is not fully understood (Poladia et al., 2006).

Kidneys grow into their final size and shape by the process of branching morphogenesis, a process that also dictates the final number of nephrons in the organ due to the reciprocal inductive interactions between the metanephric mesenchyme and the ureteric bud epithelium (Saxen, 1987). The intertwined processes of branching morphogenesis and nephrogenesis are largely guided by GDNF/RET signaling as well as FGFs and hepatocyte growth factor (HGF) and their downstream cascades, notably including the MAPK/ERK pathway (Figure 3) (Kurtzeborn et al., 2019; Sainio et al., 1997; Santos et al., 1994; Walker et al., 2016). These developmental processes and

their cellular and molecular regulation are described in detail below. Beyond basic patterning, development of the renal vasculature (Herzlinger & Hurtado, 2014; Mohamed & Sequeira-Lopez, 2019) and neuronal network (Sariola et al., 1988) is not well characterized and will not be discussed here at length because these processes were not addressed during this project.

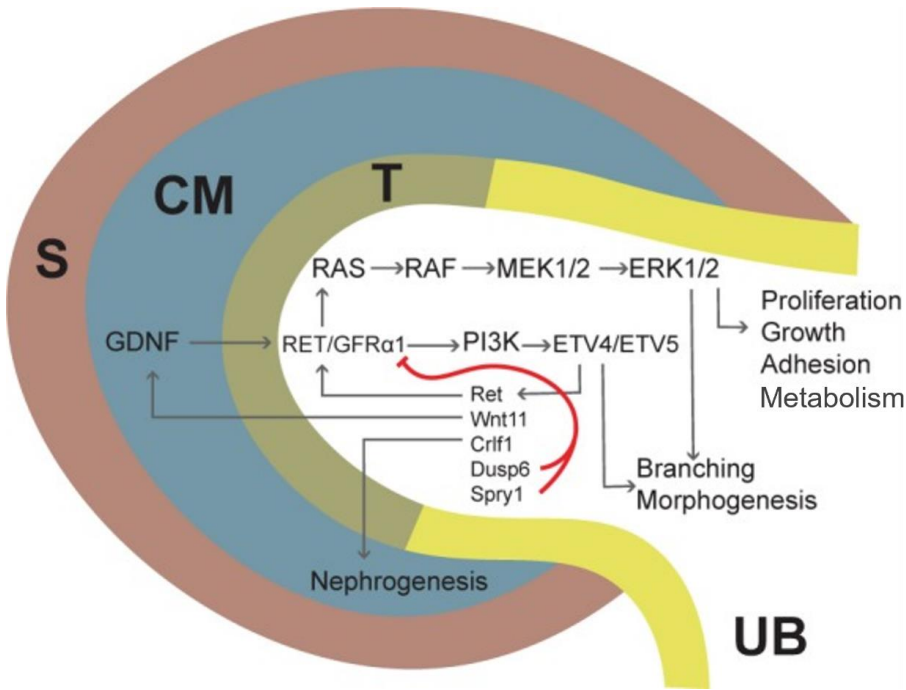


Figure 3 GDNF/RET signaling regulation in the ureteric bud (UB) tip during kidney development. *Gdnf* is expressed in the cap mesenchyme (CM) and in the stroma (S) and binds to its receptor complex RET/GFRα1 in the UB tip (T) where it sets off downstream cascades like MAPK/ERK and PI3K. Many factors important for proliferation, growth, adhesion, metabolism, and the developmental processes of branching morphogenesis and nephrogenesis, as well as factors mediating GDNF signaling itself, are activated downstream of GDNF/RET. Image used with permission (Kurtzeborn et al., 2018).

2.1.1 RENAL PROGENITOR POPULATIONS

There are four main progenitor populations in the developing metanephric kidney: the metanephric mesenchyme-derived nephron progenitors, which gives rise to the functional filtering units of the kidney, the nephrons; the ureteric bud epithelium, which gives rise to the entire epithelial collecting duct network and ureter; the stromal progenitor cells, which develop into the surrounding stroma; and the vascular endothelial progenitors (Sequeira-Lopez et al., 2015). An overview of the organization of the progenitor niche is

shown in Figure 4. The regulation of the maintenance, growth, and differentiation of the collecting duct and nephron progenitors are described in detail in sections 2.1.2 - 2.1.5. The lineage relationships of renal progenitor cells in kidney development were recently reviewed (Little et al., 2022) and are not discussed here in detail.

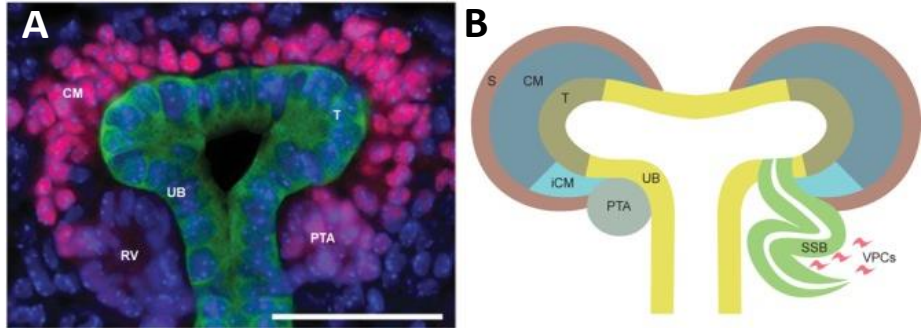


Figure 4 *In vivo* representation (A) and schematic view (B) of the embryonic renal progenitor niche made up of the UB tip (T) surrounded by the cap mesenchyme (CM) which comprises of the nephron progenitors and the surrounding stroma (S). *In vivo* representation (A) is from an E15.5 kidney stained with Calbindin (green) to mark the UB and SIX2 (magenta) to mark CM and (to less extent) the nephron precursors, pre-tubular aggregate (PTA) and renal vesicles (RV). Nuclei are in blue. Scale bar is 50 μ m. The schematic image (B) additionally shows a subset of CM which is induced for differentiation (iCM; light blue) and a nephron precursor structure in a further stage of nephrogenesis, an S-shaped body (SSB; green) with vascular endothelial progenitor cells (VPCs; pink) entering its cleft to begin the formation of the glomerular capillary network. Image adapted and used with permission (Kurtzeborn et al., 2018).

2.1.2 BRANCHING MORPHOGENESIS

In addition to the kidney, many other organs such as the lungs, pancreas, and different types of exocrine glands also develop through branching morphogenesis. The pattern of branching is dictated by signals from the surrounding mesenchyme and is specific to each organ, accounting for the vast differences in the sizes and shapes of branched organs (Lu et al., 2006). The complex, organ-specific differences in branching morphogenesis have been recently reviewed (Lang et al., 2021). Renal branching morphogenesis is guided by the reciprocal inductive interactions between the ureteric bud and metanephric mesenchyme (Costantini & Kopan, 2010; Schedl, 2007).

During kidney development, once the ureteric bud has protruded from the nephric duct and reached the metanephric mesenchyme around E11, it begins dichotomous branching that continues throughout development. Each new outgrowth of the UB balloons to form an ampulla, which then bifurcates, or less commonly trifurcates, to form new UB tips (Watanabe & Costantini, 2004). During ampulla formation, the cells of the UB tips undergo great changes in size and shape due to the dynamic mechanical forces required for the ampulla formation and branching (Kuure lab, unpublished data).

Localized proliferation in the UB tips is also required for novel branch formation and is an important mechanism for UB branching morphogenesis (Michael & Davies, 2004).

The cortical ureteric bud is made up of two molecularly distinct cell populations: the ureteric bud tips, which are comprised of the undifferentiated collecting duct progenitors, and the ureteric bud stalk (also known as “trunk”), which are committed to become collecting duct cell types (Rutledge et al., 2017; Schmidt-Ott et al., 2005). The cap mesenchyme and ureteric bud tip make up the niche that maintains their self-renewal, promotes branching, and cues the mesenchymal-to-epithelial transformation required for nephrogenesis. Surrounding this niche are FOXD1+ stromal progenitors (Kobayashi et al., 2014), which play some important roles during branching morphogenesis and nephrogenesis (Hatini et al., 1996) but are not covered here in detail because they were not studied as part of my thesis.

The importance of growth factor signaling for UB branching through, for example, GDNF and FGFs is well demonstrated (Costantini, 2010; Jain et al., 2006; Sariola & Saarma, 1999; Walker et al., 2016), but the downstream targets that ultimately mediate their functions are still not well researched. An intricate balance of GDNF levels is required for initial UB outgrowth and the development of normal UB morphology. Severe renal defects occur in genetically modified mouse models lacking GDNF or with reduced GDNF, but defects are also seen in mutants with endogenously increased or expanded GDNF, verifying the importance of proper GDNF balance (Basson et al., 2005; Basson et al., 2006; Costantini & Shakya, 2006; Grieshammer et al., 2004; Kumar et al., 2015; H. Li et al., 2019). GDNF induces the expression of several canonical WNT signaling ligands which control a hierarchy of genes essential for ureteric branching (Bridgewater et al., 2008; Iglesias et al., 2007; Ola et al., 2011). *Gdnf/Ret* and *Wnt11* also cooperate in a positive feedback loop to maintain the correct balance of UB tip cells and surrounding mesenchyme required for normal branching (Majumdar et al., 2003; Michos et al., 2007). Branching morphogenesis, GDNF functions, and GDNF/WNT11 feedback are all regulated by the inhibitory effects of BMP4 (Michos et al., 2007; Miyazaki et al., 2000).

2.1.3 COLLECTING DUCT DIFFERENTIATION

The repetitive process of UB branching forms the entire collecting duct system of the adult kidney (Cebrian et al., 2004; Riccio et al., 2016; Short et al., 2014). During the branching process, several distinct cell types are already specified within the ureteric epithelial tree by E15.5. These distinct regions can be categorized as the ureteric bud tips, ureteric tree terminal branches (excluding the tip), and the cortical- and medullary collecting ducts (Thiagarajan et al., 2011). The ureteric bud tips contain highly proliferative

cells expressing *Ret* and in direct contact with the MM, and cells without *Ret* expression are excluded from the UB tip domain, as shown in chimeric studies (Chi et al., 2009; Shakya et al., 2005). Lineage tracing experiments have confirmed that these *Ret*-expressing UB tip cells are the progenitors of all the cell types of the entire collecting duct system (Riccio et al., 2016). Specific molecular signatures and their regulation in the UB tip are still being investigated, but it is known that RET signaling is required to maintain cells in the UB tip niche (Chi et al., 2009; Kuure et al., 2010; Shakya et al., 2005).

UB cells undergo morphological changes and coordinated cell migrations in response to paracrine cues from adjacent tissues to begin the formation of the collecting duct. The mature renal collecting duct is made up of functionally distinct cell types including principle cells and alpha- and beta-intercalated cells (Bagnis et al., 2001). A subpopulation of UB cells expressing the N-terminus truncated isoform of p63 are the precursors of the cortical intercalated cells (El-Dahr et al., 2017). As development proceeds, the medullary collecting duct cells mature to form the stratified urothelium and begin a fate switch from principal to intercalated cells expressing the forkhead box gene *Foxi1* (Yu et al., 2012). The intercalated and principal cell fate determination is critically regulated by FOXI1 and the Adam10/Notch signaling pathway, respectively (Al-Awqati & Schwartz, 2004; Guo et al., 2015; Jeong et al., 2009; Vidarsson et al., 2009). *Shh* is also expressed, which acts as a paracrine signal to regulate the formation of smooth muscle around the duct (Thiagarajan et al., 2011; Yu et al., 2002). These cells finally form the mature, watertight epithelium through the expression of uroplakins (Gandhi et al., 2013).

2.1.4 NEPHROGENESIS

In early development before metanephric specification, *Eya1* and *Six1* interact to regulate nephron progenitor cell establishment and to induce the development of the nephric duct (Xu & Xu, 2015). At the onset of branching morphogenesis, the homeobox protein SIX2+ nephron progenitors condense to form a cap around each RET+ ureteric bud tip (Pachnis et al., 1993; Self et al., 2006).

Nephron progenitors are indicated by their expression of the transcription factor *Six2* beginning around E10.5-11.5 (Self et al., 2006). These cells localize around the ureteric bud tips to form the cap mesenchyme, and this cap/tip region is the nephrogenic niche. The exact mechanisms regulating niche arrangement are still being resolved (Ihmann-Hella et al., 2018; Uchiyama et al., 2010). *Six2* is required for nephron progenitor self-renewal, and *Six2*-expressing cells give rise to all segments of the epithelial nephron (Kobayashi et al., 2008). In addition to *Six2*, the uncommitted, self-renewing nephron progenitors also characteristically express *Cited1* (Boyle et al., 2008).

Nephrogenesis begins in response to canonical WNT signaling when the UB secretes wingless 9b (WNT9b) which activates beta-catenin to induce expression of fibroblast growth factor 8 (FGF8), LIM homeobox 1 (LHX1), and WNT4 by the MM (Park et al., 2007). The Notch pathway with ligands Delta, Jagged, and Serrate is known to regulate the differentiation of renal cell types and can bypass Wnt signaling to induce MM development and subsequent differentiation (Mukherjee et al., 2019). Nephron progenitors accumulate in the “armpit” of the ureteric bud, forming the nephron precursor pretubular aggregate, and thus begin to undergo a mesenchymal-to-epithelial transformation (Carroll et al., 2005; Karner et al., 2009; Ramalingam et al., 2018). NP cells are gradually recruited to the pretubular aggregate, and the future fate of the cells in the nephron is determined by the timing of this recruitment (Lindström, Brandine, et al., 2018).

Morphological stages of metanephric nephron formation

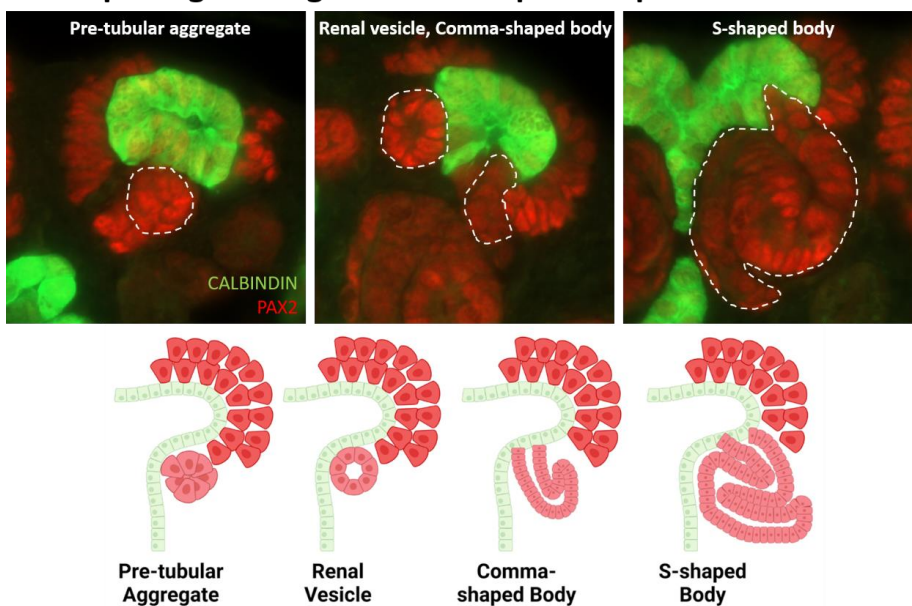


Figure 5 Distinct morphological stages of nephrogenesis: pre-tubular aggregate, renal vesicle, comma-shaped, and s-shaped bodies. Ureteric bud epithelium is in green (Calbindin staining in immunofluorescent images). Nephron progenitors and differentiating nephron precursors are in red (stained with PAX2 in immunofluorescent images). Schematic images generated with Biorender.com.

The committed nephron progenitors in the pretubular aggregate express *Wnt4* which induces transformation into an epithelial renal vesicle (Stark et al., 1994). The renal vesicle is almost immediately polarized with divergent gene expression between its proximal and distal domains (Georgas et al.,

2011). The nephron continues to form through changes in morphology from renal vesicle to comma-shaped body, S-shaped body, and early capillary loop. These nephron precursor states can be seen in Figure 5. The distal S-shaped body differentiates to become the distal and connecting tubules, merges with the ureteric bud to create a continuous lumen, and begins the formation of the glomerulus (O'Brien, 2019). The proximal and medial S-shaped body segments also contribute to the glomerulus formation and additionally give rise to the proximal tubule and loop of Henle (Saxen, 1987). Notch2 is required for the differentiation of proximal nephron structures including the podocytes and proximal tubules (Cheng et al., 2007). During nephron patterning, differential expression of cadherins also plays a role as the renal vesicle differentiates into the P-cadherin-expressing glomerulus, the cadherin-6-expressing proximal segments, and the E-cadherin-expressing distal segments (Roy et al., 2015). Several intermediate progenitor states exist during nephrogenesis as the nephron progenitors give rise to all the cell types of the mature nephron, but these intermediary states and their interactions are not well studied. However, it was shown by clonal analysis that nephrons develop from nephron progenitor cells that segment into different regions formed as local clonal expansions, indicating that different nephron segments likely form from distinct nephron progenitor sub-populations (Rinkevich et al., 2014).

In mammals, nephrogenesis ceases with the exhaustion of nephron progenitor cells around the time of birth (approximately postnatal day 3 (P3) in mice) (Hartman et al., 2007; Rumballe et al., 2011). This final exhaustion occurs as a global commitment of nephron progenitors to epithelial differentiation and does not involve selective apoptosis nor changes in cell proliferation (Hartman et al., 2007). Understanding the cellular and molecular mechanisms behind nephrogenesis cessation is important because no new nephrons are formed after depletion of the fetal nephron progenitor pool, so the final nephron number and lifelong function depend on the duration of nephrogenesis (Cebrian et al., 2014). Low nephron number has been linked to the development of hypertension, chronic kidney disease, and kidney failure later in life (Romagnani et al., 2017).

2.1.5 REGULATION OF NEPHRON PROGENITORS AND NEPHRON ENDOWMENT

During development, the nephron number is determined and remains constant throughout life (Charlton et al., 2014). Several molecular and environmental factors can affect nephron endowment, but the exact mechanisms that determine final nephron number are not completely understood. The most studied among these is maternal nutrition (Bagby, 2007; Woods, 2007), while maternal disease and toxin exposure can also affect nephron number. Here, I will focus on the molecular regulation of nephron

endowment as that is the concern of this thesis. For a more detailed account of nephron endowment regulation, see the recent review (Perl et al., 2022) and the detailed comparison of related human and animal studies (Luyckx et al., 2019).

It is essential that the population of NP cells be maintained during the period of active UB branching in order to establish a sufficient final nephron number. NP depletion experiments demonstrated that the size of the NP population critically determines nephron number (Cebrian et al., 2014). Several transcriptional regulators are involved in NP maintenance. The most well-known among these is probably SIX2, which is needed for the self-renewal of NPs and for the prevention of their premature differentiation. *Six2* maintains the nephron progenitor population by opposing *Wnt4*-mediated epithelialization (Self et al., 2006). *SALL1* has a similar function to SIX2, as *Sall1*-null NPs are prematurely depleted (Basta et al., 2014; Kanda et al., 2014).

Paired box gene 2 (PAX2) is another major player in the regulation of NP maintenance. It plays a role in the initial specification of renal progenitors from the intermediate mesoderm (Ranghini & Dressler, 2015), and it is required for the regulation of nephron differentiation (Narlis et al., 2007). PAX2 also establishes the boundary between nephrons and interstitium and acts to maintain NP cell identity by repressing interstitial identity (Naiman et al., 2017). *PAX2* mutations in humans can lead to conditions such as renal hypodysplasia, renal coloboma syndrome, and oligomeganephronia (Chang et al., 2022; Eccles & Schimmenti, 1999; Salomon et al., 2001). In contrast to its requirement *in vivo*, it has been shown that PAX2 is dispensable for nephron formation in iPS cells, indicating that PAX2 may function differently in an *in vitro* setting (Kaku et al., 2017).

Other significant molecular regulators of NP maintenance include p53, OSR1, EYA1, N-MYC, and c-MYC. The classical role of p53 is to restrain cell proliferation, but rather than enhanced proliferation upon p53-depletion in NPs, proliferation was actually impeded. This was shown to be due to a need for p53 in the metabolic fitness of NPs and therefore a requirement of p53 for NP maintenance (Li et al., 2015). OSR1 acts synergistically with SIX2 to maintain NPs by antagonizing WNT-driven NP differentiation through the stabilization of TCF-Groucho transcriptional repressor complexes (Jingyue Xu et al., 2014). EYA1 also interacts with SIX2 to control NP self-renewal. *Eya1* expression is required for expression of *Six2*, and loss of *Eya1* leads to premature differentiation of NPs (Jinshu Xu et al., 2014). Myc family of proto-oncogenes are required for renal progenitor cell proliferation and organ growth (Bates, 2000; Couillard & Trudel, 2009), and they have been shown to form a complex with SIX2 and EYA1 in NPs (Jinshu Xu et al., 2014). The kidney uses WNT9b signaling as a way to monitor SIX2 levels in NPs, and cells with reduced SIX2 are induced to differentiate upon receipt of a WNT9b signal

(Karner et al., 2011; Kiefer et al., 2012). R-spondins are also required for NP maintenance and differentiation where they act to enhance WNT signaling (Vidal et al., 2020). Additionally, MYC cooperates with the canonical WNT signaling component β -catenin to regulate the NP renewal program by activating WNT progenitor renewal target genes (Pan et al., 2017). Furthermore, WNT11 plays a role in determining nephron endowment by affecting polarity and motility of NP cells (O'Brien et al., 2018).

