THE ROLE OF CITED FAMILY PROTEINS IN THE DEVELOPING KIDNEY

By

Scott Clifford Boyle

Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

in

Cell and Developmental Biology

August, 2007

Nashville, Tennessee

Approved: 15 May 2007

Mark de Caestecker

Chris Wright, Chair

Scott Baldwin

Ray Harris

Guoqiang Gu

To Abatha,
Thank You.

ACKNOWLEDGEMENTS

The work presented in this dissertation was preformed in the Department of Cell and Developmental Biology, Vanderbilt University School of Medicine between 2002 and 2007. I would like to thank the Department for their continued support during this time and for providing such an exciting and collaborative environment in which to work. I would also like to thank the Program in Developmental Biology for their support and strong commitment to the training of developmental biologists. My participation with this enthusiastic group has greatly enriched my scholastic progress during my time at Vanderbilt.

I would like to begin by thanking my mentor, Mark de Caestecker. Your passion for science is unmatched and continues to motivate me, even when I think you are off the deep end. You have been an instrumental part of my scientific development, and the lessons I have learned during my time in your lab will go with me throughout my career. I feel privileged to have trained under you, but am more proud to call you my friend. I look forward to continuing our never ending discussion about kidney development and hope for much productive collaboration in the future.

There are many people in my life without whom I would not have reached this point; I should start at the beginning.

I would like to sincerely thank my father and mother, Kevin and Mary Boyle. Your unwavering support and commitment has brought me to this point, and for that I am forever indebted to you. Education was always the priority

when we were growing up and two mantras have stuck with me; 'Work hard' and 'Do your best.' I believe I have done both over the course of my education, and I hope I have made you proud. As a proper scientist, I should not neglect to thank you for the DNA, it has served me well.

I would also like to thank my sister Amanda. I'm not sure we could be more different, yet I feel proud to call you one of my best friends. You have always been so unconditionally supportive, and I look forward to the relationship our families will share in the future.

To my amazing wife, Abatha. It's no secret to anyone who knows us that there is little chance I would have gotten to this point without you. My life has been different since the day we met, and I'll never know how to thank you for your support and sacrifice over the past six years. Our entire marriage has been this PhD; I hope that I have made you proud. I thank you for all of the nights that I came home completely defeated by science, and you reminded me; "This is why they call it research"…and… "If it was supposed to be easy, everyone would do it". I don't say it enough, Thank You.

I would also thank you for your collaboration on the best developmental biology project I have done over the past six years, Nate. He has been such an amazing part of this experience, and I can't wait for what the future holds. Thank you, Nate for being the ultimate antidote to bad days, and reminding me why what I do is important.

To Wayne, Debbie, Emily, and Hamil; Thank you for making me part of your family, and showing me all of the time that the most interesting science happens outside of the lab.

I would like to thank all of the friends I have made at Vanderbilt during my time here, especially Ryan, Audra and Quinn Pooley. This was an amazing shared experience for our families and our lives are forever linked. To David 'Felix' Frank, who has been a mentor and a friend. I have learned so much from you, and will be forever grateful for all of the pints and all of the science. To Drew Misfeldt; building the BACs was a purely collaborative endeavor that I could not have accomplished alone, thank you. To Melissa Langworthy, I have enjoyed our shared interest in the kidney and our friendship. And to Josh Rosenberg, fellow music snob. Thanks for being the guy I never have to talk science with.

I would especially like to thank my committee; Chris Wright, Scott Baldwin, Ray Harris, and Guoqiang Gu. Our meetings have always energized my efforts and provided me countless avenues for thought. I thank you for your support and appreciate your commitment to my scientific development.

I would like to thank all members of the de Caestecker lab past and present, including (but not limited to) David Frank, Genbin Shi, Lynda Anderson, Becky Breeding, Bo Lovvorn, Shaun Opperman, Jen Westrup, Monique Brink, and Amir Abathi.

Of course, this work would not have been possible without the contributions of our collaborators, both in terms of shared interest and reagents. I

would especially like to thank Alan Perantoni, Toshi Shioda, Sallie Dunwoodie, Duncan Sparrow, Shoumo Bhattacharya and Doug Mortlock. I would also like to extend my thanks to the Scott Baldwin lab. You have been my one stop shop for all things developmental biology. I appreciate your collaborative spirit and unending willingness to help. I would especially like to thank Kevin Tompkins for pronuclear injections to create the *Cited1-CreER*^{T2} mice.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABREVIATIONS

BAC	bacterial artificial chromosome
BP	basepair
CAM	chloramphenicol
CM	condensed mesenchyme
E__	embryonic day
ECM	extra cellular matrix
eGFP	enhanced green fluorescent protein
FAK	focal adhesion kinase
Gdnf	glial derived neurotropic factor
IM	intermediate mesoderm
IRES	internal ribosomal entry site
KB	kilobase
µL	microliter
µM	micromolar
MM	metanephric mesenchyme
NB	newborn
ND	nephric duct
nM	nanomolar
ORF	open reading frame
O/N	overnight
P___	postnatal day
PFGE	pulse field gel electrophoresis
PTA	pre-tubular aggregate
RBC	red blood cell
RT	room temperature
RV	renal vesicle
SID	smad-interacting domain
SM	stromal mesenchyme
TGFβ	transforming growth factor beta.
UB	ureteric bud
UGS	urogenital system
UTR	untranslated region
WT	wildtype

CHAPTER I

INTRODUCTION

Overview

Development of the mammalian kidney is based upon the establishment of a reciprocal tissue interaction between the ureteric bud (UB) and the metanephric mesenchyme (MM) (for review see (Dressler, 2006). The UB is an epithelial tube that emerges from the nephric duct (ND) around embryonic day 10.5 (E10.5) in the mouse, invading the MM, an overlying population of renal progenitor cells in the nephrogenic cord. Invasion of the UB leads to patterning of the MM into the condensed mesenchyme, which surrounds UB tips and branch points, and stromal mesenchyme, a more loosely associated group of cells that border the condensed mesenchyme. Cross talk between the UB, condensed mesenchyme and stroma drives conversion of progenitor cells into nephronic epithelia and provides for the continued growth and iterative branching of the UB. Much work over the past decade has demonstrated that these events are controlled and integrated at the transcriptional level (for review see(Boyle and de Caestecker, 2006; Schmidt-Ott et al., 2006; Yu et al., 2004).

In a screen to identify factors which regulate this process, the transcriptional cofactor Cited1 was found to be expressed in the MM and downregulated as these cells undergo epithelial differentiation in response to UB-derived inductive signals (Plisov *et al*., 2000). Cited1 (**C**bp-P300 **I**nteracting **T**ransactivators with **E/D** rich tails) is the

founding member of a family of non-DNA binding transcriptional cofactors that includes Cited2 and Cited4 in mammals and an additional member, Cited3, in lower vertebrates.

The primary focus of my research has been to investigate the expression and function of Cited family proteins during kidney development in the mouse. An additional aim has been to exploit the *Cited1* expression domain to fate map renal progenitor cells and create a broadly applicable tool to study gene function in the MM. Chapter one will describe kidney development in the mouse and highlight key regulatory networks responsible for early specification and inductive events. It will go on to discuss the identification of Cited1 as a gene regulated during MM differentiation and highlight what is known about this family of transcriptional co-factors. Focusing initially on Cited1, chapter two will include detailed expression profiles of Cited proteins in the developing kidney and present data that demonstrate that these genes are not required for nephrogenesis. I have also used *in vitro* methods to address the function of Cited1, and chapter three will demonstrate a role in regulation of cell adhesion and migration. Chapter four will describe the creation of a *Cited1-CreER*^{T2} transgenic animal, which I have used to trace progenitor cell lineage in the developing kidney and uncover patterns of cell fate. This mouse is also a powerful tool for conditional gene deletion in the MM. In chapter five I will conclude by discussing how my work is relevant to the field of kidney development and highlight potential future experiments that will allow for better understanding of this process.

Kidney development in the mouse

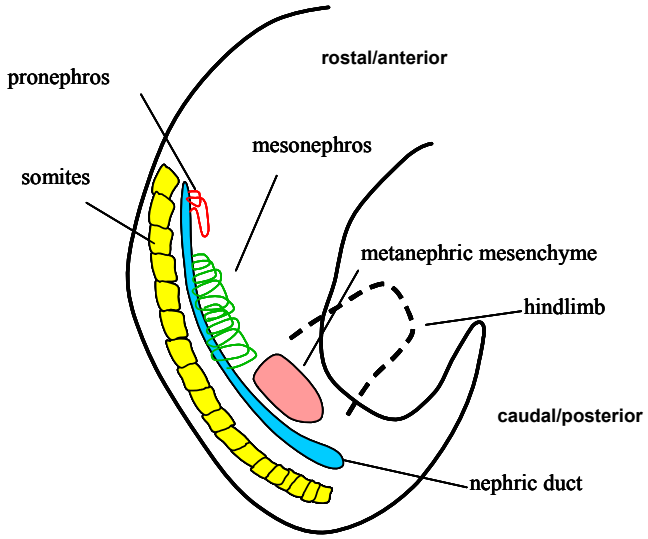
The kidney has received extensive attention from developmental biologists since the nature of its reciprocal tissue interactions was first described more than fifty years ago (Grobstein, 1953; Vize *et al*., 2002). There are three kidney structures which arise during mammalian development (Fig. 1A). The pronephros and mesonephros develop first and are transient structures in mammals that serve as excretory organs during *in utero* development. Subsequently the permanent kidney, or metanephros, is formed in the posterior aspect of the intermediate mesoderm (IM), in between the lateral and somitic mesoderm (Fig. 1B).