Nephron progenitors are also maintained as a self-renewing population by growth factor signaling through FGFs and their antagonists BMPs. These growth factors probably act as upstream regulators for the transcription factors mentioned in the previous paragraphs, but specifics of this type of regulation are not fully understood yet. FGFs work together with WNT and Notch signals to promote the timely differentiation of NPs into the different nephron segments (Ahn et al., 2013; Barak et al., 2012; Brown et al., 2013; Shuang Chen et al., 2015; Dudley et al., 1999; Dudley et al., 1995; Grieshammer et al., 2005; Motamedi et al., 2014; O'Brien & McMahon, 2014; Ohuchi et al., 2000; Perantoni et al., 2005; Qiao et al., 1999; Tomita et al., 2013). *Fgf8* was recently shown to prevent apoptosis in cortical nephron progenitors by regulation of pro-apoptotic factors BAX and BAK, and it is required for expression of *Lhx1* (Anderson et al., 2023; Grieshammer et al., 2005), a transcription factor required for tubule formation during nephron development (Kobayashi et al., 2005). The FGF-induced expansion of NPs is of great interest for the field of kidney organoids where the aim is to generate *in vitro* models to mimic *in vivo* kidney development and disease states (Kimber & Woolf, 2018; Murray & Woolf, 2014). The phosphatidylinositol 3-kinase (PI3K)/Akt pathway is also required for NP self-renewal, and reduced pathway activity leads to differentiation induced by β -catenin (Lindström et al., 2015). The PI3K pathway may also regulate self-renewal through its promotion of glycolysis, as the self-renewal of nephron progenitors is also regulated by intermediary metabolism (Liu et al., 2017), described in more detail in section 1.4.1. A detailed account of the molecules involved in NP cell commitment can be read elsewhere (O'Brien, 2019).

The role of epigenetic mechanisms in the regulation of nephron progenitors has become increasingly apparent in the past decade (Chan & Li, 2021; Dressler & Patel, 2015; Hilliard & El-Dahr, 2016; Huang et al., 2020). NP differentiation and nephrogenesis are guided by changes in DNA methylation and histone modifications that regulate gene transcription. DNA methyltransferase 1 (DNMT1) catalyzes DNA methylation in the nephrogenic mesenchyme and plays roles in nephron formation, NP self-renewal, and nephron endowment (Wanner et al., 2019). In addition, DNMT1 has been linked to the proper expression of many genes critical for kidney development including *Cited1*, *Eya1*, *Hoxc8*, *Hoxd11*, *Meox2*, *Osr1*, *Six2*, *Wnt4*, and *Wt1* (Wanner et al., 2019). Histone modification is also important in NPs where

levels of H3K9me2 and H3K27me3 are elevated to repress transcription until active differentiation (McLaughlin et al., 2013). Upon differentiation, repressive H3K9/K27me3 levels are downregulated while activating H3K4me3 remains high, a condition which activates the expression of *Pax2*, *Jag1*, and *Lhx1* (encoding for LIM-class homeodomain 1 or LIM1, a cell fate-specifying transcription factor which activates expression of Bruno-like1 (*Brn1*) and *EphA4* to regulate renal vesicle patterning (Chen et al., 2006)) (McLaughlin et al., 2014). Other NP differentiation-associated genes including *Pax8*, *Lef1*, and *Ccnd1* are activated predominantly by the gain of H3K4me3 alone (McLaughlin et al., 2013). Several histone methyltransferases and histone demethylases also play roles in kidney development and NP regulation, and details of these can be found elsewhere (Chan & Li, 2021; McLaughlin et al., 2014). In addition, histone arginine methylations have been implicated in the regulation of NP gene expression (McLaughlin et al., 2014), and histone deacetylases also regulate many important nephrogenic genes (Chen et al., 2011).

Even after the rapid phase of UB branching slows down at around E15.5 (Cebrian et al., 2004; Short et al., 2014), nephron progenitors and nephrogenesis remain active until P3 in a way that is no longer tied to UB branching (Hartman et al., 2007; Rumballe et al., 2011; Short et al., 2014). There is a final postnatal burst of nephrogenesis when the balance of signals in NPs switches from those signals maintaining the cells in a progenitor state to signals inducing nephron differentiation causing NPs to lose their self-renewal capacity and undergo accelerated differentiation (Hartman et al., 2007). The specifics of nephrogenesis cessation are still unknown, but changes in metabolism and an increase in blood flow and oxygen to the nephrogenic niche have been proposed as possible mechanisms regulating cessation (Brunskill et al., 2011; Rymer et al., 2014). Molecularly, aging NPs have increased mTOR and reduced FGF20, which could contribute to the cessation of nephrogenesis (Shuang Chen et al., 2015). In addition, a reduction in the activity of hamartin (TSC1), an mTOR-complex inhibitor and regulator of cell growth and proliferation, can prolong nephrogenesis past its normal cessation in an mTOR-independent manner. This indicates that hamartin may play a role in nephron progenitor aging (Volovelsky et al., 2018). Moreover, *Tcs1* coordinates changes in the transcriptome of NPs, increasing the cells' perception of WNT signals to end nephrogenesis (Jarmas et al., 2021). It has also been shown that mice overexpressing *Lin28* or with inactivated *Let-7* are able to prolong their nephrogenic programs (Urbach et al., 2014; Yermalovich et al., 2019). A better understanding of nephron progenitor regulation could facilitate reinitiated or prolonged nephrogenesis and thus lead to increased nephron number (Hendry et al., 2011).

2.2 RECEPTOR TYROSINE KINASE SIGNALING IN THE REGULATION OF KIDNEY DEVELOPMENT

It is widely accepted that receptor tyrosine kinase (RTK)-activated signaling is a major regulator of kidney development, and several receptor tyrosine kinases have known functions during kidney development: notably, GDNF/RET, FGFs, met/HGF, and Eph/Ephrin (Costantini, 2010; Kurtzeborn et al., 2018; Santos et al., 1994; Song et al., 2011; Walker et al., 2016; Weiss & Kispert, 2016). RTKs are activated by a myriad of ligands such as growth and neurotrophic factors, hormones, and cytokines. Upon ligand binding to the extracellular domain of an RTK on the cell surface, the receptors dimerize, autophosphorylate, and activate intracellular signaling cascades (Lemmon & Schlessinger, 2010). The main intracellular cascades activated downstream of extracellular stimuli acting through RTKs include RAS/RAF/MEK/ERK, PI3K/AKT/mTOR, and PLC γ /PKC (Jiménez et al., 2012; Regad, 2015). Our lab and this thesis are specifically focused on the RAS/RAF/MEK/ERK (commonly known as MAPK/ERK) signaling cascade.

2.2.1 MAPK/ERK SIGNALING OVERVIEW

RTKs acting through the GTPase RAS activate four separate MAPK cascades (Figure 6). Each cascade transmits the signal through a mitogen-activated protein (MAP) kinase kinase kinase (MAP3K), then a MAP kinase kinase (MAP2K), and finally a MAP kinase (MAPK). Here, I focus on the first identified and probably best characterized of these: the RAS/RAF/MEK/ERK cascade, often referred to as simply *the* MAPK or ERK pathway, although other MAPK pathways exist. In mammals, this pathway consists of three RAF protein kinases (RAF1, A-RAF, and B-RAF), two mitogen-activated protein kinase kinases (MEK1 and MEK2), and two extracellular signal-regulated kinases (ERK1 and ERK2) (Chang & Karin, 2001). RAS proteins can additionally induce activation of the PI3K pathway (Johnson et al., 1997; Tan & Kim, 1999), and it is not fully understood how the activation of different pathways is molecularly determined, which goes to show the high complexity of these signaling networks.

Upon phosphorylation by MEK1/2, activated ERK1/2 have many cytosolic and nuclear targets and functions (Roskoski, 2012a, 2012b). The speedy nuclear translocation of ERK1/2 in response to cellular stimulation has been implicated in the fast regulation of cell cycle progression and DNA replication (Brunet et al., 1999; Chambard et al., 2007). In embryonic stem cells (ESCs), ERK1/2 signaling regulates pluripotency through control of genomic stability, self-renewal, and differentiation (H. Chen et al., 2015; Ma et al., 2016). Mutations in MAPK/ERK cascade components *RAS*, *B-RAF*, *RAF1*, *MEK1*, or *MEK2* can lead to growth-factor independent cancer cell survival and

unconstrained cell proliferation, demonstrating the importance of the pathway in tissue homeostasis (Caunt et al., 2015; Shrestha et al., 2016). The MAPK/ERK pathway is tightly controlled at multiple levels, and mutations in negative regulators of the pathway (*NF1*, for example) also contribute to tumor progression (Brown et al., 2014; Kidger et al., 2018).

Besides its molecular-level regulation, signaling dynamics including the duration and strength of the signal play a huge role in the function of ERK1/2 (Marshall, 1995). This is nicely demonstrated in *Drosophila melanogaster* where ERK1/2 activity participates in cell fate determination and interestingly, the total ERK load rather than the amplitude or duration of the ERK activity is the determining factor controlling cell fate (Johnson & Toettcher, 2019). Following DNA damage, a pulsatile activation pattern of MAPK/ERK is required to sustain the pulsatile activation of p53, known for the non-permanent arrest of the cells in the G2 cell cycle phase where DNA damage repair can occur (De et al., 2020). Sustained ERK1 activation is also required for cell cycle progression through G1 where ERK1 is needed for the continual expression of *cyclin D1* (Weber et al., 1997). The duration of ERK activation can be controlled by a MEK1-MEK2 heterodimer which is subject to negative feedback from ERK itself through the phosphorylation of MEK1 at Thr292. Without this negative feedback mechanism, ERK activation is prolonged (Catalanotti et al., 2009). Changes in ERK activation dynamics can also lead to differences in downstream gene expression and cell fate outcomes (Marshall, 1995; Wilson et al., 2017; Yamamoto et al., 2006).

The other three parallel MAPK pathways activated through RAS (ERK5, JNK, and p38 cascades) are detailed in Figure 6 (Chang & Karin, 2001; Kurtzeborn et al., 2019; Nishida & Gotoh, 1993). The pathogenic roles of these three MAPK pathways in adult kidneys are well researched (Adhikary et al., 2004; Badshah et al., 2014; Grynberg et al., 2017; Sakai et al., 2002), but their functions in kidney development have hardly been explored. The ERK5 pathway is mainly activated through means other than RTKs, such as cytokines, ischemia, and hypoxia. While ERK5 does function during embryogenesis, for example in vascular and biliary duct development (Regan et al., 2002; Sato et al., 2005), its role in kidney development has not been examined. In addition to RTK-mediated signaling, JNK and p38 pathways respond to stress signals like pro-inflammatory cytokines tumor necrosis factor (TNF)- α and interleukin (IL)-1 β (Kim & Choi, 2010). During kidney development, JNK is involved with BMP7-regulated nephron progenitor proliferation (Blank et al., 2009) and is highly expressed in the adult rat kidney (Omori et al., 2000). High levels of p38 have been detected in embryonic rat kidneys where it seems to have roles in inflammation, cell growth, and nephrogenesis (Awazu et al., 2002; Omori et al., 2000).

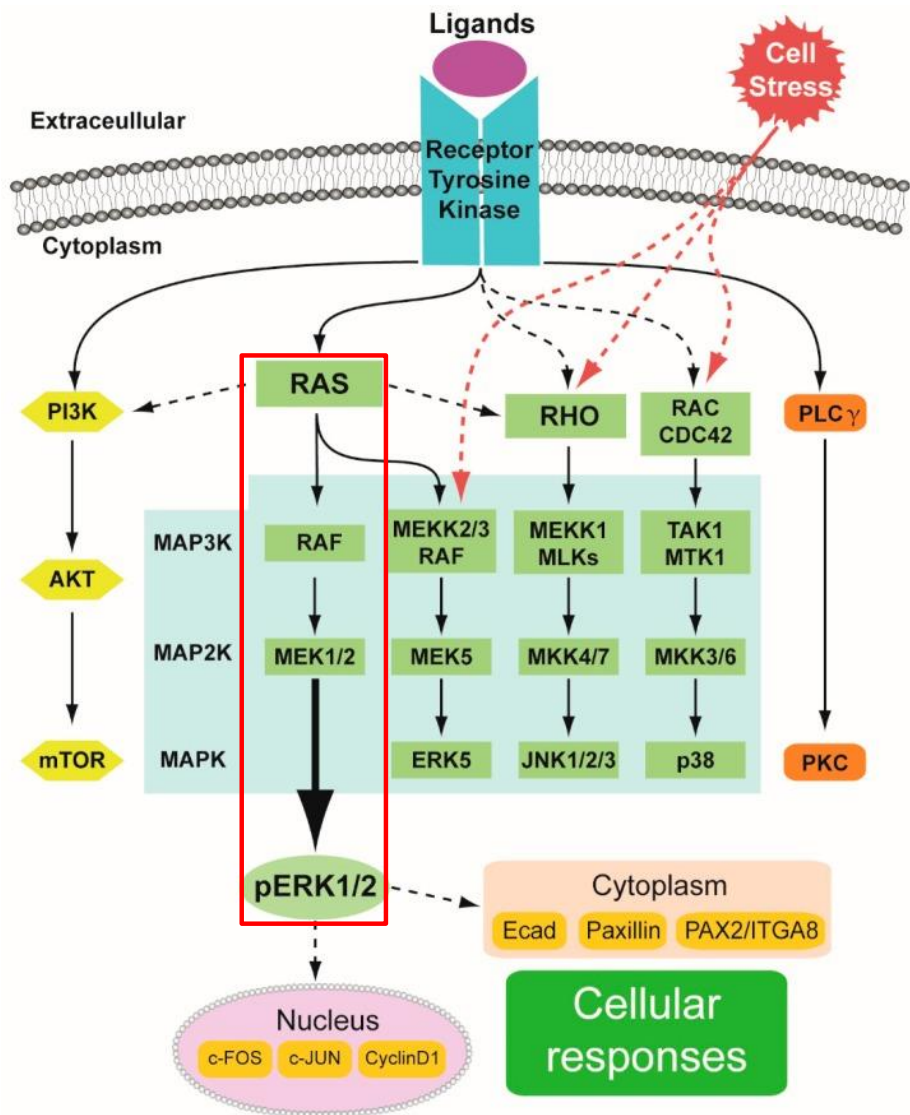


Figure 6 Overview of MAPK/ERK pathways and other pathways activated downstream of RTKs. The MAPK/ERK pathway mainly studied in this thesis is outlined in the red box. Image adapted and used with permission (Kurtzeborn et al., 2019).

During the development of many organs and tissues, the MAPK/ERK pathway plays significant roles including regulating cellular proliferation, differentiation, and survival (Boucherat et al., 2014; McKay & Morrison, 2007; Newbern et al., 2008; Scholl et al., 2007). It coordinates the activities of various signaling pathways and plays critical roles in determining the size, patterning, and function of the kidney by regulating ureteric bud branching morphogenesis and nephrogenesis (Ihermann-Hella et al., 2018; Ihermann-

Hella et al., 2014; Kurtzeborn et al., 2019). The extent of MAPK/ERK functions at the cellular and molecular levels during renal development are not fully known, so here I will discuss what we do know and what information is still missing.

2.2.2 MAPK/ERK MOUSE MODELS

The characterization of the many MAPK/ERK functions *in vivo* has been enhanced by the genetic targeting of pathway components. Constitutive loss-of-function of pathway components such as *B-raf*, *Raf1*, or *Erk2* leads to early embryonic lethality (Galabova-Kovacs, Matzen, et al., 2006; Hatano et al., 2003; Mikula et al., 2001; Wojnowski et al., 1998; Wojnowski et al., 1997). Because of this, gain-of-function and conditional allele mouse models have proven useful for examining MAPK/ERK *in vivo* (Bueno et al., 2000; Galabova-Kovacs, Kolbus, et al., 2006; Krens et al., 2006). Below, I will discuss the genetic models of *Mek1* and *Mek2*, which have been utilized in the study of renal differentiation by others and in this thesis project. I will additionally discuss the closely related *Erk1* and *Erk2* models.

Inactivation of the *Mek1* gene proved to be embryonic lethal in mice. *Mek1* mutant mice were not viable due to decreased endothelial cells and angiogenesis in the labyrinthine region of the placenta during development (Giroux et al., 1999). The placental defect could be rescued by tetraploid complementation of the *Mek1*-deficient embryos, leading to their viability and suggesting that although MEK1 is required for placental development, it is not compulsory for the development of the embryo itself (Bissonauth et al., 2006). On the contrary, genetic deletion of *Mek2* proved that it is dispensable for mouse development with normal embryogenesis and viable offspring (Belanger et al., 2003).

Double heterozygous mutants of *Mek1/2* exhibit a placental phenotype similar to *Mek1* mutants, suggesting that both MEK1 and MEK2 are essential for placental development, even though the functions of MEK1 appear to predominate (Nadeau & Charron, 2014; Nadeau et al., 2009). In a more recent genetic experiment, the functional redundancy of MEK1 and MEK2 was shown by *Mek2* knock-in under the control of *Mek1* endogenous regulatory elements. The genetic replacement of *Mek1* with *Mek2* rescued the *Mek1*-null phenotype and supported normal embryonic development. This experiment also demonstrates an essential gene dosage and therefore protein threshold effect where the total amount of MEK, rather than a specific amount of MEK1 versus MEK2 is required for functional ERK signaling (Aoidi et al., 2016). Despite some functional redundancy, it has also been shown that the roles of MEK1 and MEK2 in the phosphorylation of ERK are not interchangeable. MEK1 can negatively regulate MEK2-dependent ERK activation, and loss of MEK1 can actually increase ERK phosphorylation (Catalanotti et al., 2009).

Much the same as the *Mek1/2* mutant mice models, embryonic lethality caused by placental defects occurs in mice with *Erk2* genetic inactivation, while *Erk1* mutants appear to develop normally (Hatano et al., 2003; Pagès et al., 1999; Saba-El-Leil et al., 2003; Yao et al., 2003). Although *Erk1* mutants develop relatively normally, ERK1 has important roles in long-term memory function, control of hyperactivity, and resistance to the development of asthma, obesity, and skin papilloma (Bost et al., 2005; Bourcier et al., 2006; Goplen et al., 2012; Selcher et al., 2001). To advance the understanding of the functions of *Erk2* in mice, hypomorph and hemizygote mutants have been employed to overcome the embryonic lethality of homozygous deletion of *Erk2*. These models have proven useful to reveal a requirement of ERK2 for learning and memory (Satoh et al., 2007).

Resembling the partial functional redundancy of the upstream kinases MEK1 and MEK2, ERK1 and ERK2 also have some overlapping roles. However, due to differences in expression levels (ERK2 is more abundant in most tissues), it has been difficult for researchers to draw conclusions about the physiologically distinct and tissue-specific functions of ERK1/2. Both proteins seem to be of equal importance for growth signaling and normal proliferation. Several genetic experiments have detected the importance of both ERK1 and ERK2 during developmental processes like hematopoietic stem cell maintenance, placental differentiation, neurogenesis, and craniofacial development (Chan et al., 2013; Fischer et al., 2005; Satoh et al., 2011; Staser et al., 2012).

2.2.3 MAPK/ERK REGULATES URETERIC BUD OUTGROWTH AND BRANCHING MORPHOGENESIS

The importance of MAPK/ERK signaling during kidney development was originally demonstrated in *ex vivo* kidney cultures with inhibited MEK protein function. These experiments revealed decreased UB tip cell proliferation and atypical branch formation (Fisher et al., 2001; Hida et al., 2002; Watanabe & Costantini, 2004). The requirements of MAPK/ERK for UB branching morphogenesis and nephron development were further verified and explored *in vivo* in our lab (Ihermann-Hella et al., 2018; Ihermann-Hella et al., 2014).

At the onset of metanephric kidney development, there is a localized increase in MAPK/ERK signal in the area of the nephric duct where the initial UB outgrowth will occur, and this expression of ERK is lost if RET is inactivated in the nephric duct (Chi et al., 2009). MAPK/ERK activation is substantial yet heterogeneous in the UB epithelium and surrounding mesenchyme after the initial UB outgrowth. Within the UB, active ERK becomes restricted to the collecting duct progenitors in the UB tip region at the onset of branching morphogenesis (Ihermann-Hella et al., 2018; Ihermann-Hella et al., 2014). When ERK activation strength was measured

using a Förster resonance energy transfer (FRET)-based biosensor, ERK activity was lower in UB tips than in nephron progenitors and precursors, and ERK activity was almost non-existent in the UB trunks. This lower ERK activation strength in the UB tips compared to NPs could be explained by the heterogeneous activation pattern observed in the UB tips (Ithermann-Hella et al., 2018).

The MAPK/ERK pathway was first recognized as an important regulator of UB branching morphogenesis during experiments that genetically disrupted the RET docking sites phosphotyrosine 1062 (Y1062) and Y1015, sites required for downstream activation of MAPK/ERK and PI3K (de Graaff et al., 2001; Hoshi et al., 2012; Jain et al., 2006; Jain et al., 2010; Jijiwa et al., 2004; Wong et al., 2005). This work showed that downstream of RET, MAPK/ERK and PI3K cascades likely play critical roles in the regulation of UB branching (Costantini, 2010), however, these studies did not specifically focus on the unexplored functions of MAPK/ERK signaling in UB branching morphogenesis.

The first experiments to elucidate the tissue-specific functions of MAPK/ERK in the UB epithelium utilized conditional genetic inactivation of *Mek1* and conventional deletion of *Mek2*. UB-specific loss of *Mek1* had no effect on kidney development, similar to observations made in *Mek1* knockout embryos and epidermal keratinocytes (Bissonauth et al., 2006; Scholl et al., 2007). Reducing the gene dosage of *Mek1/2* by double heterozygosity or loss of any combination of three out of four *Mek* alleles had a limited effect on renal differentiation or UB branching, providing further evidence for the high functional redundancy between MEK1 and MEK2. In order to further study the functions of MAPK/ERK specifically in the UB, our lab generated a genetically modified mouse model where both *Mek1* alleles are conditionally deleted in a *Mek2* knockout background (details can be found in the Methods section). Deletion of all four *Mek1/2* alleles in the UB still allowed the initial UB outgrowth to occur normally, but subsequent branching morphogenesis was defective with a substantial decrease in novel branch formation. Interestingly, although the formation of new branches was inhibited, the UB stalks were still able to grow, leading to an elongation-only phenotype (Ithermann-Hella et al., 2014). Even though the UB tissue-specific dko of *Mek1/2* allows for UB outgrowth, this is likely due to the genetic model where Cre may function late or incompletely in deleting *Mek1*, as MEK-inhibited kidneys cultured with excess GDNF do not allow for the initial UB outgrowth to occur.

When our lab examined the cellular and molecular causes of the branching defect in MAPK/ERK-deficient UBs, it was theorized that the defect was likely caused by two mechanisms. First, it was determined that the regulation of cell cycle progression was aberrant in the absence of MAPK/ERK, a finding that makes sense considering MAPK/ERK signaling is a well-known regulator of

the cell cycle (Chambard et al., 2007; Terada et al., 1999; Wilkinson & Millar, 2000; Yamamoto et al., 2006). Second, substantial defects in the control of cellular adhesions were observed. MAPK/ERK-deficient UBs were unable to increase levels of cyclin D1 needed for progression from G1-to-S phase during cell proliferation (Terada et al., 1999). This proliferation defect led to fewer mitotic cells and a subsequent reduction of the total number of UB cells. Simultaneously, UB cells lacking MAPK/ERK have immature focal adhesions and exhibit abnormal adherens junctions with increased and partially displaced E-cadherin in the cell membranes (Ihmann-Hella et al., 2014). Thus, the MAPK/ERK cascade not only governs cell proliferation by its regulation of the cell cycle but also is required for maintenance of the cell adhesion dynamics required for branching morphogenesis and kidney growth. The study of the specific mechanisms through which MAPK/ERK is regulating UB cell adhesion is ongoing in our lab by myself and colleagues.

MAPK/ERK has a very dynamic activation pattern in the developing UB (Ihmann-Hella et al., 2018), suggesting the importance of the strict regulation of MAPK/ERK signal strength and duration for specifying cellular events during kidney development. This is exemplified by results from experiments with excess endogenous GDNF upstream of MAPK/ERK, where UB tips are abnormally expanded and have short trunks, a phenotype which is rescued upon MEK inhibition (H. Li et al., 2019). This phenotype is opposite that observed in MAPK/ERK-deficient UBs that have fewer tips and elongated trunks, all indicating that GDNF, acting through the MAPK/ERK cascade, positively regulates the expansion of UB tip/collecting duct progenitor cells (H. Li et al., 2019).

The requirement for accurate control of MAPK/ERK activation during UB branching is further supported by an experiment using ectopic expression in the mouse UB of a negative RTK/MAPK regulator, human sprout 2 (SPRY2), which resulted in defective branching morphogenesis (Chi et al., 2004). Further verifying the essential need for negative regulation in the control of signal strength, deletion of another Sprouty protein family member, *Sprouty1* (*Spry1*) results in the development of multiple, ectopic UBs growing out along the nephric duct (Basson et al., 2005; Basson et al., 2006). GDNF induces the expression of *Spry1* and other negative regulators of MAPK/ERK signaling including *Spred2* and *Dusp6* (Lu et al., 2009; Ola et al., 2011), confirming the role of negative feedback in UB branching, although the exact functions of SPRED2 and DUSP6 during UB branching morphogenesis remain to be determined. Interestingly, when *Gdnf* or *Ret* are genetically inactivated in a *Spry1*-null background, normal UB branching is restored, demonstrating how a fine balance is required for signaling in the development of the UB (Basson et al., 2005; Michos et al., 2010; Rozen et al., 2009).

2.2.4 BEYOND URETERIC BUD OUTGROWTH AND BRANCHING: ROLES OF GDNF IN KIDNEY MATURATION

The roles of GDNF/RET signaling during UB outgrowth and in the regulation of organ growth through branching have been well demonstrated (Cacalano et al., 1998; Costantini, 2010; Cullen-McEwen et al., 2001; Cullen-McEwen et al., 2003; Enomoto et al., 1998; Moore et al., 1996; Pichel et al., 1996; Sainio et al., 1997; Sanchez et al., 1996; Schuchardt et al., 1994). Furthermore, it was shown that loss of even one GDNF allele results in low nephron endowment, likely resulting from the decreased number of nephron progenitor niches due to the defective branching morphogenesis (Cullen-McEwen et al., 2001; Cullen-McEwen et al., 2003). GDNF/RET also regulates other aspects of urogenital development, as the proper insertion of the common nephric duct into the cloaca (the primitive bladder/urethra) has been shown to depend on signaling downstream of RET (Chia et al., 2011). Defects in the process of nephric duct extension can lead to misplaced ureters and hydronephrosis and are a common cause of pediatric kidney diseases. Although rare, *RET* mutations have also been reported in human fetuses with renal agenesis (Jeanpierre et al., 2011). The continued roles of GDNF during kidney maturation and in renal progenitors are less-well-known.

Our lab and colleagues have shown that an increase in endogenous GDNF by genetic disruption of the *Gdnf* 3' untranslated region (UTR) results in renal hypoplasia and poor kidney function (Kumar et al., 2015). Kidneys in GDNF hypermorphic mice have an initially expanded population of collecting duct progenitors around the time of UB outgrowth from the nephric duct. This expansion was shown to be at the expense of elongation of the ureteric trunks, leading to ballooned ureteric epithelium with short trunks. The mechanism by which excess GDNF regulates the development of this particular phenotype is by controlling cell cycle length and progenitor motility (H. Li et al., 2019).

2.2.5 MAPK/ERK SUSTAINS NEPHRON PROGENITORS

Along with the activation patterns in the UB, we also employed the FRET-based biosensor for ERK1/2 to live-image the signaling dynamics in the nephron progenitors and developing nephrons. We observed that ERK activation is heterogeneous throughout the nephron progenitor population and is strongest in the renal vesicles (Ihermann-Hella et al., 2018), indicating that MAPK/ERK signaling likely plays a role in the regulation of nephrogenesis in addition to branching morphogenesis. In the UB, ERK activation seems to correlate with cell cycle phase and overlaps with G1 and S phase cell cycle markers (Ihermann-Hella et al., 2014), but this was not the case with nephron progenitors and differentiating nephrons where we saw no evident connection to proliferative or non-proliferative cells.

As done previously with the UB-specific inactivation of *Mek1/2*, our lab next developed a nephron progenitor-specific *Mek1/2* double-knockout mouse model, which revealed the role of MAPK/ERK in two distinct processes during nephrogenesis. First, it was shown that MAPK/ERK is required for the maintenance and self-renewal of the NP population. Second, in line with the high levels of active ERK detected by FRET in differentiating nephron precursors, it was determined that MAPK/ERK is required to permit the progression of nephron differentiation (Ihermann-Hella et al., 2018). The phenotype observed in kidneys with MAPK/ERK-deficient NPs closely resembles the renal phenotypes detected in FGF-deficient kidneys. This suggests that MAPK/ERK could mediate the functions of, for example, FGF8/9/20 in NP maintenance, but MAPK/ERK likely acts downstream of FGF8 signaling in differentiating nephron precursors (Barak et al., 2012; Grieshammer et al., 2005; Perantoni et al., 2005; Sharma et al., 2022).

Upon inactivation of MAPK/ERK in the NP population, cells with the NP niche are disorganized and exhibit changes in the expression of integrin alpha 8 (ITGA8), a known mediator of kidney development and a major player in cell-matrix adhesion and signaling (Ihermann-Hella et al., 2018). Due to the changes observed in ITGA8, it is likely that MAPK/ERK has some role in mediating communication between NPs and the extracellular matrix. Cell-intrinsic changes were also observed in MAPK/ERK-deficient NPs. For example, reduced levels of PAX2 were observed in the NPs, and although the molecular regulation of PAX2 by MAPK/ERK is not yet determined, we were able to show that PAX2 in the NPs is required for the normal maintenance of ITGA8 levels (Ihermann-Hella et al., 2018). Such a link has also been suggested by other's experiments in the developing kidney (Naiman et al., 2017). Similarly, FGF signaling through FGFR1/2 was shown to maintain NP self-renewal and also PAX2 expression in NP cells (Barak et al., 2012; Poladia et al., 2006).

Upon chemical inhibition of MEK1/2 in rat embryonic kidneys, a reduction in nephrogenesis was detected (Hida et al., 2002). In line with this finding, we observed that mouse pups with NP-specific loss of *Mek1/2* are born with almost no nephrons. Nephron development in these mutants is normal until the renal vesicle stage where nephrogenesis is halted in almost all cases. Even though the first steps of nephrogenesis up until the renal vesicle stage appear to occur normally in MAPK/ERK-deficient NPs, an abnormal, undefined nephrogenic mass of PAX2+ cells that resembles a pretubular aggregate sometimes occurs. There is no increase in apoptosis in this population, and there is similarly no increase in the total amount of renal vesicles present in the developing kidney, so further studies are needed to determine the exact consequences of this phenotype (Ihermann-Hella et al., 2018).

Expression of *Fgf8* occurs at the same stage as nephrogenesis is arrested in MAPK/ERK-deficient NPs, the renal vesicle stage. Deletion of *Fgf8* causes a

similar defect in nephron differentiation (Grieshammer et al., 2005; Perantoni et al., 2005), and FGF8 has been shown to regulate the condensation of NPs around the UB through a chemokinetic effect (Sharma et al., 2022). MAPK/ERK signaling appears to function in renal vesicles to activate distal expression of the WNT mediator LEF1, cell cycle regulator cyclin D1, and the NOTCH ligand JAG1. MAPK/ERK is also required for proximal upregulation of WT1 in renal vesicles. These results indicate that the lack of MAPK/ERK limits nephron differentiation progression rather than the cells being specified for nephron segment identities (Ithmann-Hella et al., 2018). MAPK/ERK signaling, therefore, primes nephron precursor cells to make them receptive to molecular signals required to propel them to differentiate further through WNT and NOTCH pathways. Although, the exact molecular and cellular mechanisms by which this occurs are still under investigation.

2.3 CONGENITAL ANOMALIES OF THE KIDNEY

Congenital anomalies of the kidney and urinary tract (collectively known as CAKUT) are some of the most commonly occurring fetal malformations, affecting 1 in 500 live births, and their prevalence is increasing in Europe according to the EUROCAT European Surveillance of Congenital Anomalies (https://eu-rd-platform.jrc.ec.europa.eu/eurocat_en, 2020). There are several types of CAKUT, with clinical presentations ranging in severity from completely asymptomatic to those progressing to chronic kidney disease and ultimately renal failure. These anomalies include hypoplastic kidney, hydronephrosis, vesicoureteral reflux, duplicated kidney, horseshoe kidney, multicystic dysplastic kidney, the pediatric renal cancer called Wilms' tumor, and even total renal agenesis. Renal agenesis typically occurs when the body fails to establish the cell populations needed for kidney development or when there is a failure to induce UB outgrowth from the UB.

CAKUT arise during embryogenesis from abnormalities in renal differentiation. Some cases are sporadic, while others have a genetic or familial component. Although some potentially causative genetic mutations are identified, the genetic causes remain mostly unknown (Caruana & Bertram, 2015). Some environmental factors like maternal diabetes or drug exposure during gestation may also increase the risk of CAKUT. Treatment depends on the specific anomaly and may include surgical intervention, medication, or monitoring of kidney function. CAKUT can have long-term consequences, including chronic kidney disease and hypertension. Early detection and management of CAKUT are important for improving patient outcomes and preventing complications.

2.3.1 WILMS' TUMOR AND ITS LINK TO MAPK/ERK SIGNALING

Pediatric Wilms' tumor, also known as nephroblastoma, is the most common renal cancer in children. The exact cause of Wilms' tumor is not yet fully understood, but several factors have been identified that may contribute to its development, including both environmental and genetic factors. Its occurrence is linked to many syndromes, and its known genetic causes are heterogeneous and can occur spontaneously or by inheritance from a parent (Hastie, 2017). Wilms' tumor likely arises from undifferentiated mesenchyme remaining in the kidney after birth, and it can grow and spread to other parts of the body if left untreated. Wilms' tumor is of particular interest to our lab because its development has been linked to aberrant ERK activation in a subset of human tumors where pERK1/2 is upregulated (Subbiah et al., 2013) as well as in a mouse model of Wilms' tumor (Hu et al., 2011).

Common known causes of Wilms' tumor include aberrant expression of *WT1* (encoding the protein called Wilms tumor 1) (Kreidberg et al., 1993) and insulin-like growth factor 2 (IGF2) (Ogawa et al., 1993). Increased IGF2 expression in nephron progenitors of Wilms' tumor has been demonstrated through different mechanisms (Chen et al., 2018; Hunter et al., 2018) and has been shown to augment phosphorylation of ERK1/2 (Hu et al., 2011). Thus, increased IGF2 may be a common process through which MM is maintained in an undifferentiated state in postnatal kidneys. Additionally, LIN28B is overexpressed in many instances of human Wilms' tumor (Urbach et al., 2014), and LIN28 can be phosphorylated by MAPK/ERK in pluripotent stem cells to regulate their pluripotency (Tsanov et al., 2017). The *Lin28/let-7* axis regulates the timing of cessation of nephrogenesis during mouse kidney development (Yermalovich et al., 2019), and overexpression of *Lin28* blocks the final wave of NP differentiation and induces Wilms' tumorigenesis (Urbach et al., 2014). Although there are links between MAPK/ERK activation and Wilms' tumorigenesis, the full relevance of MAPK/ERK in the process remains to be revealed.

2.4 CELL FATE DECISION MAKING: SELF-RENEWAL VERSUS DIFFERENTIATION

Stem cells are unspecialized cells that can differentiate into many different cell types and have the capacity for unlimited replication. Similarly, progenitor cells are undifferentiated, but they are lineage-restricted and/or they have a limited capacity to proliferate. There is still some controversy over the exact definitions and differences between stem and progenitor cells, and the terms are often used interchangeably. Within the context of the developing kidney, I will refer to the undifferentiated cell populations as progenitors, whereas here, I will use the broader term 'stem cells'. Stem and progenitor cell fate is guided by the genetic program of the cell as well as signals from the cell's

microenvironment including intercellular signaling, growth factors, and cytokines. Several developmentally conserved signaling pathways are known to control fate determination. Notably, these include Wnt, Notch, Sonic hedgehog, and Smad pathways (Blank et al., 2008). The surrounding extracellular matrix and overall physiochemical environment of the stem cell niche also affect the cell fate determination. This is guided not only by the chemical composition of the ECM but also through the ECM topography, matrix stiffness, mechanical forces, oxygen tension, and electrical cues. The role of ECM and mechanical forces in regulating stem cell fate has been extensively studied, partly due to the potential applications in bioengineering materials to direct cell fate *in vitro* (Griffin et al., 2015; Guilak et al., 2009; Hu et al., 2022; Kumar et al., 2017; Lutolf et al., 2009; Watt & Huck, 2013).

Of particular interest for this thesis, metabolism also plays crucial roles in stem cell regulation by providing energy and building blocks for cellular proliferation, differentiation, and migration, which are all key processes in the formation of functional organs from stem cells (Agathocleous & Harris, 2013). Metabolism can also affect the expression of genes involved in self-renewal and during differentiation (Ryall et al., 2015). Section 2.5 below provides further details into the roles of metabolism during organogenesis and in stem cells.

2.5 THE ROLES OF METABOLISM DURING ORGANOGENESIS AND DIFFERENTIATION

During organogenesis, metabolic signals influence the energy state of stem cells and regulate the availability of specific metabolic intermediates and cofactors that can affect the activation or repression of specific genes and alter the balance between self-renewal and differentiation. For example, changes in glycolysis (the pathway which converts glucose to pyruvate) due to glucose availability can modulate the activity of pathways involved in regulating stem cell function, such as the Wnt and Notch signaling pathways (Anagnostou & Shepherd, 2008; Nishad et al., 2020). Metabolism can further regulate gene expression by controlling chromatin dynamics and epigenetics, as substrates and cofactors for most epigenetic enzymes are derived from metabolic pathways (Kinnaird et al., 2016; Reid et al., 2017). Additionally, metabolic signals can influence the redox state of the cell and the balance of pro-oxidant and antioxidant molecules, which also impacts stem cell fate (Liang & Ghaffari, 2014). Oxidative phosphorylation (OXPHOS), which produces energy in the form of adenosine triphosphate (ATP) through the oxidation of nutrients in the mitochondria, is activated during differentiation, and its suppression is required for stem cell self-renewal (Ito & Suda, 2014; Khacho & Slack, 2017). Additional ATP-independent mitochondrial signaling also plays a role in stem cell maintenance (Khacho & Slack, 2017). Disruptions in

2.5.1 WARBURG EFFECT IN STEM CELLS

In cells under normal oxygen conditions, glucose is usually converted to pyruvate then to acetyl-CoA which is oxidized in the mitochondrial tricarboxylic acid (TCA) cycle to ultimately generate 32 ATP molecules per glucose molecule consumed during OXPHOS. In hypoxic conditions, cells convert glucose to lactate, a process that produces only 2 ATP molecules per glucose. The Warburg effect is a metabolic switch from OXPHOS to glycolysis with lactate production, even in the presence of oxygen, that was originally described as occurring when normal cells transform into cancer cells (Warburg et al., 1927). A simplified overview of glycolysis and the TCA cycle can be seen in Figure 8. Much more recently, this effect has also been shown in non-cancerous cell types which are highly active, rapidly growing, or dividing. These include immune cells (Peng et al., 2016; Xu et al., 2021) and, interestingly, also stem cells (Folmes et al., 2011; Ito & Suda, 2014).

The role of aerobic glycolysis in the regulation of differentiation has since been described in several stem cell types (Agathocleous et al., 2012; Chung et al., 2010; Kondoh et al., 2007; Shum et al., 2016; Varum et al., 2011; Zhang et al., 2013; Zheng et al., 2016). For example, it has been shown that undifferentiated ESCs have high rates of glycolysis with lactate production and exhibit a gradual shift to OXPHOS as they become specialized and terminally differentiate (Agathocleous et al., 2012; Kondoh et al., 2007; Varum et al., 2011; Zheng et al., 2016). Indeed, the reverse is also true, as indicated by studies demonstrating a metabolic switch from OXPHOS to glycolysis during the reprogramming of somatic cells to iPSCs (Folmes et al., 2011; Panopoulos et al., 2012). To further support evidence for the role of the Warburg effect in pluripotency regulation, it has also been shown that two major positive regulators of glycolysis, the transcription factors hypoxia-inducible factors (HIFs) and c-Myc (Dang et al., 2009; Lum et al., 2007), are essential for pluripotency (Folmes et al., 2013; Zhou et al., 2012). Altogether, this further shows the importance of the metabolic switch between OXPHOS and glycolysis for cellular reprogramming and differentiation.

Even though the Warburg effect has been described in many types of proliferative stem and cancer cells, the reason it occurs is still up for debate (DeBerardinis & Chandel, 2020). It is likely that the metabolic switch is needed to shift the cell from an energy producing state to a biosynthetic state since the biosynthesis of cellular components like amino acids, nucleotids, lipids, and NADPH is important for cellular proliferation. Indeed, during the Warburg effect, some of the input glucose is utilized by the pentose phosphate pathway (PPP) to generate NADPH and ribose-5-phosphate, essential for cell proliferation as these are required for DNA duplication and RNA synthesis (Cantor & Sabatini, 2012; DeBerardinis et al., 2008; Vander Heiden et al., 2009).

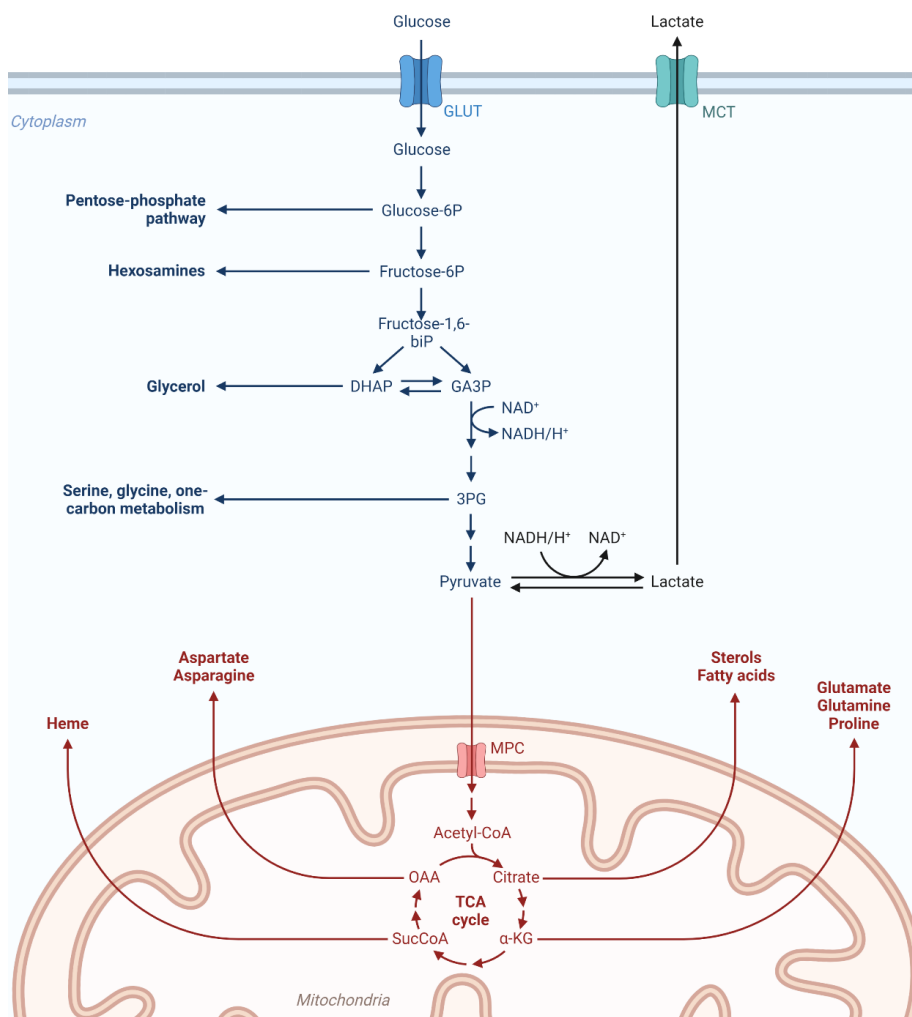


Figure 8 Glycolysis and TCA cycle metabolism. The Warburg effect is a switch from OXPHOS to glycolysis with lactate production. Glycolysis is shown in blue while pyruvate oxidation and TCA cycle are in red. In black is the final steps of the Warburg effect, the production and secretion of lactate. Image adapted from the “Warburg Effect” template at Biorender.com (2023; <http://app.biorender.com/biorender-templates>).