The first structural evidence of nephrogenesis in the mouse arises around embryonic day 8 (E8), with the formation of two parallel epithelial tubes known as the nephric ducts (ND). This process occurs through mesenchyme to epithelial differentiation, and is dependent on factors secreted by the overlying surface ectoderm and somites (Obara-Ishihara *et al*., 1999). Once formed, the ducts extend towards the posterior, or caudal, pole of the embryo, forming the core component of the developing urogenital system (UGS). The period between E8 and E9.5 is marked by formation of the pronephros and mesonephros, relying on many of the same induction mechanisms and genetic programs required for metanephric development (Vize *et al*., 1997).

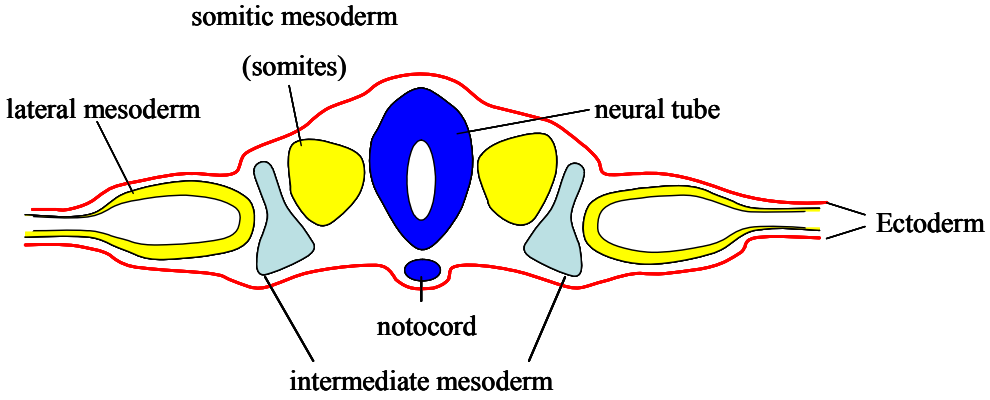
The earliest changes associated with development of the metanephric kidney begin around E10.5, as cells within the posterior region of the IM aggregate to form a structure known as the metanephric mesenchyme (MM). This group of cells is believed to contain precursors for epithelial, stromal and vascular elements of the adult kidney, but has not been definitively fate mapped. Formation of the MM is associated with the

Figure 1. ***Schematic of early kidney development in mice.*** **A.** Sagital view of E10 mouse embryo, just prior to UB outgrowth **B.** Cross section of E8 mouse embryo demonstrating relative position of the IM, site of the presumptive nephrogenic field. **C.** Outgrowth, invasion and early branching of the UB. The MM is patterned into stromal and condensed elements in response to UB invasion.

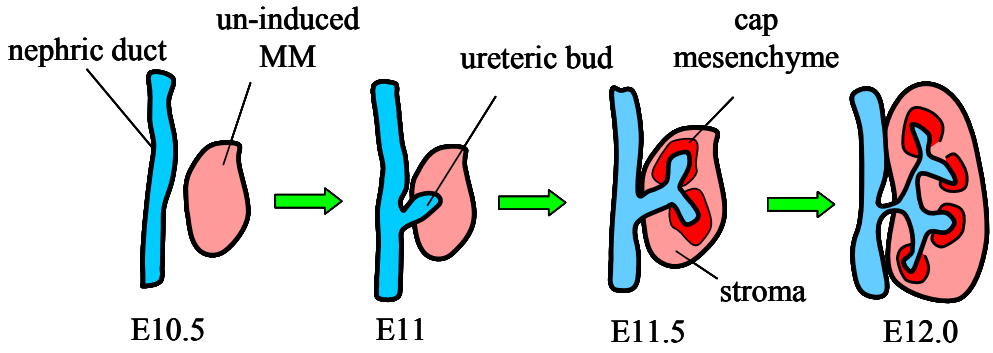
A


rostal/anterior
pronephros
mesonephros
somites
metanephric mesenchyme
hindlimb
caudal/posterior
nephric duct

B


somitic mesoderm
(somites)
lateral mesoderm
neural tube
Ectoderm
notocord
intermediate mesoderm

C

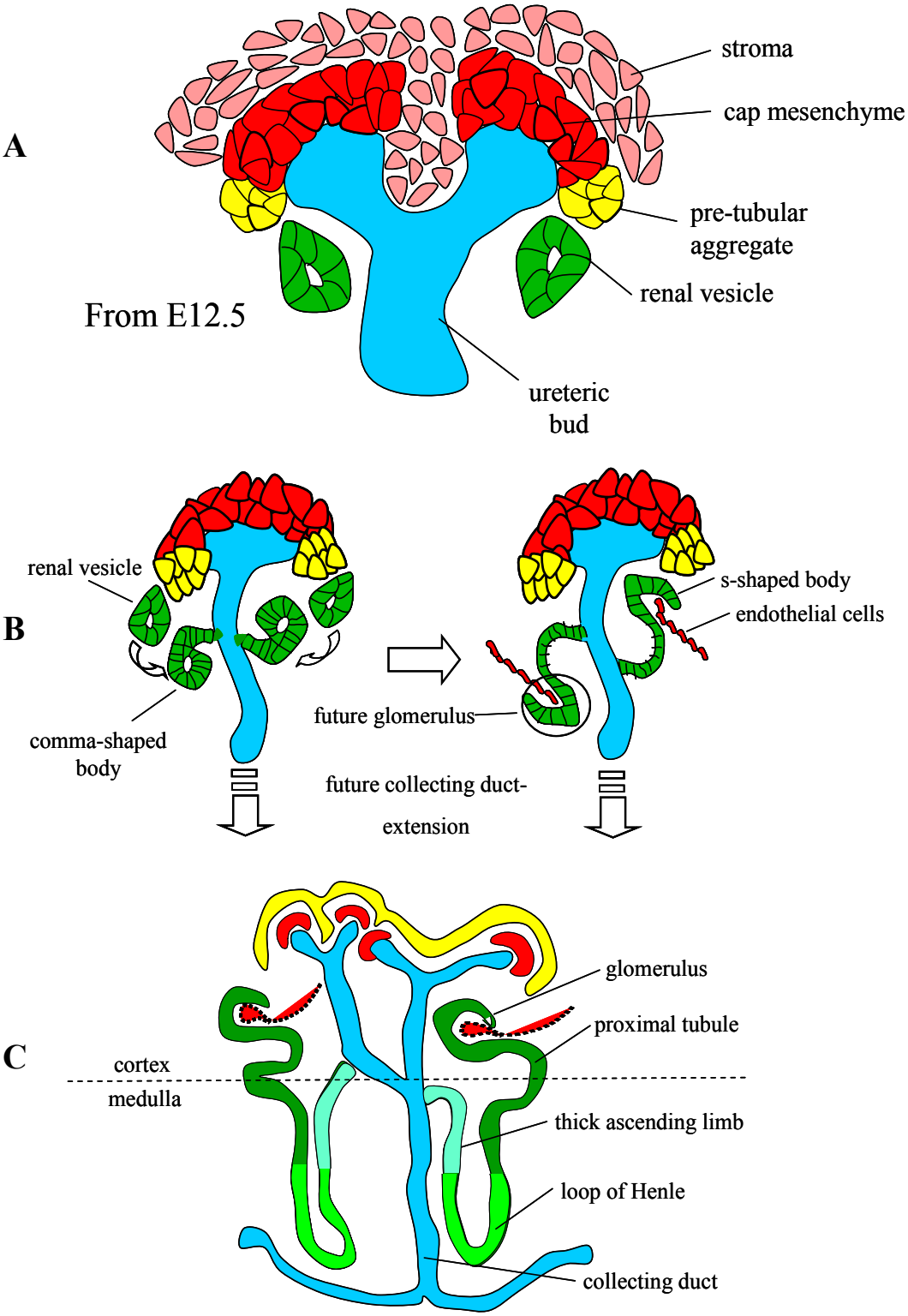

nephric duct
un-induced MM
ureteric bud
cap mesenchyme
stroma
E10.5
E11
E11.5
E12.0

expression of a distinct profile of transcription factors within the posterior portion of the IM. This culminates in the expression of the secreted growth factor Gdnf which activates its receptors Ret/Gfrα1 to induce an outgrowth from the ND known as the ureteric bud (UB). This epithelial structure invades the overlying MM, establishing the reciprocal tissue interaction that characterizes kidney development (Fig. 1C; (Cacalano et al., 1998; Moore et al., 1996; Pichel et al., 1996; Sainio et al., 1997; Schuchardt et al., 1996). All subsequent developmental events within the metanephric kidney are dependent on the coordinated cellular response resulting from invasion of the UB into the MM.

Initially, the UB secretes factors which rescue the MM from apoptosis and induce patterning of the MM. In response, MM cells condense around the UB tip to form a structure known as the cap metanephric mesenchyme. These cells represent a subset of the MM and are believed to give rise to most of the specialized epithelial cells within the mature nephron. This population is distinguishable from the surrounding, loosely packed stromal mesenchyme, which plays an important role in support and patterning of differentiating cap cells (Fig. 1C, 2A; (Jena et al., 1997; Levinson and Mendelsohn, 2003). MM patterning is dependent on UB invasion, but the specific signaling pathways that promote the segregation of cap and stromal mesenchyme are not known. It is, in fact, unclear whether establishment of the stromal compartment reflects migration of exogenous cells into the metanephric region, segregation and expansion of precursor cells within the MM, or both.

Factors secreted by the UB initiate a program of stepwise differentiation in cap mesenchyme cells. This start of this process is characterized by the formation of small clusters of cells contiguous with the ventral aspect of the cap structure, called pre-tubular

Figure 2. ***Schematic of MM patterning, epithelial differentiation and nephron patterning in the developing kidney.*** **A.** Characteristic branched UB and associated patterned MM. Early epithelial differentiation is marked by the formation of the PTA and the RV. **B.** Formation of the comma- and S-shaped bodies. Fusion with the UB and endothelial invasion are key events during this stage. **C.** Elongation and patterning of collecting system and distal nephron segments. Continued formation of new nephrons in the outer, nephrogenic zone.