2.5.2 PROLINE METABOLISM AND CELL FATE

The non-essential amino acid L-proline is derived from the amino acid L-glutamate. Pyrroline-5-carboxylate reductase (PYCR) enzymes catalyze the final step in the proline biosynthesis pathway where L-proline is synthesized from pyrroline-5-carboxylate. PYCR1 and PYCR2 function in the mitochondria, while PYCR3 (also known as PYCRL) is cytosolic and synthesizes L-proline from different precursors than PYCR1/2 (Bogner et al., 2021; Christensen et al., 2017; De Ingeniis et al., 2012). The human *PYCR1*

gene is one of the most over-expressed metabolic genes in cancers where it has been shown to sustain cancer cell survival and proliferation (Alaqbi et al., 2022; Bogner et al., 2021; Burke et al., 2020; D'Aniello et al., 2020; Geng et al., 2021; Hu, 2021; Tanner et al., 2018). The proline biosynthesis pathway is critical in cancer and stem cells because it regulates protein and nucleotide synthesis, mitochondrial function, redox balance, pyridine ratios, and signaling pathways such as the mTOR and the amino acid response (AAR) pathways (Burke et al., 2020; D'Aniello et al., 2020; Ding et al., 2020; Tran et al., 2021). Deficiencies in proline metabolism during development can lead to congenital disorders including cutis laxa, which can also occur with renal manifestations (Aguilera et al., 2016; de Koning, 2017; Patriarca et al., 2021).

Proline metabolism also has a regulatory role in stem cell pluripotency. Blocking proline transport into ESCs maintains their pluripotent state and prevents their differentiation, and in hPSCs, the blocking of proline also impairs spontaneous differentiation (Tan et al., 2011; Washington et al., 2010). Supplementation of ESC cultures with proline promotes the cells to adopt a primitive ectoderm-like morphology and gene expression state (Washington et al., 2010), and it promotes the differentiation of naïve ESCs into more committed epiblast-like cells (Casalino et al., 2011). Additionally, proline metabolism is essential for neuronal differentiation, as shown by recent genetic knockout studies of *Pycr1* and *Pycr2* in mice (Escande-Beillard et al., 2020; Stum et al., 2021).

2.5.3 PYRUVATE METABOLISM AND CELL FATE

Pyruvate is an output of glycolysis and has been implicated in specific functions during stem cell regulation (Corbet, 2018; Ramos-Ibeas et al., 2017; Schell et al., 2017). Pyruvate is a particularly interesting and complex metabolite because it can trigger several different metabolic pathways downstream of glycolysis, depending on which enzyme acts upon it. Pyruvate dehydrogenase metabolizes pyruvate to acetyl-CoA to allow entry to the TCA cycle where pyruvate can be further oxidized. Pyruvate can also be metabolized to lactate by lactate dehydrogenase to support high glycolytic rates through its ability to regenerate NAD⁺. Furthermore, pyruvate can become oxaloacetate when acted upon by pyruvate carboxylase and can act to replenish intermediates of the TCA cycle required for anabolic pathways. In differentiated cells, pyruvate is mainly catabolized in the TCA cycle, while in pluripotent stem cells, most pyruvate is metabolized to lactate (Tsogtbaatar et al., 2020). Pyruvate is essential for embryonic development and contributes to the cell-fate switches in ESCs (Butcher et al., 1998; Conaghan et al., 1993; Moussaieff et al., 2015). Pyruvate supplementation of ESCs in culture has also been shown to enhance OXPHOS and suppress glycolysis while having no effect on the expression of pluripotency markers. This addition of pyruvate

and the associated metabolic shift activated AMPK/mTOR in the ESCs and supported mesodermal differentiation (Song et al., 2019). Thus, the regulation of glycolytic flux by pyruvate is important for the maintenance of pluripotency.

2.5.4 NEPHRON PROGENITOR METABOLISM

Nephron progenitors rely on high rates of glycolysis during active proliferation and self-renewal to support the high energy and nucleotide demands during their maintenance (Liu et al., 2017; Suda et al., 2011). In early (E13.5) nephron progenitors, high glycolysis and ATP production support self-renewal, while in older (E19.5) and committed nephron progenitors, OXPHOS takes over, and cells differentiate and exit the niche. Additionally, inhibition of glycolysis promotes nephron differentiation (Liu et al., 2017). The control of this age-dependent metabolic switch in nephron progenitors is not fully understood. It has been theorized that expression of HIF, which regulates several metabolism-related genes, may be involved in the control of cell fate through mitochondrial regulation (Cargill & Sims-Lucas, 2020). It has also been shown that the glycolysis-regulated self-renewal of nephron progenitors occurs through the regulation of the cholesterol biosynthesis pathway, and inhibition of either glycolysis or cholesterol synthesis results in nephron progenitor differentiation (Tortelote et al., 2020).

The tumor suppressor p53, which inhibits the PPP (Jiang et al., 2011), is expressed in NPs where it is required for their cell cycle regulation and metabolic fitness (Berkers et al., 2013; Li et al., 2015). Dysregulation of the PPP may have a larger effect on renal development as NP-specific knockout of *p53* results in altered NP self-renewal and nephron deficit (Li et al., 2015), while conventional *p53* knockout results in ectopic UB formation from the Wolffian duct (Saifudeen et al., 2009). Although, p53 may have a cell-type specific role because its depletion in NPs was shown to suppress glucose metabolism (Li et al., 2015).

In addition to the roles of metabolism in the regulation of nephron progenitor self-renewal and differentiation, the metabolic trajectories during nephrogenesis have also been defined. Utilizing spatial dynamic metabolomics, Wang, *et al.* recently showed that nephron epithelium develops by the following metabolic trajectory: As the renal vesicle differentiates towards the S-shaped body and proximal tubule, there is a shift from glycolysis to fatty acid β -oxidation. In the same study, it was shown that in organoids derived from hiPSCs, the cells are metabolically immature and incapable of using long-chain fatty acids for energy and that the addition of butyrate enhanced proximal tubular epithelial differentiation (Wang et al., 2022).

2.5.5 LINKS BETWEEN MAPK/ERK SIGNALING AND CELLULAR METABOLISM

The MAPK/ERK signaling pathway is involved in cellular metabolism regulation at several levels by controlling gene expression, protein synthesis, and energy utilization. Here, I will briefly list some of the described functions of MAPK/ERK signaling in metabolic pathways, with the caveat that these functions may be context-specific (i.e., described in cancer models, non-renal tissues, adult tissues, etc.) and may not necessarily apply directly to renal progenitors. The MAPK/ERK pathway can regulate the expression and activity of enzymes involved in metabolic processes such as glycolysis, OXPHOS, and fatty acid oxidation (Cha et al., 2021; Jiao et al., 2013; Papa et al., 2019), and ERK1/2 has been shown to promote the Warburg effect (Cha et al., 2021; Yang et al., 2012), which is activated in cancer cells as well as stem cells. Aberrations in MAPK/ERK signaling are a common cause of cancer, and it is unclear whether this could be a cause or a consequence of its regulation of the Warburg effect. Because this thesis is focused on development rather than cancer, I will not discuss this in further detail.

MAPK/ERK signaling is a known stimulator of glycolysis and may contribute to the glycolytic flux that occurs in nephron progenitors to promote their self-renewal during kidney development (Liu et al., 2017). MAPK/ERK can regulate glycolysis-related enzymes including hexokinase (Kim et al., 2007; Marko et al., 2010), phosphofructokinase (Gómez et al., 2012), and pyruvate kinase (Shiroki et al., 2017; Wong et al., 2020). This regulation occurs largely through the activities of c-Myc, a transcription factor expressed downstream of MAPK/ERK signaling (Dang et al., 2008; David et al., 2010; Lee et al., 2010; Shim et al., 1997). Of note, the pyruvate kinase isozyme M2 (PKM2) is expressed in the embryonic kidney, and its reduced activity can protect against kidney injury (Yang et al., 2012; Zhou et al., 2019). MAPK/ERK can further regulate glucose metabolism by controlling expression and activity of insulin receptor substrate (Sugano et al., 2006) and protein kinase B (Akt) (Shin et al., 2015), key components of the insulin signaling pathway. Of particular note, MAPK/ERK signaling modulates the activity of the tumor suppressor and major metabolic regulator, p53 (De et al., 2020; Lin et al., 1998; She et al., 2000). Through its direct activation and inhibition of a plethora of protein and other molecular targets, p53 can regulate several metabolic processes including glucose, fatty acid, and amino acid metabolism as well as control of reactive oxygen species, TCA cycle, and OXPHOS (Simabuco et al., 2018).

The function of mitochondria can also be regulated by MAPK/ERK through the regulation of expression of genes related to OXPHOS, including ATP synthase (Monick et al., 2008; Yung et al., 2004) and cytochrome c oxidase (Sun et al., 2002). In addition to glycolysis and OXPHOS, MAPK/ERK signaling can also regulate the expression of genes involved in

gluconeogenesis, such as glucose-6-phosphatase (Feng et al., 2012; Schmoll et al., 2001) and phosphoenolpyruvate carboxykinase (Winiarska et al., 2015) and genes involved in fatty acid synthesis and oxidation, like acetyl-CoA carboxylase, fatty acid synthase, and carnitine palmitoyltransferase (Bian et al., 2015; Jiao et al., 2013). In addition to known gluconeogenesis-related genes, ERK can also mediate the expression of Ets1, an inhibitor of gluconeogenesis that functions by acetylating the transcription factor FOXO1 (K. Li et al., 2019).

Under physiological conditions in the kidney cortex and in primary renal cell cultures as well as in an ischemia/reperfusion kidney injury model, ERK1/2 can directly inhibit mitochondrial function by decreasing mitochondrial biogenesis through the phosphorylation of upstream regulators of peroxisome proliferator-activated receptor γ coactivator-1 α (PGC-1 α), a master regulator of mitochondrial biosynthesis (Collier et al., 2016; Haq et al., 2013). MAPK/ERK regulation of mitochondrial biogenesis was also shown in other acute kidney injury models (Smith et al., 2015), and MAPK/ERK activation can also play a role in the process of mitophagy (Dagda et al., 2008; Zhu et al., 2007). ERK1/2 was also shown to regulate NAD metabolism during acute kidney injury (Collier & Schnellmann, 2020). Overall, the MAPK/ERK pathway plays critical roles in the regulation of cellular metabolism by modulating gene expression and in the coordination of cellular metabolism with physiological and environmental cues.

2.6 KIDNEY REGENERATION AND ADULT PLASTICITY

In most mammals, including the human and house mouse (*Mus musculus*), the adult kidney is a non-regenerating organ where damaged or injured nephrons are incapable of functionality repair or regeneration beyond basic cellular regeneration in portions of existing nephrons after tubular damage. Rather than forming new nephrons, adult kidneys adapt to nephron loss through compensatory nephron hypertrophy and hypertension, subsequently contributing to systemic hypertension (Hayslett, 1979). Due to the lack of regenerative potential, birth defects or injuries causing a reduced nephron number result in a permanent nephron deficit and a subsequent increased risk of developing kidney disease.

Nephron neogenesis has been described in many vertebrates, including fish (Bates et al., 2018; Davidson, 2014; Reimschuessel, 2001; Watanabe et al., 2009), amphibians (Droz & McLaughlin, 2017), and reptiles (Camarata et al., 2016), in contrast to most adult mammals. One exclusion to this is the mammalian African spiny mouse (*Acomys wilsoni*), which is able to fully regenerate its kidneys after injury without fibrosis (Okamura et al., 2021). The field of kidney injury, repair, and regeneration is vast and mostly outside the scope of this thesis. However, it is important that I touch on the matter to

provide a current state of the art for the ongoing research to highlight the relevance of my work in the field. Also, the importance of furthering the basic knowledge generated by developmental biology studies for potential clinical applications should be noted.

A significant amount of research on kidney regeneration has focused on stem cell and gene therapies. Researchers are exploring the use of stem cells to replace damaged or diseased cells and promote the growth of new, healthy kidney tissue, several models of which are currently showing promise in clinical trials (Ahmadi et al., 2020; Bussolati & Camussi, 2015; Chen et al., 2023; Imberti et al., 2015; Suzuki et al., 2016). Gene therapy is being evaluated to introduce genes into the kidney to promote rejuvenation and regeneration, although this has not yet proven successful due to difficulties in targeting gene products to the kidney (Davis & Park, 2019; Peek & Wilson, 2022). Scientists are also exploring the use of nanoparticles to improve the delivery of drugs and other therapeutic agents directly to damaged kidney tissue with the potential to target specific areas of the kidney and promote regeneration, however, this technology is still in its infancy (X. Huang et al., 2021; Y. Huang et al., 2021; Merlin & Li, 2022). The lack of a thorough understanding of renal progenitor cell regulation is a major hindrance to the development of these applications where progenitor cell expansion and lineage commitment toward differentiated cell types of the kidney must be precisely controlled. Researchers are elucidating more and more about the molecular regulation of these processes, but the full picture of progenitor regulation has yet to form.

Further studies have focused on tissue engineering techniques to create functional kidney tissue using a combination of cells, scaffolds, and growth factors (Cintron Pregosin et al., 2021; Katari et al., 2015). This approach has the potential to replace damaged or diseased kidneys with engineered fully functional ones. Recently, bioprinting technology has been utilized to create 3D-printed kidney tissues. This approach has the potential to create custom-made kidney tissue that is tailored to the specific needs of each patient, although the technology has not yet reached a point of clinical realization (Maharjan et al., 2022; Turunen et al., 2018; Wragg et al., 2019).

In addition to the methods mentioned here, it is also of great importance that we continue to expand our knowledge of the cellular and molecular mechanisms guiding embryonic kidney development to understand how and why mammalian kidneys lose their regenerative capacity and whether there is a possibility to regain this capacity in adult kidneys. Recent advances in the understanding of human kidney development and its similarities to that in mouse (Cullen-McEwen et al., 2016; Lindström, McMahon, et al., 2018) further strengthen the relevance of our and other's studies in model organism. Moreover, involvement of the MAPK/ERK pathway has been confirmed in multiple vertebrate tissue and organ regenerative processes (although not yet in kidney) (Wen et al., 2022), even further demonstrating the importance of

our work for the field of regenerative medicine. Overall, the study of kidney regeneration is still in its early stages, but there is hope that these approaches will lead to new treatments for human kidney disease and injury in the future.

2.7 KIDNEY ORGANOID MODELS

Kidney organoids are three-dimensional miniaturized versions of kidneys that are derived from stem cells. In addition to stem cell-derived kidney organoids, other *in vitro* models called tubuloids, which are epithelial organoids derived from adult tissues, are being used for similar purposes. These organoids and tubuloids are increasingly being used as models for studying kidney development, disease, and drug discovery, and their potential has been reviewed extensively over the past couple of years (Jensen & Little, 2023; Khoshdel-Rad et al., 2022; Khoshdel Rad et al., 2020; Nunez-Nescolarde et al., 2022; Romero-Guevara et al., 2020; Stein et al., 2021). Several groups have optimized the protocols for generating kidney organoids by differentiating human pluripotent stem cells into intermediate mesoderm and then further differentiating them into kidney organoids using different combinations of growth factors and extracellular matrix components. Researchers have characterized kidney organoids using a variety of techniques including gene expression analysis, immunohistochemistry, and electron microscopy. These studies have shown that kidney organoids recapitulate many aspects of kidney development and function, including the formation of nephrons, glomeruli, and tubules. However, current models remain immature and many lack physiologically relevant features such as vasculature, issues which are only recently starting to be tackled (Bas-Cristóbal Menéndez et al., 2022; Koning et al., 2020; van den Berg et al., 2018), which makes them far from providing a solution for kidney diseases in the near future.

Kidney organoids have recently been used to model a variety of kidney diseases including polycystic kidney disease (Cruz et al., 2017; Xu et al., 2022), Alport syndrome (Morais et al., 2022), and nephrotic syndrome (Jansen et al., 2022). Organoids with deletion of WT1 have even been used to model Wilms' tumor (Waehle et al., 2021). These organoid models not only provide insights into disease mechanisms but have also been utilized for drug screening. Additional studies have identified potential therapeutic agents by using a kidney organoid model of diabetic nephropathy to test for nephrotoxicity and efficacy (Garreta et al., 2022). Organoid models incorporating other cells types, such as endothelial and immune cells (Menéndez et al., 2022), have also been developed. However, although co-cultures of organoids with immune cells have been set up, they have not yet been established in kidney organoids. Co-culture models can be used to study interactions between different cell types in the kidney (or other organs) and the effects of drugs on these interactions. Recently, bioprinting is also being used to improve kidney

organoids and to improve their reproducibility (Fransen et al., 2021; Humphreys, 2021; Lawlor et al., 2021). Although there is still much room for improvement and optimization, overall, the development of kidney organoid models has provided a powerful tool for studying kidney development, disease, and drug discovery. Advances in our knowledge of the molecular mechanisms behind kidney development will provide new information for the continued improvement of renal organoid models. For more on this, recent in-depth analyses of the current status and applications of kidney organoids can be read elsewhere (Dorison et al., 2022; Khoshdel-Rad et al., 2022; Trush & Takasato, 2022).

2.8 FUTURE PERSPECTIVES

Despite our ever-increasing knowledge of the biological intricacies of renal development and disease and the many recent advances in the fields of kidney regeneration, stem cell technologies, and organoid models, there remains no feasible cure for serious renal complications such as chronic kidney disease and end-stage renal disease. The current treatment options remain mostly limited to dialysis and transplantation, which are insufficient and costly, not to mention the serious lack of available donor organs.

Researchers have recently performed the first xenotransplantations using transgenic pigs as donors of kidneys for human transplantation. Brain-dead human recipients received genetically modified pig kidney xenografts which were shown to be viable and functional for approximately a two-to-three day time course with no signs of rejection (Montgomery et al., 2022; Porrett et al., 2022). Although these studies seem promising, they are still in the early stages. So far, there have been very few human recipients of kidney xenografts, and the studies are majorly limited by their short duration and unknown implications of the molecular milieu of brain death on renal recovery.

Kidney organoids hold a lot of promise not only for the future of kidney research but also for possible clinical applications, including personalized medicine using iPSCs derived from patients. A long-term goal is to generate transplantable, laboratory-grown kidneys, but there are many obstacles to overcome before this is realized. One of the major pitfalls for the development of kidney organoids has been the lack of necessary physiological inputs, namely from a lack of vascularization and innervation. Recent studies have begun to address these issues (Bas-Cristóbal Menéndez et al., 2022; Koning et al., 2020; van den Berg et al., 2018), but further research is needed before these techniques are perfected. Other limitations include off-target cell contamination and high variability between experiments (Phipson et al., 2019; Wu et al., 2018), and these will also need to be addressed before kidney organoids can reach their full potential.

There is also a recent spotlight on the use of precision medicine approaches for kidney disease patients (Fliser & Wanner, 2021; Sun et al., 2017; Tye et al.,

2021), kidney transplant recipients (Mann et al., 2019), and children suffering from congenital anomalies of the kidney and urinary tract (Westland et al., 2021). The use of personalized medicine, tailored to the individual patient's genetics, lifestyle, and disease profile can help optimize kidney disease management and improve patient outcomes. In line with this, the Kidney Precision Medicine Project (kpmp.org) was launched in 2017 to create a kidney tissue atlas and identify the critical cells, pathways, and targets for novel kidney disease therapies. Although this ambitious project is making great strides, there still remains a dearth of knowledge about the complex molecular and cellular processes dictating disease onset and progression.

Overall, the future of kidney disease diagnosis and treatment looks promising, with a variety of potential new therapies and interventions that can improve outcomes for patients. These strategies will only continue to improve as we pursue the remaining unknown factors dictating renal development and disease.

3 STUDY AIMS

The overarching aim of my doctoral project is to characterize the cellular and molecular roles of RTK signaling in the developing kidney. I focus on the developmental processes of ureteric bud branching morphogenesis and nephrogenesis in the embryonic kidney. My thesis dissects the tissue-specific functions of MAPK/ERK signaling, on one hand downstream of GDNF/RET in renal collecting duct progenitors and, on the other hand, downstream of FGF signaling in nephron progenitors.