A
stroma
cap mesenchyme
pre-tubular aggregate
renal vesicle
From E12.5
ureteric bud
B
renal vesicle
s-shaped body
endothelial cells
future glomerulus
comma-shaped body
future collecting duct-extension
C
glomerulus
proximal tubule
cortex
medulla
thick ascending limb
loop of Henle
collecting duct

aggregates (PTA; Fig. 2A; (Sariola, 2002). These cells undergo a burst of proliferation before they begin to express junctional and basement membrane markers, forming early polarized epithelial structures known as renal vesicles (RV). Having established UB invasion, MM derived signals then provide for continued growth and symmetrical branching of the UB (Fig 1C, 2C). This in turn promotes patterning and differentiation of the overlying cap mesenchyme within the outer, nephrogenic zone of the developing kidney. At the same time, PTA structures undergo further polarization as they form RVs lined with columnar epithelium. These vesicles undergo a series of invaginations and become first comma- and then S-shaped bodies, fusing with the extending UB to form a continuous lumen (Fig. 2B).

These events mark the initiation of a program of proximal and distal patterning that gives rise to distinct tubular segments and glomeruli, and are associated with recruitment of stromal and vascular cell components. Distally patterned elements elongate into the medulla and also connect the nephron to the UB-derived collecting system (Fig. 2C). UB branching and nephron differentiation occur throughout embryonic development, but 85% of the branching events are complete by E16.5. Detailed morphometric analysis indicates that there is a second, temporally distinct phase of nephrogenesis after E16.5 associated with a marked increase in tubular growth and patterning of nephron segments (Fig. 2C; (Cebrian et al., 2004). This results in expansion of the renal cortex and patterning of medullary regions of the adult kidney.

The iterative nature of these events suggest that there are likely to be populations of multi-potent stem cells within the cap mesenchyme and UB tips that provide a continuous supply of progenitor cells as nephrogenesis progresses. These cells have yet

to be positively identified. Furthermore, it is in the comma-shaped body (possibly as early as the RV), that the differential expression of markers which delineate specific segments in the adult nephron is first observed (Cheng *et al*., 2007; Kobayashi *et al*., 2005). This is important because it suggests that cell fate may already be determined at this early stage. This also raises questions about the commitment stage of cap mesenchyme cells. Is an individual cell within the cap predetermined to become say, a glomerular podocyte, or is fate determined at a later stage by positional cues in primitive nephrons?

Molecular programs which regulate kidney development in the mouse

Much of our understanding of the process described above comes from global gene deletion studies in the mouse. Work over the past decade has demonstrated the requirement of several secreted growth factors, transcription factors and cell surface receptors for proper development of the kidney (Table 1, Fig. 3). This part of the thesis will discuss, at the molecular level, some of the programs which are crucial *in vivo* for specification and induction during the early phases of metanephric development. The purpose of this section is not to discuss every protein that has been implicated in kidney development, but rather to cover aspects of this process which form the foundation of my work. This section will not address the molecular mechanisms within the UB that regulate branching morphogenesis. For additional information on this subject the reader is referred to (Costantini and Shakya, 2006; Dressler, 2006; Shakya *et al*., 2005). Specifically, I aim to highlight:

1) The transcriptional control of kidney development. As the principal focus of my research has been a family of transcriptional co-factors, I will demonstrate how early events during nephrogenesis are regulated by complex networks of evolutionarily conserved transcription factors and co-factors. Important cell surface receptors and secreted molecules will be covered as well.

2) The concept of functional redundancy between members of a gene family. As an example of this paradigm, I will discuss the role of Pax2 and Pax8 in specification of the ND. A primary aim of my work has been to determine if Cited family proteins can compensate for one another during kidney development.

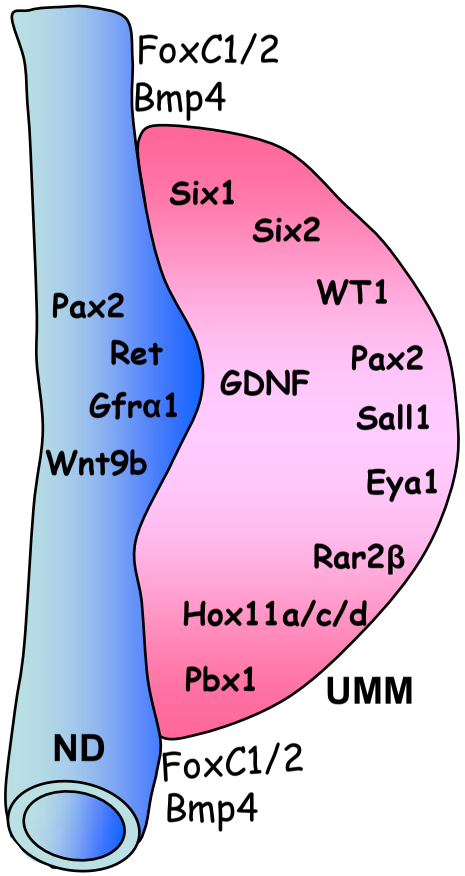
3) The difficulties in studying broader aspects of kidney development inherent in global gene deletion strategies. In addition to its usefulness for lineage tracing, our generation of a *Cited1-CreER*^{T2} transgenic mouse is a direct response to this problem and will allow for temporal control of gene deletion in the cap mesenchyme, circumventing the requirement for a given factor in early events.

Table 1. ***Genes required for early metanephric development.*** All factors have been shown to play a critical role during kidney development *in vivo*. SGF – secreted growth factor, TF- transcription factor, CSR – cell surface receptor. Competence (MM comp?) refers to the ability of isolated MM to differentiate when exposed to an inductive signal, such as isolated UB or spinal cord.

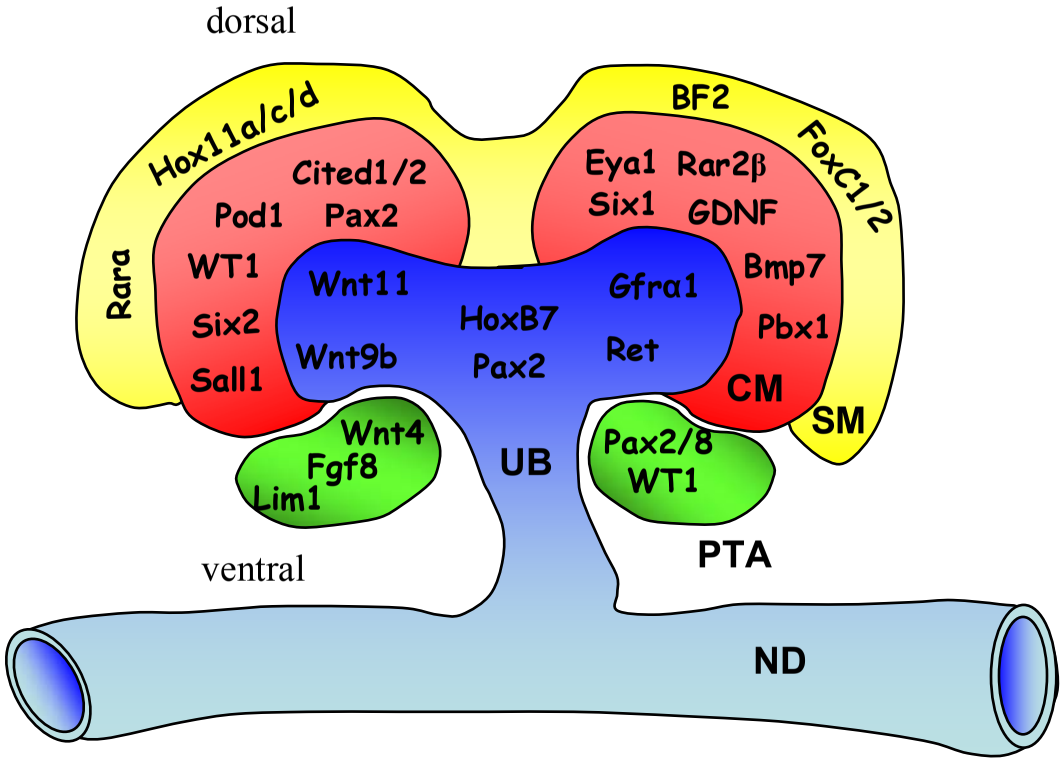
Gene	Type	Early Expression	Kidney Phenotype	Molecular Phenotype	MM Comp?	Notes
BMP4	SF	E10.5 mesenchyme surrounding ND E11.5 mesenchyme surround stalk of UB	Hypoplaisa, cystic (Hets/Hypomorph)	Pax2, c-Ret present but with reduced expression domains *Gdnf* normal, *WNT11* reduced in explant system	N/A	Global deletion results in lethality ar gastrulation. Promotes UB outgrowth and stromal MM expansion in vitro.
BMP7	SF	E11.5 UB, MM E12 PTA	Bilateral displasia, hydroureter	Wnt4, Pax2, Pax8, WT1 all initially expressed, but progressively lost	Yes	Required for survival of MM
Eya1	TF	E8.5 IM E10.5 ND, U-MM E11.0 Cap MM	Agenesis, MM not specified from IM	*Six1*, *Six2, Pax2, Gdnf* lost	MM not formed	Tx co-factor. Only mutant identified in which MM does not form. No MM may be the cause of GDNF loss, but there is also evidence for direct regulation (ref)
FGF8	SGF	E11.5 Ventral MM (Presumptive PTA) E12 PTA	Severe hypoplasia, arrest at RV stage Cap MM apoptosis	*WT1, Pax2, NMyc, Gata3* initially normal but lost as MM depleted *Wnt4* and *Lim1* reduced in PTA	No (can be rescued w/FGF8 bead)	Conditional deletion w/ T-cre (mesodermal lineages) to avoid lethal gastrulation defect. Survival signal specific for cap, stroma not affected.
FoxC1/2	TF	E9.5 MN E10.U-MM E11.0 Stromal MM	Duplex kidney	*Eya1, Gdnf* expanded	Yes	Define GDNF expression boundaries, specifying site of UB outgrowth.
GDNF	SGF	E11 MM	Agenesis, no UB outgrowth	N/A	Yes	Can promote UB outgrowth in vitro
Gfrα1	CSR	E10 ND	Agenesis, no UB outgrowth	N/A	?	Ret co-receptor
Hoxa/c/d11	TF	E10.5 U-MM E11.0 Stromal MM	Agenesis (triple knockout)	*Gdnf, Six2* absent *Eya1* normal	?	No phenotype in individual mutants
LIM1	TF	E12 PTA	Hypoplasia, non functional	Brn1, Dll1 lost in RV	Yes	Conditional deletion w/ Rar2β Cre to avoid early IM defects. Mosaic analysis shows Lim -/- cells cannot contribute to …

Gene	Type	Expression	Kidney Phenotype	Molecular Phenotype	MM Comp?	Notes
Pax2	TF	E8.5 IM, ND E10.5 U-MM E11.0 UB- MM	Agenesis, failure of UB outgrowth	*Gdnf* absent	No	Pax8 can rescue early defect in ND formation, but not in MM.
Pbx1	TF	E10.5 U-MM E11.0 MM	Expansion of cap MM, UB branching reduced, Hypoplasia	*Pax2, Wt1* expanded *Brn1, Pod1* (stroma) normal	Reduced	
Ret	CSR	E9 ND E11-UB; strongest in tips	Agenesis. ND is formed, limited capacity for UB outgrowth	N/A	N/A	Mosaic studies show Ret -/- cells cannot contribute to UB tips (Ref)
Rarα/β2	CSR	E11.5 Stromal MM	Hypoplasia, agenesis	*Pax2, Wt1, Gdnf, Bmp7* normal	Yes	
Sall1	TF	E9.5 MN, ND E10.5 U-MM E11.0 condensed MM	Agenesis (variable), failure of UB outgrowth	*Pax2, Wt1, Eya1, Gndf* reduced	Yes	Molecular phenotype likely results from hypoplastic MM and not direct regulation.
Six1	TF	E10.5 U-MM E11.0 condensed MM	Agenesis, Failure of UB outgrowth	*Pax2, Six2, Sall1* absent *Eya1, Gdnf, Wt1* normal	No	UB outgrowth fails despite detectable Gdnf.
Six2	TF	E10.5U-MM E11 condensed MM	Severe hypopalsia, few differentiated epithelial structures	WNT4↑ in ectopic PTA	Yes	Maintains MM; ectopic PTA form on dorsal side of UB in K/O
Wnt4	SGF	E12 PTA	Agenesis, PTA forms but does not progress	Fgf8, Pax8 initially expressed in PTA	No	UB invades and MM condenses. Can induce differentiation of isolated MM *in vitro*
Wnt9b	SGF	E10.5 ND, E11 ventral aspect of UB branches	Agenesis/ severe hypoplasia	FGF8, Pax8, Wnt4 absent	Yes	UB invades and MM condenses, but PTA does not form
Wt1	TF	E10.5 U-MM	Agenesis	*Six2, Gndf, Pax2* normal	No	Pax2 mRNA is present, protein is not.

Figure 3. ***Expression of notable factors required for proper kidney development*** **. A.** E10.5, Expression of key factors in the UB and uninduced MM (UMM) prior to UB outgrowth and invasion. **B.** E12.5. Expression of key factors in UB, condensed mesenchyme (CM), stromal mesenchyme (SM), and pre-tubular aggregate (PTA). Location of gene name within given structure does not indicate a specific location of expression.


FoxC1/2
Bmp4
Six1
Six2
WT1
Pax2
Ret
Gfrα1
Wnt9b
GDNF
Pax2
Sall1
Eya1
Rar2β
Hox11a/c/d
Pbx1
UMM
ND
FoxC1/2
Bmp4

E10.5


dorsal
Hox11a/c/d
Rara
Cited1/2
Pod1
Pax2
WT1
Six2
Sall1
BF2
FoxC1/2
Eya1
Rar2β
Six1
GDNF
Bmp7
Pbx1
CM
SM
Wnt11
Wnt9b
HoxB7
Pax2
Gfrα1
Ret
Wnt4
Fgf8
Lim1
UB
Pax2/8
WT1
PTA
ventral
ND

E12.5

Regulation of nephric duct formation by Lim1, Pax2 and Pax8

Lim1, also known as Lhx1, is a homeodomain DNA binding transcription factor that is initially detected in the visceral endoderm during gastrulation, and is differentially expressed in multiple tissues during development (Karavanov *et al*., 1998). In the developing UGS, expression is initiated in the lateral plate and IM at E8.5. As the pro- and mesonephros are formed within the IM, expression is lost in the lateral plate and becomes restricted to the mesonephric tubules and the ND. Pax2 and Pax8 are two closely related members of a family of paired-box DNA binding transcription factors that have largely overlapping expression domains in the developing kidney (Bouchard *et al*., 2002). Much like Lim1, Pax2 is expressed early in the IM that gives rise to the renal anlage, and both Pax2 and Pax8 are expressed in the ND as it extends posteriorly towards the cloaca (Bouchard *et al*., 2002; Dressler *et al*., 1990).

Global deletion of *Lim1* is usually embryonic lethal due to a defect in formation of the placenta (Shawlot and Behringer, 1995). The few mice that do survive until birth lack all IM derived structures, including the kidneys (Tsang *et al*., 2000). More detailed analysis of these mutant mice indicated that *Pax2* is initially expressed at low levels in the anterior portion of the intermediate mesoderm, but fails to extend posteriorly into the nephrogenic region. Analysis of *Hoxb6*, which marks a wider area of mesoderm including the IM, ND and lateral plate, reveals markedly reduced expression in *Lim1* mutant mice. This decrease is seen only in the IM, the presumptive site of ND and pro/mesonephros formation, while expression of *Hoxb6* is unaffected in the lateral plate. Taken together, these data suggest that Lim1 is specifically required for the differentiation of early nephrogenic structures within the IM.

Like *Lim1*, deletion of *Pax2* results in loss of the mesonephric tubules and the metanephric kidney (Bouchard *et al*., 2002; Torres *et al*., 1995). In fact, marked kidney defects are observed in *Pax2* heterozygotes, indicating that gene dosage is an important component of its function (Torres *et al*., 1995). Unlike *Lim1* mutants however, the ND forms in *Pax2* mutants, but fails to extend caudally to the level of metanephric development (Torres *et al*., 1995). Based on its wider expression in the IM, it is surprising that the ND forms at all in the absence of Pax2. The fact that Pax8 is also expressed in the ND raises the possibility that it can functionally compensate for loss of *Pax2* in this structure. Consistent with this, deletion of both *Pax2* and *Pax8* results in a complete failure of ND formation (Bouchard *et al*., 2002). Interestingly, deletion of *Pax8* alone does not disrupt formation of the meso- or metanephric kidney (Mansouri *et al*., 1998), suggesting that loss of function may be completely compensated for by Pax2 in the anterior intermediate mesoderm. The relationship between Lim1 and the Pax gene products during these early stages of kidney development is less clear. Most likely, the absence of *Pax2* in the posterior intermediate mesoderm of *Lim1* mutants reflects the failure of these differentiated structures to form, and not direct regulation of *Pax2* expression by Lim1 (Tsang *et al*., 2000). However, our understanding of these early events in kidney development is limited to these descriptive studies, so that the precise relationship between these genes remains unclear.

The Eya1/Pax2/Six1 transcriptional circuit

Aside from formation and extension of the ND, establishment of the interaction between the MM and the UB is initially dependent on specification of the MM from the

IM and its subsequent ability to stimulate emergence of the UB. Eya1, Pax2, and Six1 are part of a network of transcriptional regulators that have been well characterized in *drosophila* eye development (Brodbeck and Englert, 2004; Treisman, 1999). Remarkably, this network has been evolutionarily conserved and plays a critical role in both specification of the MM and stimulation of UB outgrowth.

Specification of the metanephric mesenchyme

While the precise architecture of transcriptional networks in the MM is poorly understood, it is clear that Eya1 plays an essential role in regulating early events during nephrogenesis (Sajithlal *et al*., 2005; Xu *et al*., 1999; Xu *et al*., 2002). Eya1 is one of four mammalian orthologues of *Drosophila eyes absent* (*eya*), and is expressed in the IM from E8.5 and more specifically in the ND and the uninduced MM at E10.5. As in the *Drosophila* eye, the expression domain of Eya1 in the MM overlaps with that of other members of the Eya/Six/Pax network. Mammalian *so* orthologues Six1 and Six2 are expressed in the MM prior to UB invasion, as are the *toy* and *ey* orthologues Pax2 and Pax8 (Bouchard *et al*., 2002; Xu *et al*., 2003). The *dac* orthologue, Dach1 is expressed in early nephronic epithelia of the developing kidney (Ayres *et al*., 2001), however, there are no published data relating to its functional role in renal development. Initial studies indicated that *Eya1* null mutant mice died at birth with renal agenesis (Xu *et al*., 1999; Xu *et al*., 2002). More detailed analysis has demonstrated that these mice have a normal ND and mesonephros, but fail to form the distinct MM aggregate at E10.5 in the posterior region of the IM (Sajithlal *et al*., 2005). To date, this is the only mutant identified that results in the failure of the MM to be specified from the IM. A caveat to these studies is

that Eya1 is a non-DNA binding transcriptional co-factor that must interact with other DNA binding factors in order to activate or repress transcription. This suggests that Eya1 expression alone is unlikely to be sufficient to specify the MM. Given the biochemical data demonstrating functional and physical interactions between Eya and Six family proteins (Li *et al*., 2003; Ohto *et al*., 1999), it is possible that Eya1 acts by modifying the transcriptional activity of Six family members in the MM. Indeed, Six1 is co-expressed with Eya1 in the MM and *Six1* null mice also fail to form kidneys (Xu *et al*., 2003), suggesting that specification may require the assembly of Six1/Eya1 complexes in the presumptive MM, however this has yet to be definitively shown.