More specifically, my aims are:

- 1) To identify gene and metabolite targets downstream of the MAPK/ERK pathway and elucidate their roles in renal progenitor cell maintenance and differentiation (Article I and II);
- 2) To utilize a systems biology approach to integrate results from transcriptomic and metabolomics analyses of MAPK/ERK-deficient nephron progenitors to functionally demonstrate how MAPK/ERK regulates metabolism during nephron progenitor maintenance (Article I and II);
- 3) To characterize the effects of excess fetal GDNF on nephron progenitors and define the role of GDNF activity levels in the specification of nephron progenitor lifespan (Article III).

4 MATERIALS AND METHODS

A wide range of different materials and methods were used to conduct the research of this thesis project, with many methods benefiting from the talent and expertise of co-authors and collaborators. The table below summarizes the main methods used in these studies. Details of the methods can be found in the original publications. The methods central to this thesis and requiring further discussion are described below in more detail.

Table 1 *List of methods used in the accompanying articles.*

Method	Article
Genetically modified mouse models	I, II, III
Genotyping (PCR)	I, II, III
Microdissection (collection of tissues and primary cells)	I, II, III
<i>Ex vivo</i> kidney culture	I, II, III
Cell culture	I, II
Tissue processing for histological samples	I, II, III
Immunofluorescence and Hematoxylin-Eosin staining	I, II, III
<i>In situ</i> hybridization	III
Microscopy (widefield, confocal)	I, II, III
Image analysis and quantification	I, II, III
Tissue-specific cell isolation/FACS	I, II
RNA isolation	I, III
RNA sequencing	I
Reverse transcription and quantitative PCR (qPCR)	I, III
Gene ontology and pathway analysis	I, II
Metabolite extraction from cells	I, II
Liquid chromatography-mass spectrometry (LC-MS)	I, II
ELISA	III
Mitochondrial DNA copy number measurement	I
Seahorse analysis of mitochondrial metabolism	I
Analysis and integration of omics data	I, II
Statistical analysis	I, III

4.1 ETHICAL STATEMENTS AND LICENSES

The National Animal Experiment Board of Finland authorized the animal care and research procedures, which were carried out in accordance with the Code of Ethical Conduct for Animal Experimentation as well as European Union Directive 2010/EU/63 and using the 3R principle (aiming towards

replacement, reduction, and refinement). Mice were housed in individually ventilated cages at optimal humidity and temperature with free access to food and water at the University of Helsinki's accredited Laboratory Animal Center under internal license KEK22-016.

4.2 GENETICALLY MODIFIED MOUSE MODELS

4.2.1 TISSUE-SPECIFIC MEK1/2 DELETION IN URETERIC BUD AND NEPHRON PROGENITORS

Mice with *Mek1* deletion are not viable as the loss of *Mek1* is embryonic lethal (Bissonauth et al., 2006; Giroux et al., 1999). As such, this prevents developmental biology studies, including kidney development in the conventional *Mek1* knockout model. Due to this, we employed a tissue-specific approach to remove *Mek1*. We did this in a *Mek2*-null background, since *Mek1* and *Mek2* have some redundant functions and *Mek2* is dispensable for mouse development with *Mek2*^{-/-} mice being phenotypically normal (Belanger et al., 2003). To achieve tissue-specific deletion of *Mek1/2* in the developing UB and NPs, we utilized a combination of a conventional knockout of *Mek2* (*Mek2*^{-/-}) (Belanger et al., 2003) with conditional (floxed) *Mek1* alleles (*Mek1*^{fl/fl}) (Scholl et al., 2007) under the control of tissue-specific promoters. For *Mek1* deletion in the UB/Wolffian duct lineage, we used the *Hoxb7* promoter to drive the expression of cre recombinase and green fluorescent protein (GFP) (*Hoxb7CreGFP*) (Ihermann-Hella et al., 2014; Zhao et al., 2004). Similarly, we employed the *Six2* promoter for NP-specific deletion of *Mek1* and GFP expression (*Six2*-Tet-off-eGFP-Cre or “*Six2*-TGC^{tg/+}”) (Ihermann-Hella et al., 2018; Kobayashi et al., 2008). All mice had a mixed genetic background on C57Bl6/Rcc and 129/SvEv.

Our lab first generated the UB-specific conditional *Mek1* knockout transgenic mice by breeding the *Hoxb7CreGFP* mouse line with the *Mek1*^{fl/fl} line (Ihermann-Hella et al., 2014; Scholl et al., 2007; Zhao et al., 2004). Embryonic kidneys in these *Hoxb7Cre;Mek1*^{fl/fl} mice appeared to develop normally (Ihermann-Hella et al., 2014), likely due to the compensatory function of *Mek2*, and indicated a need to genetically abrogate *Mek2* in addition to *Mek1* in order to abolish ERK phosphorylation and elucidate the full effects of loss of MAPK/ERK signaling on kidney development. UB-specific reduction of gene dosages of *Mek1/2*, even the loss of any combination of three out of four *Mek1/2* alleles, allowed for relatively normal kidney development. This resulted in the generation of the full *Mek1/2* knockout *Hoxb7Cre;Mek1*^{fl/fl};*Mek2*^{-/-} line which we refer to simply as *Hoxb7* double-knockout (dko) (Ihermann-Hella et al., 2014).

When the *Six2-TGC* transgenic mice were first generated, the authors described that some of the *Six2*⁺ cells failed to express cre recombinase and the associated GFP (Kobayashi et al., 2008). We showed that when crossed

with *Mek1* floxed mice, protein levels of MEK1 were diminished in nearly all the NPs and nephron precursors by E13.5 (Ihermann-Hella et al., 2018). Combined breeding with the *Mek2* conditional knockout (*Six2-TGC^{tg/+};Mek1^{fl/fl};Mek2^{-/-}*) depleted MAPK/ERK activity in NPs and descendant renal vesicles. We refer to the NP-specific loss of all four *Mek1/2* alleles as “Six2 dko”. As with the *Hoxb7* dko, full loss of all four *Mek1/2* alleles in NPs is required to abrogate MAPK/ERK activity in the Six2+ cells (Ihermann-Hella et al., 2018).

4.2.2 OTHER GENETICALLY MODIFIED MOUSE MODELS

Mice with a hypermorphic *Gdnf* allele (*Gdnf^{hyper}*) were generated by disrupting the transcription of the *Gdnf* three prime untranslated region (3'-UTR). This was accomplished through the insertion of a neo-selection cassette into the wild type *Gdnf* 3'UTR with a bovine growth hormone polyadenylation signal after the stop-codon in the *Gdnf* endogenous locus. This results in mice with impaired *Gdnf* mRNA regulation causing an increased expression of GDNF that is not spatially changed. Homozygous mutation leads to kidney defects which cause postnatal lethality at around three weeks, while heterozygous mutants (*Gdnf^{flwt/hyper}*) appear healthy with only nominal reduction in kidney size observed (Kumar et al., 2015).

Generation of *Wnt11* knockout mice was first conducted by homologous recombination in ES cells derived from 129Sv mouse strain (Majumdar et al., 2003). This model was updated by crossing the *Wnt11^{-/-}* 129Sv mice with mice from a C57Bl6 background in order to overcome the high mortality observed in the original *Wnt11^{-/-}* in 129Sv background and allow for longer-term studies of kidney function in the absence of *Wnt11* since some of these mutant mice live until adulthood (Nagy et al., 2016).

Due to the increased canonical WNT signaling we observed in *Gdnf^{hyper/hyper}* mice, we crossed the *Wnt11* knockout allele with the *Gdnf^{hyper}* background to generate a combined *Gdnf^{hyper};Wnt11^{-/-}* model. Breeding heterozygous *Gdnf^{flwt/hyper}* and *Wnt11^{+/-}* failed to generate any double homozygous knockouts (*Gdnf^{hyper/hyper};Wnt11^{-/-}*), indicating embryonic lethality. Because of this, we focused our studies on *Gdnf^{hyper/hyerr};Wnt11^{+/-}* mice.

Mutant mice with targeted *Fgf20* gene inactivation were originally created by insertion of a β -galactosidase (β Gal) cDNA into exon 1 of *Fgf20* (Huh, et al. 2012). Compound *Fgf9;Fgf20* mutant mice (*Fgf9^{+/+};Fgf20^{+/-}*, *Fgf9^{+/+};Fgf20^{-/-}*, *Fgf9^{+/-};Fgf20^{-/-}*) were generated as previously described (Barak, et al. 2012). Double homozygous mutants (*Fgf9^{-/-};Fgf20^{-/-}*) exhibit renal agenesis and were therefore not examined in our study (Barak, et al. 2012).

Mice with the conventional deletion of the UB-expressed GDNF-target *Crlf1* (previously called the NR6 gene) were originally generated by homologous recombination in ES cells by replacing the gene's immunoglobulin-like and hemopoietin domains with a G418-resistant

cassette. Homozygous *Cr1f1*^{-/-} mice do not show compromised embryonic survival, but the conventional loss of *Cr1f1* is lethal during the first 24 hours of life (Alexander et al., 1999).

Mice with double-knockout of pyrroline-5-carboxylate reductases 1 (*Pycr1*) and 2 (*Pycr2*) were generated previously by crossing *Pycr1*^{-/-} mice with *Pycr2*^{+/-} mice (Escande-Beillard, et al. 2020; Stum, et al. 2021). The resulting double *Pycr1/2* mutants proved to be subviable. *Pycr1* mutant mice were created by the Knockout Mouse Program (KOMP) at the Jackson Laboratory, and the *Pycr2* mutant mice were generated through the ENU mutagenesis program, also at Jackson Laboratory (Stum et al., 2021).

4.3 EX VIVO EMBRYONIC KIDNEY CULTURES

Ex vivo organ cultures are an important tool for developmental biology studies because they allow tissue observation and manipulation in ways that would not be possible *in vivo*. The first kidney culture experiments were performed already in the 1950's (Grobstein, 1953), and while many aspects of the original methods have remained in use, they have evolved over time to match the current state of the art.

In the studies of this thesis project, I utilized a Trowel-type setup that employs an air-liquid interface during kidney culture on top of a mesh filter. This method was first described by Lauri Saxén (Saxen & Lehtonen, 1987), and updated protocols with detailed instructions for the microdissection of embryonic kidneys and subsequent culture, similar to those used in these studies, have been recently published (Aresh & Peuckert, 2017; Ihermann-Hella & Kuure, 2019).

4.4 CELL LINES

Whenever possible, we utilized primary cells from embryonic kidneys for experimentation. However, due to the relatively low amount of NP cells in the early embryonic kidney, it was necessary to occasionally utilize a metanephric mesenchyme-derived cell line. These cells are referred to as mK4 cells, and they represent induced metanephric mesenchyme capable of undergoing epithelial conversion/nephrogenesis. This clonal cell line was originally generated from transgenic mice with the Simian Virus 40 T-antigen (*SV40 Tag*) gene, which causes developmental arrest and immortalization of the cells, under the control of the *Hoxa11* promotor (Valerius et al., 2002).

5 RESULTS

5.1 IDENTIFICATION OF MAPK/ERK TRANSCRIPTIONAL TARGETS IN RENAL PROGENITOR CELL POPULATIONS (ARTICLES I & II)

Previous studies in our lab as well as my own work have shown that MAPK/ERK signaling regulates UB development as well as the preservation and differentiation of NPs. The full spectrum of transcriptional regulation by MAPK/ERK in these tissues, however, is unclear. In this study, we used control mice along with two tissue-specific MAPK/ERK knockout mouse models to perform RNA sequencing (RNA-Seq) on UB epithelial and NP cells to identify MAPK/ERK targets within these progenitor populations. Henceforth, I will refer to these as UB dko and NP dko, where dko stands for double knockout (i.e., both *Mek1* and *Mek2* are knocked out in the tissue; further information regarding mouse models can be found in the Methods section). To identify primary rather than secondary MAPK/ERK targets, RNA was obtained from embryonic renal tissues at developmental stages when the MAPK/ERK pathway activity was primarily reduced, and morphological abnormalities were first appearing. Using our knowledge of the phenotypes resulting from the UB- and NP-specific deletion of MAPK/ERK, we chose to isolate RNA from UBs in E12.5 kidneys and from NPs in E13.5 kidneys.

Following RNA-Seq, we first performed principal component analysis (PCA). PCA revealed that both UB and NP control and dko samples highly clustered within their respective populations but are obviously different from one another. Levels of gene expression in dko populations were normalized against those in controls. Differential expression analysis was done using the DESeq2 tool in the R/Bioconductor package to compare control and dko samples.

The total numbers of transcripts detected by RNA-Seq were 55,335 in the UB and 52,636 in the NPs. To discover which RNAs were most abundant in the UB and NP dko cells, we set statistical thresholds using the following cutoff values: adjusted P-value (P_{adj}) less than 0.05 and an expression magnitude threshold of $|\log_2\text{foldchange}| \geq 1$. Consequently, this left us with 5053 (2341 upregulated; 2713 downregulated) significantly differentially expressed genes (DEGs) in the dko NPs and 1004 (632 upregulated; 372 downregulated) in the dko UB cells. The cellular heterogeneity within the NP population is likely reflected in the fact that there were so many DEGs detected in this population.

5.2 TRANSCRIPTIONAL TARGETS OF MAPK/ERK ARE HIGHLY TISSUE SPECIFIC (ARTICLE I)

We next performed gene ontology (GO) analysis for the UB and NP dko cells to identify alterations in gene expression at a broad level in each population. GO analysis using the ToppFun application from ToppGene Suite (<https://toppgene.cchmc.org>) revealed 595 significantly affected ($p < 0.05$) biological processes in the UB dko and 1035 in the NP dko cells. Additionally, 80 cellular components were altered in the UB dko and 257 were altered in the NP dko. Finally, we looked at changes in molecular functions, and we found that 29 were altered in the UB dko and 144 in the NP dko. Results of the GO analysis suggest that there are several different roles played by the MAPK/ERK pathway in both renal progenitor populations examined.

Consistent with the phenotype of MAPK/ERK-deficient UBs that was previously described by our lab, including molecular-level changes and deficiency in branching morphogenesis, we now observed that many of the DEGs and related changed biological processes in the UB dko participate in proliferation and cell motility, response to stimuli, and epithelium development. The major disrupted biological processes in the NP dko cells were related to metabolism, cell cycle, and protein localization and transport. Analysis of the variations in molecular functions found changes in enzymes like kinases and transferases in both UB and NP dkos, while in the UB dko alone, there was altered DNA binding, and in the NP dko, RNA binding, ribonucleotide biology, and purine binding were altered. The tissue-specific fidelity of MAPK/ERK deficiency was further demonstrated by the analysis of cellular components. In the NP dko, DEGs were involved with adhesions, mitochondria, and catalytic complexes, while in the UB dko, DEGs were mainly related to the structure of organelles such as the Golgi, endosomes, and cell membranes and junctions.

Upon comparison of the UB and NP dko datasets, we discovered only 362 DEGs common between the datasets. This accounts for 36% of the total DEGs in the UB dko and only 7% of the NP dko DEGs. This further indicates that changes in gene expression resulting from the loss of MAPK/ERK signaling are largely tissue-specific as the majority of transcriptional changes are seen in only one or the other examined tissue.

5.3 DIFFERENTIALLY EXPRESSED GENES COMMON TO BOTH MAPK/ERK-DEFICIENT URETERIC BUD AND NEPHRON PROGENITOR POPULATIONS REFLECT EPIGENETIC CHANGES (ARTICLE I)

Although the identified MAPK/ERK targets were highly tissue-specific, a subset of 362 of the identified genes were differentially expressed (DE) in both the UB and the NPs. Of these shared genes, 193 transcripts (53%) were

upregulated in both cell populations, 157 (43%) were downregulated in both populations, and only 12 genes (3% of shared genes) were inversely DE between the UB and NPs. Within the group of 12 inversely expressed genes, 11 have confirmed protein-coding activity. Interestingly, two of these genes are MAPK/ERK inhibitors with recognized roles during kidney organogenesis: *Sprouty1* (*Spry1*) was downregulated in the MAPK/ERK deficient UB and upregulated in the NPs, while *Cavin1* was upregulated in the UB and downregulated in the NPs. Other kidney development-related genes, *Cadherin 6* and *Sox9* were also inversely expressed between the UB and NP populations.

The majority of MAPK/ERK targets common to both the UB and NP dkos encode proteins that participate in proliferation, cell cycle, DNA and RNA binding, growth factor response, and organ development and branching morphogenesis, as predicted given previously reported MAPK/ERK functions. GO analysis of the 193 upregulated DEGs common to both tissues found relatively few genes fitting into any single biological or molecular process category, suggesting substantial variety among upregulated DEGs that reflect MAPK/ERK targets. Analysis of the 157 common downregulated genes corresponds well with the biological processes and molecular functions discovered in the total shared genes. This shows that the MAPK/ERK pathway has a significant and probably ubiquitous regulatory function in processes such as cell cycle, proliferation, growth factor response, organ development, and DNA/RNA binding.

The majority of the common downregulated DEGs, comprising 13% of the shared genes, are histones, and GO analysis revealed that the most impacted biological processes are chromosomal condensation and nucleosome assembly. Overall, in UB dko, 25 genes encoding histone cluster components, histone chaperones, or histone-associated kinases are downregulated, whereas in NP dko, there are 69 histone-related DEGs. *Hist1h2br* (H2B clustered histone 24) in UB dko and *Hist1h2ai* (H2A clustered histone 13) in NP dko are the most downregulated genes in each dataset. Indeed, there is a significant histone cluster abnormality in the NP dko where 57 DEGs encode histone cluster components, of which 28 of the genes exhibit a 16-fold difference in expression and rank among the top 50 downregulated genes, compared to the control. Notably, the gene encoding the protein responsible for most of the DNA methylation in mouse cells, DNA (cytosine-5)-methyltransferase 1 (*Dnmt1*) is downregulated twofold in both UB and NP dko populations. Additionally of note, in the NP dko, *Dot1l*, encoding for histone H3 methyltransferase is downregulated.

Next, we examined how broadly the MAPK/ERK cascade controls the genes identified by RNA-Seq. The first step was to isolate RNA from complete E12.5 kidneys grown in control and MAPK/ERK inhibition conditions to use for quantitative PCR (qPCR) on 20 DEGs chosen for their significant DE and known relevance in signal transduction and/or kidney development. The full list of genes used can be seen in Figure 9. *Dusp6*, encoding a protein related to negative regulation of ERK and *Etv4*, with a known function in kidney

development, was found to be significantly DE in MAPK/ERK-inhibited whole kidneys. We confirmed RNA-Seq identified DEGs by qPCR in isolated UB and NP populations by revealing a similar pattern of DE of genes related to RTK signaling (*Wnt11*, *Etv4*), cell growth (*Ppan*), UB tip identity (*Wnt11*), metabolism (*Acot7*, *Adsl*), and chromatin regulation (*Dnmt1*, *Hist1h2br*). Finally, we wanted to examine how universal were these gene expression changes caused by MAPK/ERK deficiency, so we expanded our qPCR analysis to RNA isolated from embryonic lung and adult liver tissue. Many of the DEGs were also changed in the embryonic lung, but not in the adult liver, likely because of the molecular and morphologic similarities between the embryonic kidneys and lungs (Article I, Additional file 8: Table S7).

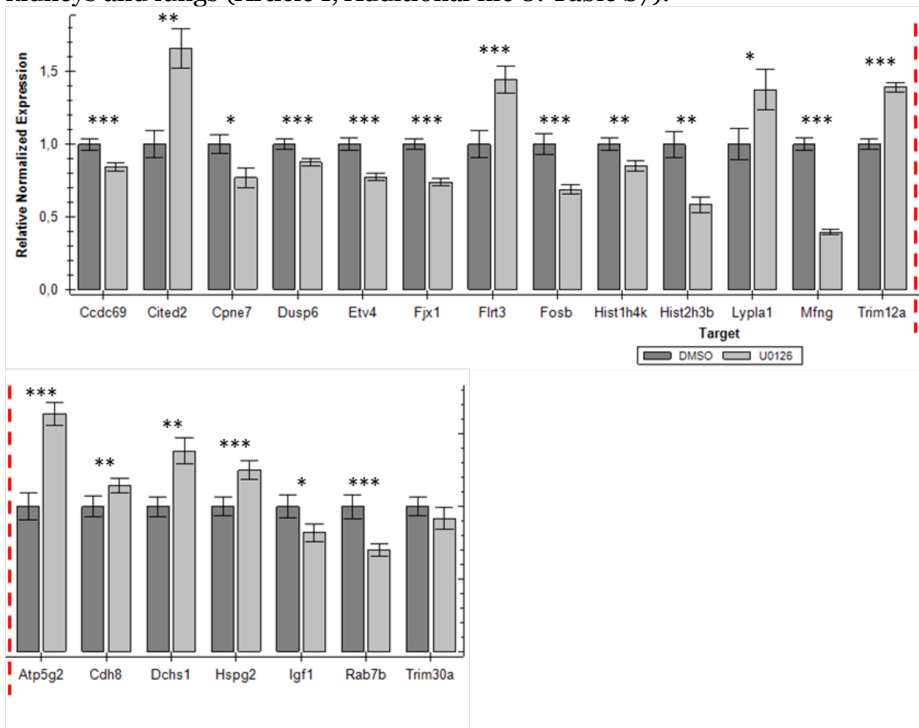


Figure 9 Gene expressions in whole E12.5 kidneys treated with MEK inhibitor U0126 versus vehicle control DMSO, as measured by qPCR. The red line separates the genes with similar expression changes in UB and/or NP tissue-specific RNA-Seq and whole kidney qPCR (top) from those with opposite expression changes (bottom). Opposite expression changes (bottom) indicates that the genes were either upregulated in RNA-Seq and downregulated in qPCR or downregulated in RNA-Seq and upregulated in qPCR analysis. Image modified and used with permission (Article I, Additional file 1: Figure S3).