Stimulation of UB outgrowth from the nephric duct

Outgrowth of the UB and its subsequent invasion of the MM is an essential induction step in development of the metanephros proper. This event is dependent both on a signal secreted by the MM (Gdnf) and the expression of its receptors (Ret/Gfrα1) in the ND. Deletion of any of these components limits the ability of the UB to emerge and results in renal agenesis (Cacalano *et al*., 1998; Sanchez *et al*., 1996; Schuchardt *et al*., 1996). It follows that deletion of genes in the uninduced MM which compromise its ability to produce Gdnf, or genes in the UB which effect expression of Ret or Gfrα1 also have this phenotype. Indeed, in most of the mouse mutants which specifically lack kidneys, alterations in this program account for the primary defect (Table 1). Because of this, establishing the transcriptional network upstream of Gdnf in the MM has received considerable attention from the field in recent years. *In vivo* and *in vitro* studies clearly demonstrate a role for Pax2 in the regulation of Gdnf expression in the MM (Brophy *et*

al., 2001). In addition to the ND, Pax2 is expressed in the MM prior to UB invasion (Dressler *et al*., 1990). The MM forms properly in *Pax2* null mice, however, it fails to express Gdnf (Brophy *et al*., 2001). Pax2 binds and activates defined elements within the Gdnf promoter suggesting that it directly regulates Gdnf expression (Brophy *et al*., 2001), but other components of this network up- and downstream of Pax2 are less clear. Based on their overlapping expression patterns and common phenotype, speculation has focused on other members of the Eya/Pax/Six network.

Initial characterization of the *Eya1* knockout mouse at E11.5 revealed that expression of *Pax2*, *Six1*, and *Gdnf* were lost in the area of the MM (Xu *et al*., 2002) while other studies have shown that Eya1 expression is independent of Pax2 (Xu *et al*., 2003). Together these data make it tempting to speculate that Eya1 acts upstream of Pax2 and Six1 in a transcriptional circuit driving Gdnf expression in the MM. However as discussed above, the MM never forms in *Eya1* null mice, making it unclear whether these effects are due to the direct regulation of this pathway by Eya1 or simply a consequence of losing the MM. Furthermore, Gdnf expression is maintained in the MM of *Six1* null mice despite the failure of the UB to emerge from the ND (Xu *et al*., 2003). It is unclear whether this is simply an effect of reduced Gdnf dosage that is not reflected by *in situ* hybridization, or if another, parallel pathway is required for UB outgrowth. For example, deletion of the *Hoxa11/Hoxc11/Hoxd11* gene cluster also results in loss of Gdnf expression in the MM and the subsequent failure of UB outgrowth, despite normal expression of Pax2 (Wellik *et al*., 2002). It is unclear if these genes regulate Gdnf expression through an independent, non-redundant pathway, or if they are part of a common transcriptional network downstream of Pax2. Studies in the developing

Drosophila eye may provide insight into these mechanisms. Overexpression of the Hox orthologue *antennapedia* in the eye inhibits transcriptional responses mediated by the Pax orthologue, *eyeless* by competitive inhibition of DNA binding (Plaza *et al*., 2001). If a similar mechanism is operative in the mammalian kidney, these findings would suggest that the Hox11 paralogues could play a direct role in modifying the transcriptional activity of Pax2. This would account for the loss of Gdnf despite maintenance of Pax2 expression in *Hoxa11/Hoxc11/Hoxd11* compound mutant mice.

Making nephrons: progenitor cell maintenance and differentiation

The formation of early epithelial structures from the MM involves a host of coordinated cellular events which not only give rise to primitive nephrons, but also replenish the MM for future rounds of differentiation. These processes are likely not mutually exclusive, though the relationship between the two is unclear. Furthermore, in many of the mouse mutants in which kidney development fails, the UB never invades the MM, complicating the study of subsequent events. This is important as a number of key early factors are expressed at later stages of nephrogenesis, and presumably play a role in these contexts. A handful of recent studies, however, have shed light on factors which are important for maintenance and differentiation of progenitor cells in the developing kidney.

Differentiation of the cap mesenchyme

The precise signals and downstream effectors which are necessary (and/or sufficient) for epithelialization of the MM have been elusive. An especially contentious

point has been the nature of the signal (thought to be UB derived) which initiates differentiation of cap mesenchyme cells. Several studies using explanted kidneys from rats have identified factors which have this property *in vitro* (Barasch *et al*., 1999; Perantoni *et al*., 1991; Plisov *et al*., 2001; Rogers *et al*., 1993), though until recently none has been shown to be required *in vivo* in mice. Numerous pieces of evidence have implicated the Wnt signaling pathway as being important for MM differentiation. Many Wnt ligands are expressed in the developing kidney, but the studies of Wnt4 and Wnt9b are the most remarkable, demonstrating a clear role for these genes in initiation and maintenance of MM epithelialization. Wnt4 is expressed in the PTA, and its deletion results in renal agenesis (Stark *et al*., 1994). Examination of early stages of kidney development in these mice demonstrates that while the UB invades the MM properly and even undergoes a few rounds of branching, no MM derived epithelial structures are present, including RVs. Furthermore, Wnt4 expressing cells can induce epithelialization of MM explants *in vitro* (Kispert *et al*., 1998). Together these data suggest that Wnt4 is both necessary and sufficient for MM differentiation, and is required during the early stages of this process. However, because Wnt4 is expressed in the PTA, but not earlier in condensed mesenchyme, it is unlikely to be the signal that initiates conversion of cap mesenchyme cells into epithelia. Recent studies have shown that UB derived Wnt9b is required *in vivo* for initiation of cap mesenchyme differentiation and formation of the PTA (Carroll *et al*., 2005). Wnt9b is expressed in the branched ends of the UB, most prominently on the ventral aspect and, like Wnt4, deletion results in renal agenesis. Formation of the PTA is normally associated with the expression of a distinct group of makers on the ventral side the cap mesenchyme including *Fgf8, Pax8 and Wnt4* (Fig 3B).

In the *Wnt9b* mutant, the UB invades the MM and branches, cap cells condense around the bud tips, but expression of this panel of makers is lost. This indicates the PTA fails to form in the absence of *Wnt9b*. Interestingly, *Pax8* and *Fgf8* are initially expressed in *Wnt4* null mice, suggesting initial differentiation of cap cells to form the PTA occurs in these animals but does not progress to the RV stage. Together these data evoke a model in which the sequential activity of Wnt9b and Wnt4 are respectively required to first initiate and then maintain epithelial differentiation of nephron precursors.

Maintenance of the cap mesenchyme

Progenitor cells within the cap mesenchyme must be continually repopulated to be available for induction by new UB branch tips and the formation of successive generations of nephrons. In general our understanding of this process is limited. One could imagine two possible models for this phenomenon. The cap mesenchyme could be repopulated by exogenous cells migrating in and condensing around UB tips, thereby filling the void left by differentiating cells. Alternatively, there could be a program within the cap mesenchyme whose job it is to oppose signals from the UB promoting differentiation, thereby allowing some cap cells to persist and repopulate this niche. The later is generally thought to be true, and fate mapping results I will present in chapter four strongly support a model by which cap cells are capable of self renewal. Furthermore, a recent report has demonstrated that the transcription factor Six2 is required for maintinence and renewal of cells within the condensed mesenchyme (Self *et al*., 2006).

Six2 is expressed in the MM prior to emergence of the UB and is restricted to the condensed mesenchyme in response to invasion. Outgrowth of the UB and condensation

of the MM are not affected in *Six2* null mice, but by e16.5 these mice have markedly smaller kidneys than wild type littermates. This is associated with disorganized epithelial structures and loss of condensed mesenchyme around UB tips; stromal cells are not affected. One possible explanation for this phenotype is that Six2 holds condensed MM cells in an undifferentiated state, and that in its absence all progenitor cells are exhausted in initial rounds of differentiation. Indeed, *Six2* null mice develop ectopic RVs on the dorsal side of the UB, as opposed to the usual location just ventral to the UB tip (Fig. 3C (Self et al., 2006). This phenotype is associated with the progressive loss of MM specific markers (such as *Eya1*) and inappropriate expression of RV markers (such as *Wnt4* and *Sfrp2*). It also appears that abnormal apoptosis of MM cells after UB invasion contributes to the loss of progenitor cells in *Six2* null mice; however given the presence of ectopic RVs it is likely that the primary function of Six2 is to oppose differentiation rather than promote survival. Importantly, these phenotypes can be rescued in kidney explants by expressing ectopic Six2 (Self *et al*., 2006).

The signals (and their origin) which act upstream of Six2 to maintain progenitor cells in an undifferentiated state are unknown, though several reports have demonstrated that Bmp7 acts as a crucial survival signal for the condensed mesenchyme (Dudley *et al*., 1999; Dudley *et al*., 1995; Luo *et al*., 1995). In the absence of *Bmp7*, early MM genes such as *Pax2* and *WT1* are initially expressed and *Wnt4* is present in the area of the PTA. These markers are progressively lost, and like the *Six2* null mice, this seems to be associated, at least in part, with apoptosis of the condensed MM. *Bmp7* null mice have not been analyzed for the presence of ectopic RVs, making it unclear if Bmp7 acts strictly

as a survival factor or if it also lies up- or downstream of Six2 in a pathway that promotes persistence of the condensed mesenchyme.

Genetic manipulation of kidney development: traditional limitations and new approaches

Additional *in vivo* genetic studies of cap mesenchyme differentiation and maintenance have been limited as many of the factors that are expressed in the MM and primitive epithelial structures are also required for early specification and/or inductive events (Table 1). Historically, this problem has been addressed *in vitro* by studying the ability of isolated MM structures from E11.5 mutant mouse embryos to undergo differentiation when exposed to potent inductive signals from spinal cord or wild type UB isolates**.** This approach has enabled us to identify several so called 'competence' factors that are required for this process (Table 1), but it is unclear from these studies whether the apparent requirement for these factors *in vitro* actually reflects their functional role in the context of intact metanephroi. A good example of this problem comes from the analysis of *Wt1* null mutant mice, in which there is complete loss of MM structures by E12.5 with no evidence of differentiation (Kreidberg *et al*., 1993). Tissue recombination experiments demonstrate that wild type spinal cord and UB structures are unable to rescue the *Wt1* null MM phenotype *in vitro* (Donovan *et al*., 1999; Kreidberg *et al*., 1993), suggesting that Wt1 could be a competence factor for MM differentiation. Loss of *Wt1* is also associated with a marked increase in apoptosis of the uninduced MM over the 24 hour period following its formation (Kreidberg *et al*., 1993). The inappropriate death of MM cells in *Wt1* null mice makes it impossible for tissue recombination experiments to determine whether the primary function of Wt1 is to act as

a survival/maintenance factor for the MM or if it has an additional role in regulating differentiation.

Many of these issues can now be circumvented using Cre/LoxP technology to delete genes in a spatially and temporally controlled manner. Creating a tool useful for such studies has been one of the primary aims of my work, and will be presented in chapter 4. A recent example of this approach in the developing kidney is the deletion of *Lim1* exclusively in MM-derived structures using *Rarb2-Cre* and *Lim1*^{LoxP} conditional mice, thus bypassing the early requirement for Lim1 in the IM (Kobayashi *et al*., 2005). These studies demonstrated that in the absence of *Lim1*, the PTA and RV form normally, but fail to undergo further differentiation or express nephron segment specific markers, Dll1 or Brn1. These findings indicate that Lim1 is not required for initial differentiation of the cap mesenchyme, but is essential for subsequent patterning of the RV into tubular structures of the nephron. An alternative approach to study later function of genes essential for early patterning is to study cell behavior *in vivo* using mosaic mice generated by introducing mutant ES cells with lineage specific markers into wild type blastocysts. This approach has recently been used to evaluate the functional role of Ret signaling in regulating UB branching morphogenesis (Shakya *et al*., 2005). By limiting the dosage of $Ret^{-/-}$ cells, the authors were able to track and study mutant cell function while preserving early renal development programs. Together, conditional deletion and mosaic studies should enable us to determine the role of a given factor in later events and the functional relationship between genetic pathways which regulate kidney development.

The CITED family of transcriptional co-factors

In a screen to discover novel factors which regulate differentiation of the MM, the transcriptional co-factor Cited1 was identified (Plisov *et al*., 2000). In this study it was shown that Cited1 is expressed in MM explants at an early stage and is downregulated as cells express markers of differentiation in response to UB conditioned medium.

Cited1 (**C**bp-P300 **I**nteracting **T**ransactivators with **E/D** rich tails) is the founding member of a family of non-DNA binding transcriptional cofactors which includes Cited2 and Cited4 in mammals and an additional member, Cited3, in lower vertebrates. These factors share a highly conserved C-terminal transactivation domain (CR2) and bind core elements of the transcriptional machinery such as P300, in addition to a variety of transcription factors (Braganca *et al*., 2003; Braganca *et al*., 2002; Plisov *et al*., 2005; Shioda *et al*., 1997; Yahata *et al*., 2001; Yahata *et al*., 2002). Cited1 also contains a Smad interacting domain (SID) not present in other family members, required for activity downstream of TGFβ signals (Plisov *et al*., 2005; Yahata *et al*., 2000). Germline deletion studies implicate Cited1 and Cited2 in a variety of developmental processes. Few studies have addressed the expression or biological function of Cited4.

During embryogenesis, Cited1 is required for proper trophoblast function, as null animals exhibit placental insufficiency causing growth defects late in gestation and death at birth (Rodriguez *et al*., 2004). In these mice, deletion of *Cited1* causes a disorganization of both maternal and embryonic blood vessels within the placenta. This effect is dependent on genetic background; *Cited1*$^{C57Bl/6}$ null mice suffer from placental insufficiency while *Cited1*^{Svj129} null animals do not. In addition, Cited1 is required for maturation of the mammary gland during puberty (Howlin *et al*., 2006). Female

Cited1^{SVJ129} null mice display abnormal branching and organization of the ductal tree at the onset of puberty. This process is known to be regulated by TGFβ and estrogen signaling, both of which can be activated by Cited1 (Plisov *et al*., 2005; Yahata *et al*., 2000; Yahata *et al*., 2001). In the absence of Cited1 there is an impairment of ductal outgrowth, consistent with reduced estrogen signaling. There are also defects in duct patterning, which is regulated by TGFβ (Howlin *et al*., 2006). This process is interesting because it represents a developmental program at work later in life and shares common themes with kidney development, such as progenitor cell differentiation and migration as well as branching morphogenesis .

In addition to its role during development, Cited1 is differentially expressed in many malignant cell types. The initial discovery of Cited1 was in a mouse melanoma cell line and it has subsequently been shown to be associated with melanogenesis (Nair *et al*., 2001; Shioda *et al*., 1996) and malignancies of the skin (Sedghizadeh *et al*., 2005). Cited1 has also been linked to malignancies of the thyroid, where it has emerged as a possible diagnostic indicator of tumor stage and prognosis (Fluge *et al*., 2006; Fryknas *et al*., 2006; Huang *et al*., 2001; Prasad *et al*., 2004).

Most interestingly, our lab has recently shown that Cited1 is highly expressed in Wilms' tumor, a relatively common pediatric malignancy of renal origin (Lovvorn *et al*, 2007, in review). Wilms' tumors are thought to arise from pockets of MM that fail to undergo epithelial differentiation called nephrogenic rests, resulting in retention of these cells in the tumor. It is in these poorly differentiated cells that Cited1 is expressed, primarily in the nucleus. These data are intriguing when considering normal and

abnormal development of the kidney, and will be discussed further at the end of Chapter two.

Cited2 is the best characterized member of this gene family. Its role as a competitive inhibitor of HIF1α has been extensively described at the biochemical level, and seems to be its primary mode of action in the developmental setting (Bhattacharya *et al*., 1999; Bhattacharya and Ratcliffe, 2003; Freedman *et al*., 2003). Deletion of *Cited2* results in embryonic lethality between E13.5 and E17.5, likely due to defects in the heart and/or placental vasculature (Bamforth *et al*., 2001; Weninger *et al*., 2005; Withington *et al*., 2006; Yin *et al*., 2002). In the developing heart, deletion of *Cited2* results in widespread abnormalities including septal defects in the atria and ventricles, a doubling of the right ventricular outlet and persistent truncus arteriosus (Bamforth *et al*., 2001). These defects are likely manifestations of abnormal left/right body axis formation in *Cited2* null mice (Bamforth *et al*., 2004; Weninger *et al*., 2005).

In addition to cardiac and placental defects, *Cited2* null mice lack adrenal glands, display abnormal cranial ganglia, and have exencephaly resulting from failed closure of the neural tube (Bamforth *et al*., 2001; Weninger *et al*., 2005; Yin *et al*., 2002). This neural tube defect is interesting because it can be rescued by administering folic acid to pregnant females (Barbera *et al*., 2002), suggesting that Cited2 is involved in the genetic pathways which underlie spina bifida in humans.