5.4 URETERIC BUD TIP CELL IDENTITY REQUIRES MAPK/ERK SIGNALING (ARTICLE I)

It is well established in the field of renal developmental biology that the tips of the UB contain progenitors that differentiate into all cell types of the mature collecting ducts. A few years ago, an analysis of UB gene expression in E16.5 kidneys was published which showed a distinct segregation of gene expression profiles between UB tips and UB stalks. This analysis revealed 116 genes enriched in the tips and 393 in the stalks. A comparison of our UB dko DEGs to this dataset of UB tip and stalk enriched genes, identified that within the UB dko, there were 39 DE tip-enriched genes and 82 stalk-enriched genes. All 39 tip genes were downregulated in UB dko, and 78 out of 82 stalk genes were upregulated. The gene identified by Rutledge, *et al.* that was the most enriched within the UB tip was *Wnt11*, and this gene was interestingly downregulated in our UB dko tissue. Other DE tip-related genes also have characterized functions during kidney development and specifically in UB branching morphogenesis. These are summarized in Table 2. This dramatic shift in gene expression from tip- to stalk-enriched genes means that the progenitor status of the UB tip cells very likely depends on MAPK/ERK activity.

To look further into the gene regulatory networks underlying the maintenance of UB tip identity, we used StemChecker software (Pinto et al., 2015) to compare our UB dko dataset to published lists of transcription factor target genes. From the genes downregulated in UB dko, there was an overrepresentation of genes targeted by *Oct4* and *Sox2*, both pluripotency master regulators. We also observed an overrepresentation of genes targeted by proliferation regulators *Myc*, *E2f4*, and *Max*. Comparison with the upregulated UB dko genes showed that they were targeted by *Ezh2*, *Ring1B*, and *Suz12*, components of polycomb complex, which function in mESCs to repress developmental regulatory genes. Overrepresentation ($p < 0.005$) was calculated by the hypergeometric test, and the enrichment was assessed against the full annotated mouse genome. This analysis added further credence to our suspicion that UB tip cells with MAPK/ERK deficiency lose their capacity for proliferation and self-renewal and ultimately their progenitor status.

Table 2 *Ureteric bud tip-enriched genes downregulated in the absence of MAPK/ERK. Of the 39 downregulated tip genes, 19 have been characterized as having functions during renal branching morphogenesis (Article I).*

Gene	References
<i>Nrtn</i>	(Davies, 1999)
<i>Etv5</i>	(Kuure et al., 2010; Lu et al., 2009)
<i>Wnt11</i>	(Majumdar et al., 2003; O'Brien et al., 2018)
<i>Etv4</i>	(Kuure et al., 2010; Lu et al., 2009)
<i>Cxcl14</i>	(Schmidt-Ott et al., 2005)

<i>Ccnd1</i>	(Ihermann-Hella et al., 2014)
<i>Adamts18</i>	(Rutledge et al., 2019)
<i>Ror2</i>	(Nishita et al., 2014)
<i>Ret</i>	(Costantini, 2012; Schuchardt et al., 1994)
<i>Spry4</i>	(Zhang et al., 2001)
<i>Hs3st3b1</i>	(Rutledge & McMahon, 2020)
<i>Sox8</i>	(Reginensi et al., 2011)
<i>Mycn</i>	(Hohenstein et al., 2015; Pan et al., 2017; Stanton et al., 1992)
<i>Gfra1</i>	(Cacalano et al., 1998; Costantini, 2012; Enomoto et al., 1998)
<i>Ctnnd2</i>	(Rutledge et al., 2017)
<i>Frem2</i>	(Jadeja et al., 2005; Kiyozumi et al., 2012)
<i>Rprm</i>	(Magella et al., 2018)
<i>Stmn1</i>	(Li et al., 2005)
<i>Spred2</i>	(Motta et al., 2021; Ullrich et al., 2006)

To check these findings *in vivo*, we next looked at the maturation of MAPK/ERK-deficient UBs in E16.5 kidneys by immunofluorescent staining of proteins in mature collecting duct cell types (Article I, Figure 5C-H'). UB tips hold the progenitor cells for all the cell types of the collecting ducts, and here we looked at markers for the intercalated cells (V-ATPaseB1/2) and principal cells (aquaporin 2 (AQP2) and Na/K-ATPase). In control kidneys, none of the examined markers were present in the cortical UB tips, while in the medullar region, AQP2 localized to principal cells and V-ATPase localized to intercalated cells. All three of the examined mature cell-type markers were seen in the cortical UB epithelium which is normally the UB tip region. Altogether, the findings of these studies strongly suggest that upon abrogation of the MAPK/ERK cascade, UB cells prematurely differentiate into the cell types of mature collecting ducts, indicating the requirement of MAPK/ERK for the progenitor/tip identity maintenance of the UB cells.

5.5 NEPHRON PROGENITORS REQUIRE MAPK/ERK FOR PROPER EXPRESSION OF MITOCHONDRIAL GENES (ARTICLES I & II)

Because we identified several mitochondria-related DE transcripts in the NP dko, we wanted to more closely characterize the mitochondria-related biological processes related to respiratory chain function. Because the majority of DEGs related to mitochondria in the NP dko are downregulated, we first had to distinguish whether MAPK/ERK deficiency in NPs actually causes a defect in mitochondrial function or if the observed decrease in gene expression was simply due to a lower number of mitochondria present in these cells. To accomplish this, we analyzed mitochondrial DNA (mtDNA) copy number in E12.5 control and MAPK/ERK-deficient kidneys. Somewhat expectedly, the number of mitochondria per cell was indeed significantly

decreased in kidneys lacking MAPK/ERK (Article I, Figure 6B), indicating a role of MAPK/ERK in the replenishment of mitochondria during NP cell division.

After we determined that NPs without MAPK/ERK activation have a reduced number of mitochondria, we wanted to know if these cells with less mitochondria were unable to properly fuel themselves. We identified that ATP-related metabolism was affected by the lack of MAPK/ERK, which further suggested that the NP dko cells were incapable of proper energy metabolism. We examined this by liquid chromatography-mass spectrometry (LC/MS) to measure actual amounts of ATP in embryonic kidneys. A significant decrease in ATP amount was detected in these cells (Article I, Figure 6C). Further analysis of mitochondrial function in MAPK/ERK-deficient NPs using Seahorse also revealed a decrease in ATP production as well as defective basal and maximal respiration and spare capacity. Altogether, we identified from our RNA-Seq data that there is a decrease in biological processes related to mitochondria in NP dko cells which we were able to functionally validate by showing a decrease in mtDNA copy number, ATP levels, and oxygen consumption (Article I, Figure 6D-E). Therefore, NPs lacking MAPK/ERK exhibit mitochondrial defects which may contribute to the impaired self-renewal capacity of these progenitor cells.

5.6 MITOCHONDRIA AND RIBOSOMES ARE THE CELLULAR COMPONENTS MOST AFFECTED BY MAPK/ERK-DEFICIENCY IN NEPHRON PROGENITORS (ARTICLES I & II)

We previously utilized a genetic approach to identify the critical roles of the MAPK/ERK pathway for progenitor maintenance and propagation specifically in the NPs of the developing kidney by inducing NP-specific inactivation of both *Mek* genes (*Six2-TGC^{tg/+};Mek1^{fl/fl};Mek2^{-/-}*, more information on this mouse model can be found in the Methods section) (Ihermann-Hella et al., 2018). MAPK/ERK deletion from NPs led to molecular defects and altered composition of the niche environment, ultimately resulting in a rapid decline of NP cells. As mentioned above in Section 5.1, we performed RNA-Seq on the MAPK/ERK deficient NPs isolated from kidneys at E13.5, which is before the rapid decline in cell numbers occurs. From the sequencing, there were more than 5000 DEGs identified.

We next performed a more in-depth GO analysis of DEGs using the Cytoscape plugin ClueGO (Bindea et al., 2009; Shannon et al., 2003). The processes most overall affected by MAPK/ERK depletion in NPs were OXPHOS and ribosome biogenesis (Article II, Figure 1). Analysis using only the downregulated genes revealed that 40% of the downregulated genes were related to mitochondrial function under GO terms 'mitochondrial inner

membrane' (25%) and 'mitochondrion' (15%). These findings corroborate the results we obtained from MAPK/ERK-deficient cells where we observed defective mitochondrial functions. Besides metabolism, we also saw a large proportion of downregulated genes related to ribosomes in MAPK/ERK-deficient NPs. Additional GO analysis performed with the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Ashburner et al., 2000; Ogata et al., 1999) was in line with the Cytoscape findings showing defective ribosomes and metabolism. We also performed pathway analysis with the upregulated genes, but they failed to cluster into distinct pathways in any significant way.

5.7 AMINO ACID METABOLISM IS DIMINISHED IN MAPK/ERK-DEFICIENT CELLS (ARTICLE II)

To further elucidate the effects of MAPK/ERK on metabolism, we performed LC/MS targeted metabolomics analysis to quantify metabolites altered by MAPK/ERK inhibition with MEK inhibitor U0126 in the embryonic kidney mesenchymal cell line (mK4, more information about cell lines can be found in the Methods section). Eight significantly changed intermediate metabolites were identified. The altered metabolites are involved in ATP production, glycolysis, and the TCA cycle, validating previously published studies as well as other work from this thesis showing that the energy source is important for NP regulation (Li et al., 2015).

We next employed untargeted tandem mass spectrometry (MS2) to broaden our identification of metabolic changes in MAPK/ERK-inhibited cells. Comparison of the resulting MS2 spectra with an MS database revealed 34 decreased metabolites. Of these altered metabolites, several were amino acids, including methionine, glutamine, leucine, proline, threonine, tryptophan, and L-aspartic acid. This was of particular interest to us because several recent studies show that amino acid metabolism can regulate stem cell maintenance and fate determination (Allmeroth et al., 2021; Kim et al., 2020; Someya et al., 2021; Zhao et al., 2012), and specifically, methionine may have a role in NP proliferation (Makayes et al., 2021). The altered metabolites identified from MS2 were further analyzed by metabolite set enrichment analysis (MSEA) using MetaboAnalyst. The most altered metabolic pathways in the MAPK/ERK-inhibited cells were purine metabolism, PPP, Warburg effect, and glycine/serine metabolism.

5.8 INTEGRATION OF TRANSCRIPTOMIC AND METABOLOMIC DATA INDICATES DEFECTIVE NEPHRON PROGENITOR FATE DETERMINATION (ARTICLE II)

We employed a systems biology approach to combine our transcriptome-level data from RNA-Seq and our metabolomics data from LC/MS to gain a comprehensive view of MAPK/ERK's regulation of metabolism. Of the altered metabolites, 28 correlated with 113 DEGs. The most affected pathways are mitochondria and energy metabolism, but amino acid metabolism is also affected at both the metabolite and transcriptional levels. A comprehensive overview of the metabolite and gene expression changes and how they relate to metabolic pathways can be found in Article II, Figure 4. Notably, the metabolism of proline, tryptophan, glutamine, and glycine is affected. Interestingly, proline metabolism is decreased in MAPK/ERK-inhibited cells, and it is known to regulate pluripotency and contribute to differentiation (Casalino et al., 2011; Escande-Beillard et al., 2020; Liu et al., 2019; Patriarca et al., 2021; Stum et al., 2021; Washington et al., 2010). Direct regulators of proline metabolism, *Pycr1* and *Pycr2* are also downregulated in NP dko cells, indicating that MAPK/ERK may regulate proline metabolism at the transcriptional level. The other amino acids with altered metabolic pathways also have recognized roles in stem/progenitor cells. For example, tryptophan can regulate stem cell proliferation and the expression of *Oct4*, the pluripotency master regulator (Cheng et al., 2015; Someya et al., 2021). Glutamine can function in cell fate reversibility (Kim et al., 2020), and glycine has a function in the specification of neurons as well as its common role in the antioxidant response (Avila et al., 2014).

Because SIX2+ NP cells are a heterogeneous population which range from completely undifferentiated (expressing *Six2* and *Cited1*) to more committed cells (expressing *Six2* without *Cited1*) (Brown et al., 2013; Brown et al., 2015; Brunskill et al., 2014; O'Brien, 2019; Self et al., 2006), we examined the different subpopulations to see if any were more susceptible to changes in MAPK/ERK signaling than others. We compared our RNA-Seq data from NP dko cells with a published single-cell RNA-Seq (scRNA-Seq) dataset. Within the *Six2*+ cells screened by scRNA-Seq, all expressed the MAPK/ERK target *p53*. Of the identified MAPK/ERK target genes related to metabolism, *Pkm2* was present in all *Six2*+ cells, *Pycr1* was expressed in 44% and *Pycr2* in 77% of *Six2*+ NP cells. We also looked at genes related to differentiation, and they were expressed in only a small percent of NPs, indicative of the known heterogeneity within the *Six2*+ cell population. The MAPK/ERK targets that we examined are expressed in a larger portion of the total *Six2*+ cell population than are the differentiation markers we looked at, which suggests that MAPK/ERK is functioning in both the undifferentiated and committed NP cells.

5.9 MORPHOLOGY OF THE NEPHRON PROGENITOR NICHE DEPENDS ON PYRUVATE AVAILABILITY (ARTICLE II)

Since pyruvate metabolism was one of the pathways affected by MAPK/ERK deficiency, we decided to examine the consequences of differing concentrations of pyruvate on renal phenotype during development. *Ex vivo* cultures of E11.5 kidneys were incubated for 72 hours with standard (1 mM) and altered pyruvate concentrations ranging from 0 to 5 mM. After 72 hours, the cultured kidneys were fixed and immunostained with calbindin (CALB1) to visualize the branching UB network whose tips comprise an extrinsic niche for NP cells (O'Brien, 2019). UB branching was severely defective with insufficient amounts of pyruvate, resulting in 50-80% less UB tips per kidney, failure of the tips to normally enlarge, and extra long UB trunk regions (Article II, Figure 6A). The phenotype we observed with low pyruvate concentrations is similar to that we see in our mouse model of UB-specific MAPK/ERK deficiency as well as in embryonic kidneys treated with MEK inhibitors (Fisher et al., 2001; Hida et al., 2002; Ihermann-Hella et al., 2014). This demonstrates that one of the ways MAPK/ERK exerts its control of renal progenitors is by regulating the energy metabolism of these cells. Further confirming the importance of metabolism in kidney progenitor regulation, we also saw that pyruvate concentrations over the standard 1 mM caused a decrease in overall UB branching and tip numbers but, interestingly, in a different pattern than we saw in kidneys cultured in low pyruvate concentrations.

5.10 NEPHRON PROGENITOR MAINTENANCE REQUIRES PROLINE METABOLISM (ARTICLE II)

One of the most interesting findings from our consolidation of metabolomic and transcriptomic changes in NP dko cells was the alteration of proline metabolism. Not only was the metabolite L-proline itself reduced in the absence of MAPK/ERK, but the expression of the proline metabolism regulatory genes *Pycr1* and *Pycr2* was also significantly reduced. To further investigate, we used a mouse model with the conventional knockout of *Pycr1*^{-/-} and *Pycr2*^{-/-} (*Pycr1/2* dko). Kidneys from these animals at P1 were histologically examined and exhibited no abnormalities in overall size or shape (Article II, Figure 6B). However, when we looked closer at the NP population within the *Pycr1/2* dko kidneys, we observed a defect in NP cell maintenance with reduced numbers of SIX2⁺ NP cells per niche and a more abundant level of SIX2 in differentiating nephron precursors, likely indicative of premature terminal differentiation of the entire NP population.

Although we suspected that premature differentiation was the cause of the loss of NPs, we wanted to validate this by assessing the actual mechanism

behind the loss of NPs in the *Pycr1/2* dko. Besides an overall acceleration of differentiation, we thought that changes in proliferation or apoptosis could also play a role. We performed immunostaining with phospho-histone H3 to mark mitotic cells and with cleaved-caspase 3 to mark apoptotic cells. We saw no differences between wildtype and *Pycr1/2* dko kidneys regarding changes in proliferation or apoptosis. Additionally, we looked at mitochondrial morphology, and also saw no significant difference in the *Pycr1/2* dko. Finally, we immunostained for two different markers of nephron differentiation, cadherin 6 (CDH6) and cyclin D1 (CD1) (Article II, Figure 7A-D). Quantification of nephron precursor structures indicated that there were significantly more in *Pycr1/2* dko kidneys than in wildtype. This was in line with our initial speculation that the final burst of NP differentiation occurs prematurely in *Pycr1/2* dko kidneys with defective proline metabolism. Thus, we demonstrated with these studies not only that MAPK/ERK cascade is required for normal proline metabolism in NPs, but that NPs need proper proline metabolism to maintain their progenitor status and prevent premature differentiation.

5.11 GDNF IS REQUIRED FOR THE NEPHROGENIC PROGRAM DURING EMBRYOGENESIS (ARTICLE III)

In embryonic and early postnatal kidneys, the spatial distribution of SIX2+ NPs has a well-characterized and stereotypical pattern in which the initial multi-cell-layered tissue thins throughout time without compromising overall niche structure (Short et al., 2014). In *Gdnf^{hyper/hyper}* kidneys, NPs are dispersed as a thinner-than-normal layer around an excessively wide UB (Article III, Figure 1A-F). Quantification of NPs in *Gdnf^{hyper/hyper}* kidneys at E11.5 demonstrated an initial rise in NP numbers, which was transiently corrected by E12.5 but substantially diminished by E14.5 (Article III, Figure 1E-G). Analysis of pHH3+ proliferating cells revealed that both endogenously raised and exogenously supplied GDNF reduce the amount of pHH3+ NPC significantly (Article III, Figure 1H). Excess GDNF, which predominantly operates in the UB where its receptors are present (Pachnis et al., 1993), causes a dramatic decrease in NP cell proliferation during early kidney development.

NP differentiation occurring prematurely or at an accelerated pace leads to an overall decrease in NP cells in their niche (Kobayashi et al., 2008; O'Brien, 2019). To evaluate the potential causes of decreased NP cells in *Gdnf^{hyper/hyper}* kidneys, we immunostained for LEF1 to identify the earliest differentiation-committed cells and cyclin D1 to indicate nephrons undergoing full epithelialization (Lindström et al., 2015). This revealed a general decrease in the number of early nephron precursors in embryonic *Gdnf^{hyper/hyper}* kidneys as well as impaired nephron differentiation (Article III, Figure 2). Measurements of glomerular density confirmed this, revealing lower overall

glomerular densities in hypodysplastic *Gdnf^{hyper/hyper}* kidneys compared to WT kidneys. Cortical glomeruli within the total measured area, and specifically cortical-to-total glomeruli ratios, were slightly higher (17%) in *Gdnf^{hyper/hyper}* than in WT (12%). This suggests that, despite the early slowing of NP self-renewal and differentiation, the kidneys exposed to higher-than-normal GDNF during organogenesis have continued postnatal nephrogenesis with a modest increase in the most cortically situated nephrons (Rumballe et al., 2011; Short et al., 2014).

5.12 THE LIFESPAN OF THE POSTNATAL NEPHRON PROGENITOR POOL CAN BE EXTENDED BY EXCESS GDNF (ARTICLE III)

Many mouse models with impaired kidney growth, caused by defective UB morphogenesis or nephron differentiation, die shortly after birth (Kuure & Sariola, 2020). Despite severe hypoplasia, aberrant UB shapes, and significantly disrupted embryonic nephrogenesis, *Gdnf^{hyper/hyper}* animals survive postnatally for up to 3 weeks (Kumar et al., 2015; H. Li et al., 2019), providing a unique opportunity to study postnatal nephrogenesis.

As previously reported in WT kidneys (O'Brien, 2019), our examination of WT NP populations at P1-P10 revealed a dramatic loss of NPs at P3, with SIX2+ cells detected primarily in differentiating nephron precursors and with only 10% of the analyzed UB tips (NP niches) surrounded by NP cells. SIX2+ NPs, on the other hand, were found in 59% of *Gdnf^{hyper/hyper}* NP niches at P3. PAX2+ NPs were also found in NP niches of *Gdnf^{hyper/hyper}* kidneys at P3, while PAX2+ cells in WT kidneys were only detected in developing nephron precursors (Article III, Figure 3).

In postnatal *Gdnf^{hyper/hyper}* kidneys, nephrogenesis was improved compared to that in embryonic kidneys, as evidenced by comparable patterns of nephron precursor in WT and *Gdnf^{hyper/hyper}* kidneys. We also discovered that approximately 18% of NP niches in *Gdnf^{hyper/hyper}* kidneys had SIX2+ cells at P4 with committed NP cells present until P6, while committed NP cells were only observed until P4 in WT kidneys (Article III, Figure 4). This reveals that the NP lifespan is not fixed *in vivo* and suggests that growth factor levels, notably GDNF levels, influence the timing of the final wave of nephron differentiation.

We theorized that the persisting NP cells we observed in postnatal *Gdnf^{hyper/hyper}* kidneys could be caused by compensatory mechanisms triggered by UB branching abnormalities resulting in renal hypoplasia (Kumar et al., 2015; H. Li et al., 2019) or by GDNF-specific effects on the NP cells themselves. We investigated postnatal NPs in various models of renal hypoplasia to try to elucidate the cause. *Fgf9;20*-deficient kidneys have embryonic NP cell layer thinning comparable to *Gdnf^{hyper/hyper}* kidneys (Barak et al., 2012), however we did not find SIX2+ or PAX2+ cells in their NP niches

at postnatal time points. To expand on this, we examined *Hoxb7Cre;Mek1^{fl/fl};Mek2^{+/-}* kidneys (Ihermann-Hella et al., 2014), which, like kidneys in *Gdnf^{hyper/hyper}* mice, are hypoplastic due to defective UB branching. This model also had no evidence of extended nephrogenesis, as indicated by the lack of SIX2+ or PAX2+ cells in the postnatal NP niches, lending credence to the notion that renal hypoplasia alone is insufficient to sustain postnatal nephrogenesis. Available data also shows that renal hypoplasia alone is insufficient to enable postnatal NP maintenance (Kuure & Sariola, 2020), implying that GDNF plays an active role in the process. We cannot, however, rule out the potential that excess growth factors other than GDNF would have the same impact.