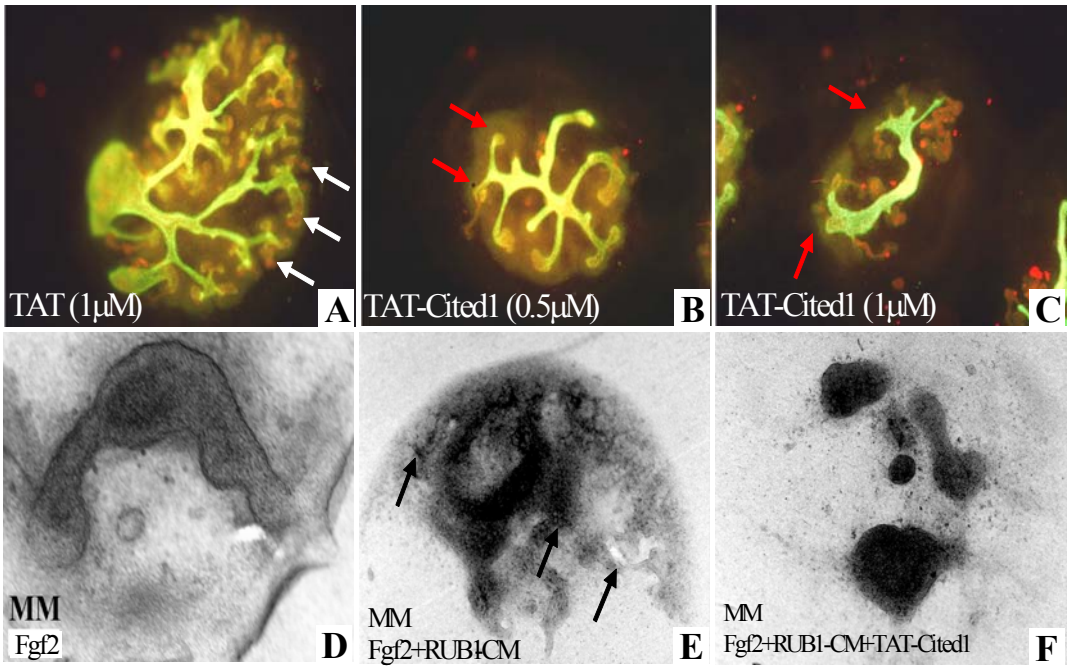
Cited1 in the developing kidney

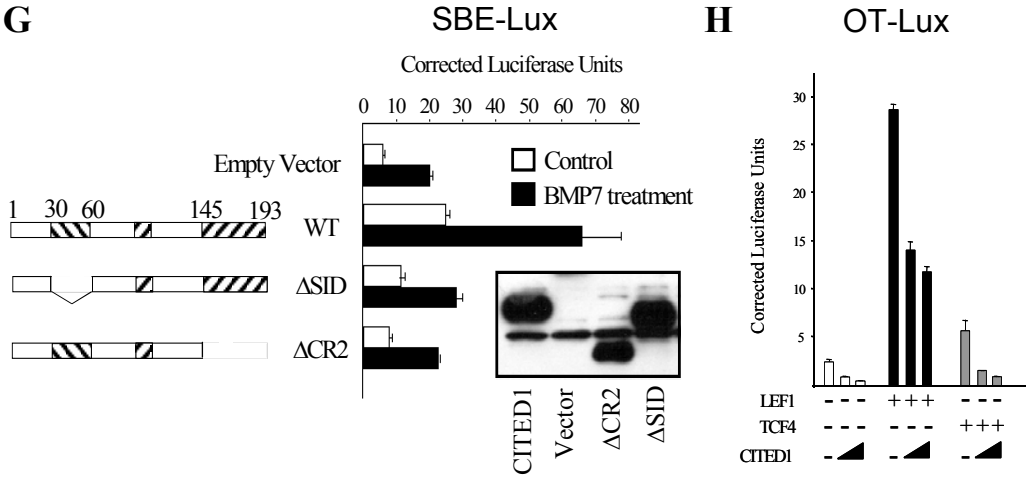
Though Cited1 has been identified as being regulated during the early stages of kidney development, its function in this setting is unknown. We have previously

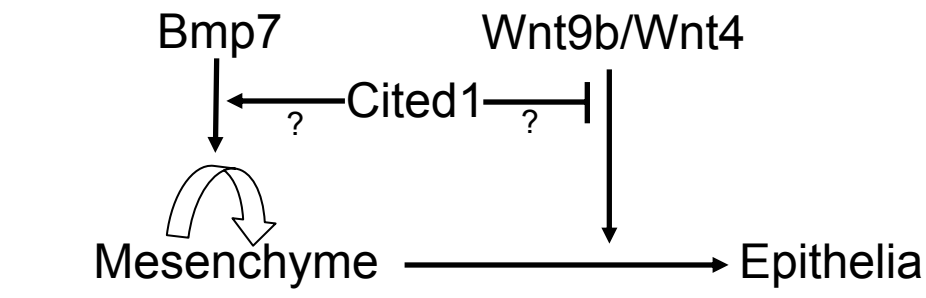
published a report investigating the role of Cited1 in the developing kidney (Plisov *et al*., 2005). These studies were primarily carried out *in vitro* and provide the foundation for studying the expression and function of Cited1 *in vivo*, which has been the principal focus of my research. As background, I will provide a summary and brief discussion of this paper, as well as a composite of key data (Fig. 4).

As it had been previously shown that differentiation of the MM was associated with downregulation of Cited1 (Plisov *et al*., 2000) we asked what effect Cited1 overexpression would have on kidney development. For these studies cultured kidney rudiments were transduced with TAT-Cited1, a fusion protein whose 'TAT' domain mediates cellular uptake. When Cited1 is overexpressed in this manner, cultured kidneys display fewer nephronic epithelia and reduced UB branching, while TAT alone has no effect (Fig 4A-C). Furthermore, in kidneys treated with TAT-Cited1 there appears to be an expansion in the size of the MM. These effects are dose dependent as higher concentrations of TAT-Cited1 exacerbate the phenotype (compare Fig. 4B to 4C). Condensation of the MM and nephron induction is dependent on UB branching, making it unclear which is responsible for the primary defects observed here. If the role of Cited1 in the MM was to directly influence the differentiation state of cap cells, overexpression may block epithelial conversion, resulting in fewer primitive nephrons and an expansion of the cap structure. This failure of MM differentiation would then perturb the normal tissue interactions required by the UB, thereby reducing total branching. Alternatively, Cited1 could modulate expression of a MM derived factor which regulates UB branching. In this case the reduced UB branching observed in TAT-Cited1 kidneys would result in fewer new nephrons being induced. One way to address

Figure 4. ***Effects of Cited1 overexpression in cultured kidneys and activity on BMP and WNT signaling pathways*** (adapted from Plisov et al., 2005.) **A-F.** TAT-Cited1 overexpression in intact metanephric rudiments (A-C) or isolated MM (D-F). **A.** Control explant incubated with TAT alone. **B,C**. Explants incubated with increasing concentrations of TAT-Cited1. White arrows – new nephronic epithelia, Red arrow – expanded MM region with absence of nephronic epithelia. **D.** Control isolated MM incubated with survival factor Fgf2. **E.** Control isolated MM induced to form tubules by addition of UB conditioned medium; Arrow- epithelial tubules **F.** Isolated MM treated with TAT-Cited1 and UB inductive medium. **G.** Effect of Cited1 on BMP7 mediated transcriptional responses. Left panel - schematic of constructs (inset – respective expression levels by western blot). Right panel – activity of Cited1 and deletion mutants on Smad responsive luciferase reporter. **H.** Effect of Cited1 on Wnt mediated transcriptional responses. **I.** Schematic model of bi-functional hypothesis of Cited1 function in the MM.


TAT (1μM)
A
TAT-Cited1 (0.5μM)
B
TAT-Cited1 (1μM)
C
MM
Fgf2
D
MM
Fgf2+RUB1-CM
E
MM
Fgf2+RUB1-CM+TAT-Cited1
F


G
SBE-Lux
H
OT-Lux
Corrected Luciferase Units
0 10 20 30 40 50 60 70 80
Empty Vector
Control
BMP7 treatment
1 30 60 145 193
WT
ΔSID
ΔCR2
CITED1
Vector
ΔCR2
ΔSID
Corrected Luciferase Units
30
25
20
15
10
5
LEF1 - - - +++ - - -
TCF4 - - - - - - +++
CITED1 - - -


I
Bmp7
Wnt9b/Wnt4
Cited1
?
?
Mesenchyme
Epithelia

this question is to use isolated MM cultures, which can be induced to form tubule structures with UB conditioned medium (Fig. 4E), thereby removing the influence of UB branching. When TAT-Cited1 is added to isolated MM and cultured in inductive medium, tubular epithelial structures do not form (Fig 4F). Together, these data imply that the primary role of Cited1 is to regulate the differentiation state of MM cells.

We then asked what biochemical properties of Cited1 could shed light on its role in the MM. It has been previously reported that Cited1 can mediate transcriptional responses downstream of TGFβ superfamily ligands (Shioda *et al*., 1998; Yahata *et al*., 2000). Reporter assays using the SBE-Lux reporter (Smad responsive) in NmuMg cells demonstrate that Cited1 can activate transcriptional responses downstream of BMP7 (Fig 4G). This effect is dependent on both the CR2 and SID domains of Cited1. Cited1 also has the capacity to repress Lef/TCF4 mediated Wnt transcriptional responses as measured by activity of the OT-Lux reporter (Fig 4H). This inhibition is dose dependent and requires the CR2 but not the SID domain (Fig. 4H, (Plisov et al., 2005).

This result is intriguing because both of these pathways have been shown to play a crucial role in regulation of early events in the MM. Both Bmp4 and Bmp7 are required for nephrogenesis (Table 1; (Dudley et al., 1999; Dudley et al., 1995; Dunn et al., 1997; Luo et al., 1995; Miyazaki et al., 2000). As discussed above, BMP7 is necessary for recruitment and survival of the condensed mesenchyme, while BMP4 has been implicated in UB outgrowth and expansion of the stromal MM and can substitute for the loss of BMP7 in the developing kidney (Oxburgh *et al*., 2005). Wnt9b and Wnt4 are required for the initiation and maintenance of epithelial differentiation, respectively, of cap mesenchyme cells (Carroll *et al*., 2005; Kispert *et al*., 1998; Stark *et al*., 1994).

Together these data suggest a number of possible models for Cited1 activity in the condensed mesenchyme (Fig. 4I). If the primary function of Cited1 in the MM is to mediate BMP-dependent responses, it could act downstream of BMP7 or BMP4 to promote maintenance or proliferation of cap cells. Alternatively, Cited1 could act as a Wnt antagonist in the MM, repressing target genes until they are needed for formation of the PTA. Downregulation of Cited1 during cap mesenchyme transition would alleviate this repression in those cells forming the PTA and allow expression of genes needed for epithelialization. This could explain the reduced nephron induction when Cited1 is overexpressed in cultured kidneys. Furthermore, Cited1 could function in both of these pathways simultaneously, making it tempting to speculate that Cited1 acts a 'gatekeeper' in the MM. In this model, downregulation of Cited1 would be a transcriptional switch that allowed for conversion of cap cells into the PTA, shutting off BMP responses important for maintenance, and de-repressing WNT targets needed for differentiation. This hypothesis is reminiscent of what has been observed in the maturing mammary gland, where it appears that Cited1 acts downstream of TGFβ and estrogen signaling simultaneously (Howlin *et al*., 2006).