5.13 ENDURING POSTNATAL NEPHRON PROGENITORS PROLONG THE NEPHROGENIC PROGRAM BEYOND ITS TYPICAL CESSATION (ARTICLE III)

Once we discovered the sustained NPs in postnatal *Gdnf^{hyper/hyper}* kidneys, we next investigated their differentiation potential (Article III, Figure 5). Proliferation in postnatal WT and *Gdnf^{hyper/hyper}* kidneys was examined by looking at Ki67 localization which demonstrated comparable patterns until P4. Ki67+ cells in the cortical differentiation zone of WT kidneys were reduced after P5. Unlike in the WT kidneys, this decrease in cortical proliferation was not seen in *Gdnf^{hyper/hyper}* kidneys, which sustained actively cycling cells in nephron precursor-like structures until P7. However, these actively cycling cells were diminished by P12.

Analysis of postnatal nephron differentiation revealed an abundance of nephron precursors in *Gdnf^{hyper/hyper}* kidneys until P7 but only up to P4 in WT kidneys (Article III, Figure 6). However, such prolonged postnatal nephrogenesis did not improve glomerular density compared to late embryonic stages (6.4 ± 2.1 versus 10.2 ± 1.2 , respectively; mean $\pm$ s.e.m.). The fraction of cortical glomeruli in *Gdnf^{hyper/hyper}* kidneys at P7 (59%) is 1.5 times greater than in WT kidneys (39%), indicating that the persistent postnatal NP pool in *Gdnf^{hyper/hyper}* kidneys generates additional nephrons after normal nephrogenesis ceases.

During their short lifespan, *Gdnf^{hyper/hyper}* mice with extended nephrogenesis in severely hypodysplastic kidneys exhibit no evidence of neoplasia or tumorigenesis (Kumar et al., 2015; Turconi et al., 2020). The embryonic drop off of NP cells in *Gdnf^{wt/hyper}* kidneys is milder than in *Gdnf^{hyper/hyper}* kidneys, and these mice have a normal lifespan with no malignancies detected in their kidneys or other organs (n=62) (Turconi et al., 2020). *Gdnf^{wt/hyper}* mice exhibit a twofold increase in GDNF expression during embryogenesis, but even this twofold increase fails to maintain the NP population in postnatal kidneys, implying a strict threshold for GDNF's ability to influence the nephrogenic program.

5.14 PERSISTENT POSTNATAL NEPHROGENESIS DEPENDS ON GDNF-INDUCED SIGNALING LEVELS (ARTICLE III)

WT kidneys lose *Gdnf* expression by P2 (www.gudmap.org), while its mRNA and protein persist in *Gdnf^{hyper/hyper}* kidneys clear until P7 (Article III, Figure 7). We hypothesized that the nephrogenesis-related effects of excess endogenous GDNF had to be regulated by GDNF-dependent molecules generated in and secreted from the UB or else by other molecules whose expression could be altered by UB enlargement alone. We reasoned this because neighboring the NP cell populations are the UB tips, which are the only sites in the embryonic kidney to express the conserved GDNF receptor complex.

At E14.5 in *Gdnf^{hyper/hyper}* kidneys, analysis of the expression of UB-derived and secreted GDNF-targets revealed considerable upregulation of *Crfl1*, *Srgn*, and *Wnt11*. *Wnt9b* expression levels were also greatly elevated. Further examination of WNT/ β -catenin signaling targets (Clevers & Nusse, 2012) in E14.5 WT and *Gdnf^{hyper/hyper}* kidneys revealed a significant upregulation of the canonical WNT target *Axin2*, whereas NP-expressed WNT targets (Karner et al., 2011) trended towards downregulation, most likely due to the overall lower amount of NP cells. These findings suggest that, around E14.5, not only heightened GDNF-induced signaling but also increased *Wnt9/11*-induced signaling, both of which have recognized involvement in NP regulation, play a role in enabling the reciprocal communication from the mutant UBs to the neighboring NP populations (Karner et al., 2011; Kiefer et al., 2012; Nagy et al., 2016; O'Brien et al., 2018).

At P5, *Gdnf^{hyper/hyper}* kidneys had upregulated expression of GDNF targets *Crfl1*, *Etv4*, *Etv5*, *Cited1*, and *Uncx4.1* but also increased expression of potential niche factor *Lama1* (Rayagiri et al., 2018), nephron differentiation inducer *Wnt4* (Stark et al., 1994), and WNT signaling agonist R-spondin 1 (*Rspo1*). This transcriptional profile shows an enhanced UB-derived influence on NPs, which appear to go through a wave of differentiation based on the decreased expression of *Fgf9* and *Fgf20* and altered SIX2+ cell location.

We wanted to see if the genes whose expression was most significantly enhanced (*Crfl1*, *Wnt11*, and *Wnt9b*) could impact the mouse nephrogenic program. In isolated rat kidney mesenchyme cells, CRLF1 causes differentiation in conjunction with its ligand cardiotrophin-like cytokine (Schmidt-Ott et al., 2005). Characterization of *Crfl1*-knockout kidneys demonstrated comparable kidney size and nephrogenesis progression to WT kidneys, implying that CRLF1 functions are dispensable *in vivo*.

We also wanted to determine the role of enhanced WNT signaling in the phenotypes produced by excess GDNF. Tankyrases 1 and 2 are required for proper kidney development and can be chemically inhibited by IWR1, a canonical WNT pathway antagonist (Chen et al., 2009; Huang et al., 2009; Karner et al., 2010). In the presence of increased GDNF, IWR1 therapy

reduced UB tip numbers and shape. Ultimately, by crossing a *Wnt11* knockout-allele to the *Gdnf^{hyper}* background, we explored if elevated *Wnt11* expression in *Gdnf^{hyper/hyper}* kidneys contributes to the protracted nephrogenic program in *Gdnf^{hyper/hyper}* animals. This cross was unsuccessful at producing double homozygote *Gdnf^{hyper/hyper};Wnt11^{-/-}* offspring (n=95; $p<0.005$ with breeding of *Gdnf^{flwt/hyper};Wnt11^{+/-}*). These findings point to embryonic lethality in *Gdnf^{hyper/hyper};Wnt11^{-/-}* mutants, forcing us to narrow our investigation to *Gdnf^{hyper/hyper};Wnt11^{+/-}* kidneys.

Removal of one *Wnt11* allele in a *Gdnf^{hyper/hyper}* background improved UB morphology and reduced kidney size even more than in *Gdnf^{hyper/hyper}* kidneys, most likely due to reduced formation of cysts in the collecting ducts consequent to the reduction of *Wnt11* dosage. However, the population of SIX2+ NP cells increased (29.8 vs 40.7 NP cells/tip at E14.5 and 20.3 vs 30.9 NP cells/tip at Po), and we observed enriched nephrogenesis in the cortical differentiation zone of *Gdnf^{hyper/hyper};Wnt11^{+/-}* kidneys (Article III, Figure 8). Therefore, our findings indicate that elevated GDNF levels amplify many UB-derived GDNF/RET targets, which, when combined with increased classical WNT signaling, regulate NP cells in embryonic kidneys.

6 DISCUSSION

6.1 TRANSCRIPTIONAL PROFILING OF MAPK/ERK-DEFICIENT KIDNEY CELLS

Although we and others have proven the importance of the MAPK/ERK signaling system in the early development of the kidney (Fisher et al., 2001; Ihermann-Hella et al., 2018; Ihermann-Hella et al., 2014; Kurtzeborn et al., 2019; Watanabe & Costantini, 2004; Willecke et al., 2011), the tissue-specific effects of MAPK/ERK signals on transcriptional and metabolic regulation remained unknown. Furthermore, MAPK/ERK activation in growing kidney tissues is very dynamic (Ihermann-Hella et al., 2018), implying heterogeneity in cellular responses. The kind of transcriptional response in the signaling cell has been intricately related to the MAPK/ERK activation pattern, where dynamic activation often leads to more effective transcription of target genes, which are nonetheless susceptible to significant post-translational control (Wilson et al., 2017). To gain deeper insights into MAPK/ERK's transcriptional targets at the genetic level, we used RNA-Seq on cells isolated from tissue-specific MAPK/ERK-deficient kidneys (UB dko and NP dko), cell populations that are made up of heterogeneous progenitors. RNA-Seq was performed at the earliest time points where we have previously confirmed depletion of MAPK/ERK activity but where kidney size was not yet affected (Ihermann-Hella et al., 2018; Ihermann-Hella et al., 2014). Our goal was to identify the principal MAPK/ERK targets in the given tissues, albeit this technique may overlook transcripts impacted by partial pathway downregulation and also catch secondary targets. Profiling at various embryonic stages might offer additional information, but owing to the intricate biology of conditional deletion of numerous genes, which would necessitate an excessive number of breeding mice, it would be time-consuming, costly, and opposed to 3R principles. Furthermore, diverse modalities and intensities of ERK activation are now known to influence unique transcriptional outputs, resulting in alternative cell fate decisions (De et al., 2020; Johnson & Toettcher, 2019; Wilson et al., 2017). A possible next step would be to perform single-cell RNA-Seq profiling of UB and NP cells with differing levels of ERK activation and at differing stages of development.

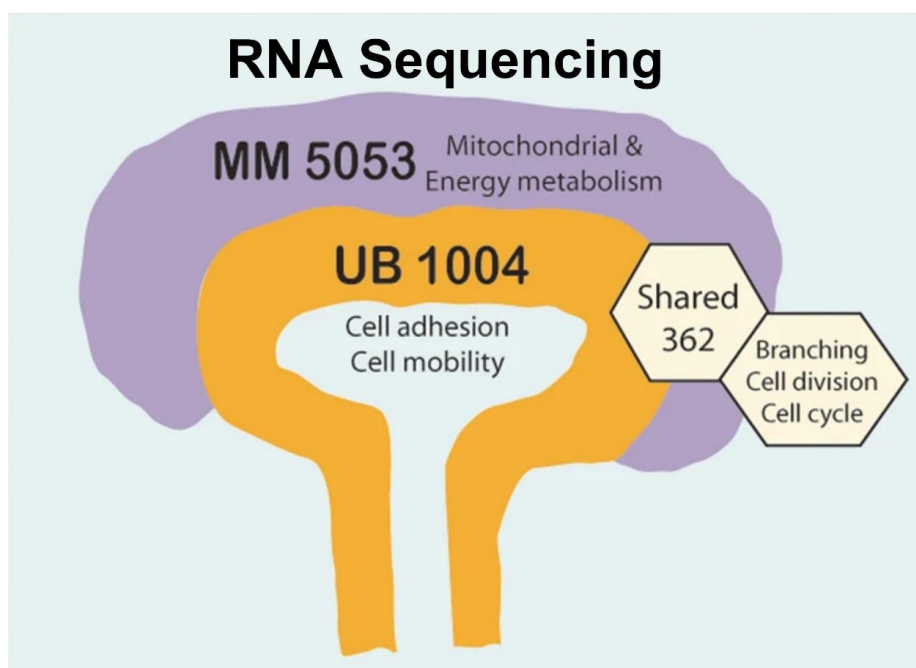


Figure 10 Schematic summary of MAPK/ERK functions in renal progenitor populations. MAPK/ERK-deficiency in UB epithelium (orange) and metanephric mesenchyme (MM) which is comprised of nephron progenitor cells (purple) resulted in significant differential expression of 1004 and 5053 genes, respectively. Gene expression alterations in the NPs were strongly related to mitochondrial and energy metabolism, whereas changes in the UB were largely related to cell adhesion and mobility, as we demonstrated via further experiments, which showed a loss of UB tip identity and premature differentiation (not depicted here). 362 genes were differentially expressed in both MAPK/ERK-deficient UB and NPs, the majority of which were related to cell cycle, cell division, and branching morphogenesis. Results from RNA-Seq experiments met the first part of Study Aim 1. Image modified and used with permission (Article I).

Microarray analyses have revealed gene expression profiles of entire wildtype kidneys at various developmental stages (Stuart et al., 2001), but the total absence of spatial characterization of gene expression is a severe limitation. Since its establishment, RNA-Seq (Mortazavi et al., 2008) has yielded significant insights into early-stage renal development (Brunskill et al., 2014; Brunskill & Potter, 2012). The work presented here establishes the first tissue-specific MAPK/ERK-deficient genetic profiles for UB and NP cells in the developing kidney. Our findings imply that MAPK/ERK targets are extremely tissue-specific, despite the pathway's relatively general control of proliferation via histone availability, adhesion, and energy consumption via the regulation of metabolic enzyme expression. Tissue-specific transcriptional responses are not surprising given that diverse growth factors, as well as the activation profile of MAPK/ERK, produce unique outcomes for target gene expression and cell fate (Bishop et al., 1994; De et al., 2020; Johnson &

Toettcher, 2019; Marshall, 1995; Wilson et al., 2017). Because NPs and UB cells are regulated by growth factor cues that are somewhat overlapping but hierarchically different (Costantini, 2010; Walker et al., 2016), and as we have previously demonstrated both sustained and dynamic MAPK/ERK activation in developing kidney tissues (Ihermann-Hella et al., 2018), target gene tissue specificity is consistent with their complex expressional control. An overview summary of the findings is depicted in Figure 10.

One of the earliest RNA-Seq studies in renal developmental biology indicated relatively extensive transcriptional heterogeneity in an otherwise homogeneous nephron progenitor compartment (Brunskill et al., 2014), which has since been verified by others (Lawlor et al., 2019; Lindström, Brandine, et al., 2018; Menon et al., 2018). Brunskill and colleagues uncovered a few DEGs that are intriguing and may be relevant to our findings. They discovered certain cells, mostly on the outside of the NP niche, that expressed both the NP marker *Six2* and the stromal marker *FoxD1* (Lin et al., 2014; Self et al., 2006), indicating that some early NP cells had multilineage capabilities (Brunskill et al., 2014). Expression of *Six2* was not substantially changed in MAPK/ERK-deficient NP cells, however, six of Brunskill's ten most upregulated genes in uninduced MM relative to induced MM were downregulated in our NP dko dataset. These include *Ctsz*, *Osr1*, five *Coq9* family members (3, 6, 7, 8a, and 8b), *Arf2* family member *Arf5*, *Cpxm1*, and *Ccm2*. MAPK/ERK-deficient NPs displayed elevated expression of *FoxD1* and other Fox genes, presumably indicating lineage confusion in NPs lacking MAPK/ERK activity.

6.2 MAPK/ERK ACTIVITY IS INVOLVED IN CHROMATIN-LEVEL REGULATION OF STEMNESS

ERK-chromatin interactions have been studied in a variety of cell and tissue types, including human and mouse ES cells, where ERK targets histone and other transcriptional regulatory genes to regulate nucleosome assembly and chromatin availability (Göke et al., 2013; Tee et al., 2014). Similar widespread modifications in nucleosome and chromatin assembly/organization were observed in the embryonic lung and placenta development (Boucherat et al., 2014; Nadeau & Charron, 2014). Furthermore, prolonged MEK inhibition or genetic loss in ES cells leads to epigenetic and genomic alterations that substantially impair developmental potential (Choi et al., 2017). We demonstrate here the changes in chromatin-regulating gene expressions indicative for interactions between MAPK/ERK and chromatin in the embryonic kidney, where histones account for 2.5% of all DEGs in UB dko (25 genes) and 1.4% of the DEGs in NP dko (69 genes), while a larger portion (13%) of the shared downregulated DEGs are involved in chromosome condensation and nucleosome assembly.

DNA replication in rapidly proliferating cells necessitates not only histone recycling but also effective histone transcription, which is aided in part by histone clustering in the genome (Almouzni & Cedar, 2016; Corpet & Almouzni, 2009). This preserves freshly produced DNA through packing, which is necessary for maintaining chromatin integrity during development and differentiation (Mendiratta et al., 2019). Histone abundance is intrinsically tied to DNA content (Armstrong & Spencer, 2021; Claude et al., 2021; MacAlpine & Almouzni, 2013), and the lower amount of histone-related gene transcription in MAPK/ERK-deficient tissues is likely related to the defective cell cycle progression we have previously identified in both mutant tissues (Ihermann-Hella et al., 2018; Ihermann-Hella et al., 2014). MAPK/ERK also directly influences transcriptional machinery and chromatin structure (Tee et al., 2014; Whitmarsh, 2007; Yue et al., 2017), suggesting another reason for the observed changes in the expression of histone-related genes.

The chromatin landscapes of NPs were recently characterized (Hilliard et al., 2019), but the mechanisms regulating the opening and closing of chromatin structures in the developing kidney are unknown. In both the UB dko and NP dko populations, the gene responsible for the majority of DNA methylation, *Dnmt1* was downregulated. Additionally downregulated in both tissues was *Uhrf1* that codes for a protein which mediates the recruitment of DNMT1 to DNA. UHRF1 attracts DNMT1 to DNA during the cell cycle S-phase to make sure that DNA methylation marks are maintained in replicating cells (Nishiyama et al., 2020). *Dnmt1*-deficient nephron progenitors are incapable of maintaining DNA methylation status and consequently cell identity (Wanner et al., 2019), and downregulation of *Dnmt1* and *Uhrf1* in response to MAPK/ERK deficiency indicates that this is the case in renal progenitors in the absence of MAPK/ERK activation as well. To further support the defective regulation of DNA methylation as a mechanism by which kidneys lacking MAPK/ERK fail to maintain their progenitors, we also noted a significant downregulation of histone 3 lysine 79 methyltransferase *Dot1l* in MAPK/ERK-deficient NPs. The functional requirement of *Dot1l* for maintenance and differentiation of NPs was recently described (Wang et al., 2021).

Our RNA-Seq of UB dko demonstrated that without MAPK/ERK, the UB tip progenitors downregulate numerous genes previously found to be UB tip-specific but concurrently upregulate many known stalk-specific genes (Rutledge et al., 2017). Additionally, GO biological process analysis found 62 DE genes engaged in cell differentiation negative regulation and 25 stem cell differentiation genes. Most of the identified DE genes in these GO processes have been described as regulators of self-renewal and progenitor status, and in cells of the developing kidney *Sema3a* (Reidy et al., 2009), *Shh* (Gill & Rosenblum, 2006; Yu et al., 2002), and *Dnmt1* (Wanner et al., 2019) are known to function as such. These findings imply that MAPK/ERK activity is

essential in the UB tips for maintenance of the tip cell identity and perhaps for collecting duct progenitor features, since the cells become transcriptionally more similar to UB stalk cells in the absence of MAPK/ERK activation. Indeed, our protein level study confirmed the transcriptional alterations observed in the UB dko and demonstrated early and ectopic differentiation of collecting duct cell types (principal and intercalated cells) in the UB tips. This need for MAPK/ERK in the UB to maintain the tip/progenitor cell identity is also supported by the fact that MAPK/ERK acts downstream of GDNF in collecting duct progenitors to mediate its regulatory effects (H. Li et al., 2019), and it explains the findings previously reported by our lab that MAPK/ERK-deficient UB cells fail to expand and form branches (Ihermann-Hella et al., 2014).

6.3 NEPHRON PROGENITOR METABOLISM CRITICALLY DEPENDS ON MAPK/ERK ACTIVITY

Transcriptional analysis of MAPK/ERK-deficient NPs revealed a surprising number of DEGs associated with proliferation and mitochondrial metabolism. Apoptosis may be caused by mitochondrial dysfunction, and this could explain why we found lower cell yields in dko populations (38% in UB and 63% in NP) when we used FACS to isolate cells for RNA-Seq. Despite that, our lab's previous research has shown that cell death is not significantly impacted in either the UB or NP-specific dko kidneys (Ihermann-Hella et al., 2018; Ihermann-Hella et al., 2014), suggesting that the decreased energy metabolism may be altering the self-renewal/differentiation balance. It has been shown that the energy source influences this balance in NPs, which differentiate early if glycolysis is inhibited (Liu et al., 2017). The overall mitochondrial quantity per cell is reduced in MAPK/ERK-deficient NP cells, which may disrupt the energy source balance in these highly proliferative cells due to decreased use of pyruvate for OXPHOS. It has been demonstrated that p53, a known MAPK/ERK target (De et al., 2020) that was likewise downregulated in our NP dko dataset, regulates energy metabolism in NPs (Li et al., 2015). The phenotype of p53-deficient NPs is strongly reminiscent of our previously described MAPK/ERK-deficient NPs where the cells are disorganized, have defects in cellular adhesion, and are rapidly diminished (Ihermann-Hella et al., 2018).

Upon our implementation of a systems biology approach where we integrated transcriptional data from MAPK/ERK-deficient NPs with metabolomics data from MAPK/ERK-inhibited cells (Figure 11), we observed defects in pyruvate and proline metabolism, processes which are linked through their roles in glycolysis and glutaminolysis. Pyruvate is an end-product of glycolysis which converts into acetyl-CoA in the mitochondria where it feeds into the TCA cycle. Proline is produced mainly from glutamate during glutaminolysis (De Ingeniis et al., 2012), a process which compensates

for low glucose by converting glutamine to glutamate to serve as an energy source (Wu et al., 2014; Yang et al., 2014). Because the main pathways for energy metabolism in mammals are glycolysis and glutaminolysis, the defects in their pathway components in MAPK/ERK-deficient cells no doubt contribute to energy deprivation in these cells.

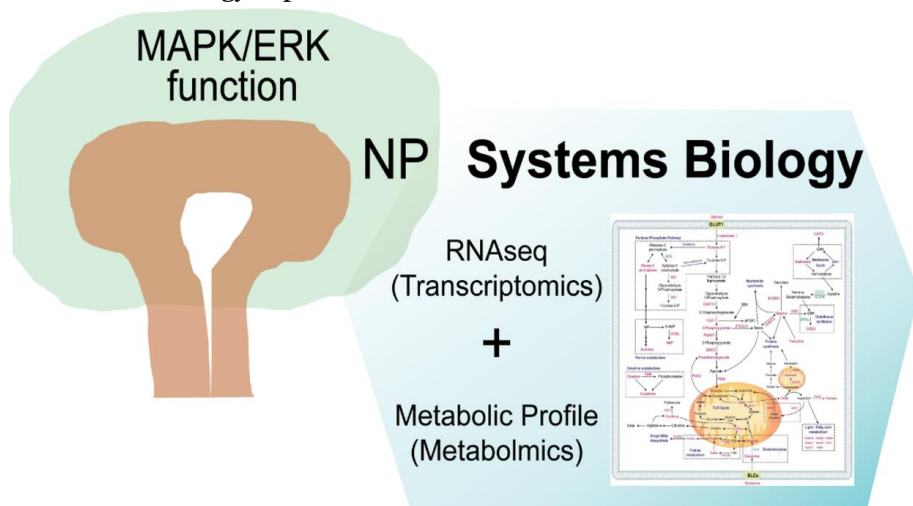


Figure 11 Schematic summary of the systems biology approach implemented to integrate transcriptomic and metabolomic results from MAPK/ERK-deficient nephron progenitors. Results from these experiments met the objectives for Study Aims 1 and 2. Image used with permission and modified from Article II.

Pyruvate production depends on the glycolytic enzyme PKM2, which is not only expressed in the embryonic kidney but is also regulated by MAPK/ERK (Gupta & Bamezai, 2010; Yang et al., 2012). In MAPK/ERK-deficient NP cells, we found that *Pkm2* is downregulated and there was a concomitant reduction in the TCA intermediate metabolite citrate. The morphology of the extrinsic niche of NP cells, namely the UB tips (O'Brien, 2019), depends on pyruvate concentration in a dose-dependent manner. Low amounts of available pyruvate, which could be caused by the defective conversion of phosphoenolpyruvate to pyruvate (as would be the case with decreased enzyme *Pkm2*), can still support the growth and elongation of the UB but with a reduction in the generation of new UB tips which form the NP niche environment and are required for the maintenance of NP cells. In contrast, high pyruvate levels did not affect UB tip morphology but still caused a decrease in the overall tip/NP niche numbers. It is very likely that pyruvate availability is therefore crucially required for the regulation of stemness in NPs, and this is supported by previous findings that pluripotency can be increased in ES cells with forced redirection of pyruvate to mitochondrial acetyl-CoA production, while glycolytic production of acetyl-CoA drives histone acetylation (Moussaieff et al., 2015).

Proline metabolism plays several different roles in the regulation of cellular homeostasis (Patriarca et al., 2021), and its involvement as a stem-cell regulator is critical to our research. Proline supplementation in ES cells promotes proliferation and differentiation into epiblasts, whereas maternal proline improves nutrient transport to support the development of pre-implantation embryos (Casalino et al., 2011; Cermola et al., 2021; Someya et al., 2021; Washington et al., 2010). Proline insufficiency causes a variety of hereditary illnesses, most of which impact development, the nervous system, and kidney function (Patriarca et al., 2021). To further examine proline metabolism in the regulation of NP maintenance, we characterized postnatal kidneys from mice deficient in *Pycr1/2*, the enzymes responsible for the final steps of proline biosynthesis. We observed that *Pycr2* can compensate for the loss of *Pycr1* in the developing kidney, results that are in line with their determined genetic interactions (Stum et al., 2021). In humans, PYCR1 deficiency causes *cutis laxa*, a condition characterized by inelastic skin, and it leads to cell death in the central nervous system of mice and in the skin of zebrafish and *Xenopus* (Escande-Beillard et al., 2020; Kuo et al., 2016; Reversade et al., 2009). However, the *Pycr1/2*-deficient mouse kidneys exhibited no increase in apoptosis. Instead, we observed premature differentiation of the NPs in postnatal kidneys lacking *Pycr1/2*. The discovery of proline as a regulator of NP cell state is a significant finding, not only because the precise activities of proline metabolism at the tissue and organ levels are unclear, but also because of its therapeutic potential as a simple to administer metabolite.

6.4 NEPHRON PROGENITOR LIFESPAN IS FLEXIBLE

The final nephron count is determined during fetal development and can vary significantly between individuals. Some of the less severe congenital anomalies of the kidney, such as reduced nephron mass caused by defects in NP maintenance or differentiation, predispose to chronic kidney disease and cardiovascular comorbidities in adult life, even though they are usually not life-threatening in childhood (Bertram et al., 2011; Luyckx & Brenner, 2010). Because NP expansion and lifespan have a large impact on nephron endowment, identifying regulatory systems that regulate NP self-renewal is critical.

Despite its negative effects on overall kidney growth, the neurotropic factor GDNF, which initiates renal development and controls UB branching morphogenesis (Costantini, 2010; Kurtzeborn et al., 2018; H. Li et al., 2019), is capable of prolonging the nephrogenic program beyond its normal termination. We demonstrate that excessive GDNF has a biphasic impact on NP self-renewal and maintenance, and we suggest that GDNF functions in *Gdnf^{hyper/hyper}* kidneys via mechanisms involving changes in cell cycle progression and the ability to detect growth factor levels.

Higher than normal GDNF levels in embryonic kidneys initially cause a rapid decline in NP numbers per niche, which is characteristic of many other mouse models with primary UB deficiency (Carroll & Das, 2013; Shaowei Chen et al., 2015; Costantini & Kopan, 2010; Liu et al., 2018). The decline of NPs observed in other published models is usually caused by their premature differentiation, which is not the case in *Gdnf^{hyper/hyper}* kidneys. Excess GDNF in the neonatal kidney, on the other hand, can keep the cell cycle active in the kidney cortex for much longer than in WT kidneys. NPs in *Gdnf^{hyper/hyper}* kidneys persist longer than in WT kidneys and continue to proliferate and form new nephrons. This opens the possibility for the use of GDNF in the future to promote continued nephrogenesis, although more studies are needed to overcome the limitations of the negative impact on UB morphogenesis and organ growth.

6.5 GDNF REGULTES NEPHRON PROGENITORS VIA ITS SECRETED TRANSCRIPTIONAL TARGETS

Our findings show that the early drop in NPs of *Gdnf^{hyper/hyper}* embryonic kidneys is caused by decreased cell proliferation rather than increased differentiation (Article III). Molecular changes found in known GDNF target genes (*Crfl1*, *Wnt11*, and *Srgn*) (Lu et al., 2009; Ola et al., 2011) and key NP maintenance signaling regulators (*Wnt9b*, *Wnt11*, and their transcriptional targets) (Karner et al., 2011; Kiefer et al., 2012; O'Brien et al., 2018) reinforce the cellular mechanism behind the decreased proliferation. Previous studies of NP cell lifespan and resilience by ablating the NP population with diphtheria toxin A or by disrupting MAPK/ERK activation specifically in NPs, both of which cause rapid decline in NP cell numbers without increasing cellular lifespan (Cebrian et al., 2014; Ihermann-Hella et al., 2018), combined with our findings suggest that NP cell proliferation inherently contributes to their lifespan determination. Moreover, as kidney development progresses, NP cells have substantially lower rates of proliferation and accelerated differentiation, and the lifespan of older NP cells can be extended by transplanting them into earlier-stage kidneys (Shuang Chen et al., 2015).

From our findings, we think it is likely that GDNF plays a role in the process by which NP cells sense their developmental age and, ultimately, determine their lifespan (Article III). GDNF expression is high at the start of normal kidney morphogenesis and during the active growth period, but it drops dramatically in late embryonic kidneys, resulting in loss of GDNF expression in early postnatal days. GDNF levels are abnormally elevated in early *Gdnf^{hyper/hyper}* kidneys, which appears to inhibit NP cell proliferation. This could be due to changes in extrinsic NP niche, i.e. the UB tips, where we detected molecular alterations, for example in the WNT signaling pathway, which is known to impact NP regulation (Karner et al., 2011; Kiefer et al., 2012;

Nagy et al., 2016; O'Brien et al., 2018). *Gdnf* mRNA and GDNF protein levels decline in postnatal *Gdnf^{hyper/hyper}* kidneys similarly as in WT kidneys, because *Gdnf* is manipulated at the post-transcriptional level, enabling endogenous temporal regulation of expression (Kumar et al., 2015). This likely reduces GDNF expression to a level that allows NP cell self-renewal and differentiation, which is identified through sustained nephrogenesis in postnatal kidneys. Such recently discovered growth factor-dependent plasticity in renal differentiation raises the prospect of its future use in regenerative medicine.

Previous experiments inhibiting BMP/SMAD1/5 signaling resulted in slightly larger kidneys with more nephrons than control kidneys (Brown et al., 2015), which provided the first hints for the potential of extending the nephrogenic period. Another study showed that lowering the amount of *Tsc1*, which encodes an mTOR inhibitor, keeps NP cells active for an extra day with no impact on embryonic NP cell numbers (Volovelsky et al., 2018). Furthermore, recent analysis of the transcriptome of NPs from *Tsc1^{+/-}* mice with increased nephron numbers revealed a role for *Tsc1* in the coordination of increased cellular perception of Wnt signals to end nephrogenesis. This study additionally found that nephron number could be increased by delaying cessation of nephrogenesis through the removal of one *Rspo3* allele in NPs (Jarmas et al., 2021). Other studies showed that prolonged nephrogenesis was obtained in mice with overexpression of *Lin28* or inactivation of the microRNA *Let-7* (Urbach et al., 2014; Yermalovich et al., 2019), models which exhibited either direct tumorigenesis or activation of the *Igf2* or *H19h* loci associated with Wilms' tumor development (Chen et al., 2018; Ogawa et al., 1993). In *Gdnf^{hyper/hyper}* kidneys, which are hypodysplastic yet functional for three weeks postnatal, no changes in pSMAD1/5 localization or levels were observed (Article III).

GDNF's role during nephrogenesis has previously been investigated by decreasing endogenous *Gdnf*, which results in decreased nephron endowment (Cullen-McEwen et al., 2001; Cullen-McEwen et al., 2003). The RET tyrosine kinase and its co-receptor GFRa1 create the canonical receptor complex for GDNF, which is only found in the UB epithelium (Costantini, 2012; Golden et al., 1999; Pachnis et al., 1993). For UB tip cells, GDNF is an important niche regulatory protein (Chi et al., 2009; Riccio et al., 2016; Shakya et al., 2005). Excess GDNF expands the collecting duct progenitors at the cost of UB trunk elongation, resulting in a phenotype of expanded UB tips and short trunks due to progenitor motility defects and shortened cell cycle (H. Li et al., 2019). During embryogenesis, the combined impact of high GDNF, biomechanical changes within the NP niche due to abnormal UB tip morphology, and the associated changes in GDNF targets and the WNT pathway appear to drive NP cells toward decreased proliferation. In postnatal *Gdnf^{hyper/hyper}* kidneys, we demonstrate that the expanded UB tip morphology improves over time but

persists in mild forms until P4. UB tip cells maintain their progenitor identity in P5 *Gdn^{hyper/hyper}* kidneys, which exhibit high levels of GDNF-regulated UB tip markers *Crlf1*, *Etv4*, *Etv5*, and *Lama1*; NP-expressed canonical WNT targets *Cited1* and *Uncx4.1*; and WNT agonist *R-spondin1* (Karner *et al.*, 2011; Lu *et al.*, 2009; Vidal *et al.*, 2020). Postnatal sustainment of the progenitor milieu in the UB tips continues its role as a NP niche to provide an environment favorable for NP self-renewal beyond P2/P3.

Normal situation

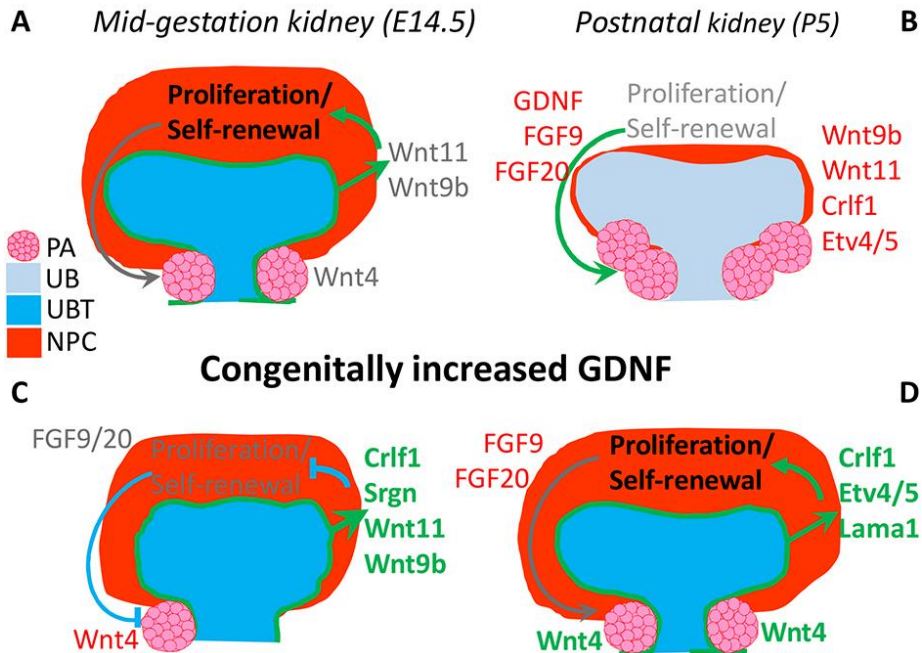


Figure 12 Schematic summary of excess GDNF effect on kidney development. (A) In a developing kidney, nephron progenitors (NPC, red) receive UB tip (UBT)-secreted (blue) signals that regulate proliferation and self-renewal versus differentiation into pretubular aggregates (PA, pink). (B) In postnatal kidneys, the UB (gray) loses its tip/progenitor identity, as indicated by a loss of tip-specific gene expression (genes shown in red letters). Concurrently, levels of GDNF and FGFs fall, leading to accelerated NP differentiation and depletion of the NP pool. (C) Excess fetal GDNF causes aberrant UBT shapes and increases expression of UBT-secreted regulatory molecules (green text). This increased expression inhibits NP cell cycle, reducing proliferation and self-renewal. Less NPCs leads to slower PA differentiation, as shown by a loss of Wnt4 expression (red text). (D) In postnatal kidneys subjected to excess GDNF, the UBT shape and niche normalize, despite the higher-than normal expression of tip-derived factors (green text). These molecular changes support normal NP proliferation, self-renewal, and differentiation, as indicated by the maintenance of a large NP population, increased Wnt4 expression, and a similar-to-wildtype amount of nephron precursors/PAs (pink). Therefore, excess GDNF under its own promotor allows nephrogenesis to continue until P7. Text colors: dark gray indicates no change in expression, red is decreased expression, and green is increased expression. These results met Study Aim 3. Image used with permission (Article III).

We identified UB-derived, secreted GDNF targets CRLF1 and WNT11, along with enhanced canonical WNT signaling and increased *Wnt9b* expression, as mediators of GDNF on NP regulation. Our findings also suggest that CRLF1's role is redundant with other cytokines, as its inactivation has no effect on renal differentiation. Many features of NPs and nephrogenesis are regulated by WNT9b and WNT11 (Carroll et al., 2005; Karner et al., 2011; Majumdar et al., 2003; Nagy et al., 2016; O'Brien et al., 2018). Upregulation of UB-expressed *Wnt9b* in *Gdnf^{hyper/hyper}* kidneys may mimic its forced expression in NPs (Kiefer et al., 2012), disrupting their balance of fate decisions and thus contributing to the mechanisms behind the imbalanced NP self-renewal in *Gdnf^{hyper/hyper}* kidneys. This is substantiated by the improvement in UB tip morphology following canonical WNT inhibition with IWR1 in kidneys exposed to high levels of GDNF. IWR1 inhibition has a significant impact on NPs, causing their dispersal and loss of adhesion to UB tips. We demonstrated that lowering UB-expressed *Wnt11* in the *Gdnf^{hyper/hyper}* background improved NP maintenance and differentiation but did not completely rescue the hypoplasia phenotype caused by the excess GDNF. This could be due to the inability to inactivate both *Wnt11* alleles in the *Gdnf^{hyper/hyper}* mice owing to the embryonic lethality of *Wnt11*^{-/-} (Nagy et al., 2016). The one remaining *Wnt11* allele may be enough to augment the GDNF cascade, as previously proposed (Majumdar et al., 2003), and thus partially prevent the complete rescue of nephrogenesis. An overview of the effects of excess GDNF during renal development compared to wildtype development is shown in Figure 12.

With our current knowledge from these studies, we cannot disregard the possibility that some of GDNF's effects on NPs are due to non-canonical GDNF signaling, as the NPs express potential alternative signaling receptors (Brodbeck et al., 2004; Davis et al., 2013; Enomoto et al., 2004; Paratcha et al., 2003). However, if we look at receptor components capable of RET-independent GDNF signaling, NCAM and GFRa1, both NCAM loss and UB-specific expression of *Gfra1*, under the control of the *Ret* promotor, preserve the normal renal phenotype (Cremer et al., 1994; Davis et al., 2013; Heuckeroth et al., 1999; Rossi et al., 1999; Sariola & Saarma, 2003). This indicates that neither NCAM nor GFRa1 are required for the regulation of nephrogenesis on their own.

Considering all our findings, MAPK/ERK presumably controls the self-renewal and maintenance of NPs by regulating energy metabolism and metabolite availability while upstream of MAPK/ERK, alterations in the signaling levels of GDNF are capable of prolonging the NP lifespan and nephrogenic program beyond its normal developmental timeline. Furthermore, MAPK/ERK is required for the maintenance of the collecting duct progenitors in the UB, likely due to its role as a mediator of signals originating from GDNF. Because of the non-regenerative status of the kidney

and the fact that low nephron number predisposes to adverse kidney health, any factors that potentially play a role in renal progenitor maintenance or that affect the rate or extent of nephrogenesis are of great interest.

7 CONCLUSIONS

In this thesis, I have presented novel mechanisms by which extracellular guidance cues are mediated in two embryonic renal tissues containing collecting duct and nephron progenitor cells, the UB epithelium and the metanephric mesenchyme. Utilizing RNA-Seq to identify MAPK/ERK transcriptional targets, this work provides novel evidence that MAPK/ERK cascade activity is required for the maintenance of the progenitor identity of cells within the UB tips where it acts to prevent premature differentiation, allowing for ongoing kidney growth via repetitive branching of the UB tips. This was verified at the protein level where we demonstrated that MAPK/ERK-deficient UB cells prematurely differentiate into different cell types of the collecting duct while losing their capacity for new branch formation and subsequent growth (Ihermann-Hella et al., 2014). This indicates that MAPK/ERK may play a role as a gatekeeper of differentiation in the UB epithelium, an important finding since very little is known about UB progenitor regulation.

MAPK/ERK activity has a dual requirement in NPs where it is needed for maintenance and differentiation (Ihermann-Hella et al., 2018). My findings in NPs show that MAPK/ERK activation is required for normal turnover of mitochondria and consequently for mitochondrial metabolism, processes that are highly important for these proliferative progenitor cells. Based on the variations in target gene profiles between UB and NP, we propose that MAPK/ERK activity plays an earlier role in the cell fate determination hierarchy in NPs than in the UB. This is supported by the fact that many other tissue-specific stem cells exhibit energy dependence similar to NPs, which, combined with the known cell cycle regulatory functions of MAPK/ERK, suggests that control of mitochondrial metabolism could be a general mechanism by which the MAPK/ERK pathway regulates cell turnover.

One goal of this research was to better understand the MAPK/ERK-dependent transcriptional and metabolic regulation of NP cell maintenance, propagation, and differentiation. NP cells with intact and inhibited MAPK/ERK activity were employed *in vitro* to investigate the NP niche-intrinsic processes of nephrogenesis guidance. Although this *in vitro* model ignores the biological intricacy of whole-kidney development, which is dependent on UB epithelial and stromal cell activities, it proved to be a useful tool to study the metabolic regulation by MAPK/ERK in NPs where we showed transcriptional and metabolic-level changes in mitochondria, ribosome and amino acid metabolism, and diminished pyruvate and proline metabolism. Although some recent studies have begun looking at the metabolic regulation of NPs, very little is actually known, which is why our work significantly benefits the understanding of the role of metabolism in NP fate determination.

Finally, we demonstrated that by genetically increasing GDNF, we can prolong the lifespan of nephron progenitors and nephrogenesis past normal cessation. Our findings suggest that GDNF, which has been and is presently being studied for Parkinson's disease etiology, may have the potential to function as a future means of improving nephron endowment. More research is required to identify potential ways to over-activate GDNF signaling without negatively impacting kidney growth. Altogether, these studies provide crucial new information on the regulation of renal progenitor cells and kidney development, information that will surely prove useful for future kidney development and regeneration studies. Our results could also be utilized for kidney organoid differentiation protocols where, for example, MEK inhibitor use could be a game changer for certain aspects of nephrogenesis, and precise control of GDNF dose, which is an essential supplement for UB differentiation in organoids, could be a critical factor.

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