While this study certainly suggests a role for Cited1 in kidney development, it primarily uses *in vitro* methods to addresses what Cited1 can do in this context. This is different from understanding what Cited1 actually does in the MM. This question forms the basis of the work presented here. The primary aim of my project has been evaluate the expression and function of Cited1 *in vivo*, using knockout mice to evaluate nephrogenesis in its absence. This work has also led to the characterization of Cited2 and Cited4 during kidney development, as well as the creation of *Cited1/2* double mutant

animals. This work is detailed in Chapter 2. I have also used *in vitro* methods to evaluate the function of Cited1, and demonstrated a role in regulation of cell migration and adhesion, presented in Chapter 3. In Chapter 4 I will describe how we exploited the Cited1 expression domain to lineage trace cap mesenchyme cells and create a unique tool for conditional gene deletion in these cells. To conclude I will discuss the impact of my work on the field of kidney development and suggest future directions for the research presented here.

CHAPTER II

CITED FAMILY PROTEINS ARE DYNAMICALLY EXPRESSED IN THE DEVELOPING KIDNEY BUT ARE NOT REQUIRED FOR NEPHROGENESIS

Introduction

To address the function of Cited family proteins in the developing kidney, we mapped their expression during nephrogenesis and evaluated the consequences of germline deletion during this process. My studies initially focused on Cited1, as this was the factor identified in the original differential display screen (Plisov *et al*., 2000). We show that Cited1 expression is initiated in the MM after invasion of the UB and becomes restricted to the cap mesenchyme, those condensed cells directly opposed to UB tips. As cap cells begin to differentiate, Cited1 is downregulated in the PTA, absent in the RV, and is not expressed in any differentiated elements of the kidney. Despite its dynamic expression pattern, we show that deletion of *Cited1* does not disrupt nephrogenesis. We hypothesized that this was due to functional redundancy through other members of this gene family. Indeed, *Cited2* and *Cited4* are also expressed in the developing kidney. *Cited2* expression overlaps with Cited1 in the condensed MM, but persists in differentiating epithelial structures, including glomeruli. Conversely, *Cited4* is expressed only in the UB, most prominently in the tips. Because of their overlapping expression patterns we focused on Cited2 as a candidate for redundancy.

To test our hypothesis, we evaluated kidney development in *Cited2* null and *Cited1/2* compound mutant animals. Deletion of *Cited2* had a modest effect on UB branching and the formation of new epithelia, but did not alter the overall growth of

cultured kidneys. Investigating kidney development is *Cited1/2* compound mutants was problematic due to a hastening of embryonic lethality observed in *Cited2* null mice. In those embryos that did survive until the onset of kidney development we did not observe major perturbations in UB branching or differentiation of new epithelia.

Expression and function of Cited4 in the developing kidney has not been previously addressed. We show that Cited4 is expressed early in the ND and later in the tips of the UB, directly opposed to Cited1 expressing cells. Like *Cited1* and *Cited2*, deletion of *Cited4* does not disrupt nephrogenesis. Based on these studies we conclude that Cited family proteins are differentially expressed in the developing kidney, but are not required for nephrogenesis.

Experimental Procedures

Animals

Generation of *Cited1*^{LacZ} (Howlin *et al*., 2006; Sado *et al*., 2000), *Cited2*$^{+/-}$ (Bamforth *et al*., 2001) and *Cited2*^{LacZ} (Barbera *et al*., 2002) mice has been previously described. *Cited4*^{LacZ} mice were made in the Toshi Shioda lab (MGH) in a manner similar to the *Cited1*^{LacZ} animals, with a β-galactosidase/neomycin cassette replacing the open reading frame (Figure 13A). Mice were genotyped using the following primers.

Cited1^{WT}
5' - TTA CTT GCA GAC CAA CAG GC
3'- TGC TTC TTT GAC CCA TTT CC

Cited1^{LacZ}
5' - TTA CTT GCA GAC CAA CAG GC
3' - GCG AGT AAC AAC CCG TCG GAT TCT CCG TGG

Cited2^{WT}
5’ - AAA GGC GCT AAG GAT AGA CAC
3’ - ATA CTG AGG TCC CTG GCA C

Cited2^{Neo}
5’ - CTA CCC GGT AGA ATT GAC CTG
3’ - TGC TGT AAG ACC TTC TTG GC

Cited2^{LacZ}
5’ - TGG CGT TAC CCA ACT TAA TCG CCT TGC AGC
3’ - GCG AGT AAC AAC CCG TCG GAT TCT CCG TGG

Cited4^{WT}
5’- TCA AGG TAG TGT CTA GCC CA
3’- TGA GCT GTT GAG AGC CAC CA

Cited4^{LacZ}
F- CCG GGT GAG ACA GTG AAC GAA TCC GAT TTA TTC
R- GCG AGT AAC AAC CCG TCG GAT TCT CCG TGG

Cited1^{LacZ} mice are maintained on the 129/SvJ background. *Cited2*$^{+/-}$ and *Cited4*^{LacZ} mice are maintained on the C57Bl/6 background, and *Cited2*$^{+/LacZ}$ mice on a mixed background. Timed pregnancies were counted with the morning of vaginal plug appearance as day 0.5.

β-Gal staining

Whole embryos or kidneys were isolated in cold PBS and fixed in 0.4% glutaraldehyde in PBS with 2mM MgCl2 and 5mM EGTA. Fixation times were determined empirically and varied from 1 to 4 hours at 4°C according to age. For whole mount staining, embryos were equilibrated in β-Gal wash (0.1M Phosphate buffer pH 7.3 containing 2mM MgCl2, 5mM EGTA, 0.02%NP40, 0.01% NaDeoxycholate) and stained O/N at 37°C in β-Gal wash containing 1mg/ml X-Gal and 5mM K^+ ferro- and ferricyanate. Embryos were cleared for photography using a glycerol gradient in PBS.

For frozen sections, fixed tissues were cryoprotected in 30% sucrose in PBS o/n, sectioned at 10 μm, and stained as described above. Sections were counterstained with eosin, dehydrated and mounted.

Immunohistochemistry

Paraffin embedded tissues were sectioned at 6μm, and subject to antigen retrieval using buffered citrate. After allowing slides to cool, tissue was blocked in 10% goat serum in PBS for 1 hour. Sections were incubated with 1° antibodies o/n at 4°c. After washing 3 x 5 minutes in PBS, sections were incubated with 2° antibodies for 1 hour at r/t, washed again 3 x 5 minutes in PBS and mounted with Vectashield containing dapi (Vector Labs).

Antibodies

Polyclonal rabbit anti-Cited1 (NeoMarkers #RB-9219) was used at 1:250. Monoclonal mouse anti-ECadherin (BD biosciences cat # 610181) was used at 1:300. Polyclonal rabbit anti-calbindin D_{28k} (Calbiochem, #PC253L) was used at 1:1000. Polyclonal rabbit anti-Pax2 (Zymed/Invitrogen #71-6000) and WT1 (Santa cruz #SC192) were used at 1:100. HRP conjugated *Tetragonolobus purpureas* lectin, (Sigma L5759) was used at 0.3mg/ml. Conjugated secondary antibodies included horse anti-mouse fluoresine (Vector Labs #FI-2000) and goat anti-rabbit Rhodamine X (Jackson Immunoresearch #111-295-144) both used at 1:250.

Organ culture isolation and staining

Metanephric rudiments were isolated at E12, and grown for 5 days on clear 0.4 μm transwell filters (corning costar #3460) in DMEM containing 10% FBS. For immunostaining, filters were fixed in cold methanol for 10 minutes, and incubated with primary antibodies diluted in 5% goat serum in PBST (PBS + 0.1% Tween 20) o/n at 4°C. Filters were washed extensively the next day at room temperature in PBST, with the final wash extending o/n at 4°c. Cultures were incubated with 2° antibodies for 1 hour at R/T, washed 3 times 1 hour in PBST and mounted with vectashield (Vector labs).

Glomerular Counts

Glomeruli were counted as previously described (Godley *et al*., 1996). Briefly, individual kidneys were isolated from adult mice (> 8weeks of age), minced into 2mm cubes and incubated in 5 ml 6M HCl at 37°C for 90 minutes. Tissue was homogenized through repeated pipetting and 25 ml H_20 added. After incubation O/N at 4°C, glomeruli in 5 X 1 ml of this solution were counted in a 35mm counting dish. Total glomerular number per kidney was extrapolated mathematically from the mean of these five counts.

RT-PCR

RNA was extracted from E15.5 kidneys using absolutely RNA microprep kit (Stratagene #400805). RT reactions were carried out using 1μg total RNA and primed with random hexamers. PCR primer sets were designed to cross intron/exon boundaries except for *Cited4* which only has 1 exon) to distinguish between cDNA and genomic DNA in case of contamination. Annealing temperature was 60°C. Primer sets:

mCited1 F – GCA CTT GAT GTC AAG GGT GG
mCited1 R – GAG AGA CAG ATC CCG GAG AC
mCited2 F – GAG CAG AAA TCG CAA AGA CG
mCited2 R- TGT TGA GCT TCT GCA GCT CG
mCited4 F – TCA AGG TAG TGT CTA GCC CA
mCited4 R – TGA GCT GTT GAG AGC CAC CA

In situ hybridization

In situ hybridization for *Cited2* was preformed as previously described. (Dunwoodie *et al*., 1998; Hogan, 1994). Digoxigenein labeled riboprobes were generated from a plasmid containing full-length mouse *Cited2*. Tissue was treated with PK for 7 minutes, and probes were hybridized O/N at 55°C. After extensive washing tissues were incubated with AP-conjugated α digoxigenin antibody (roche # 1093274) at 1:1000, O/N at 4°C. Tissue was developed with BM purple AP substrate (Roche # 1442074).

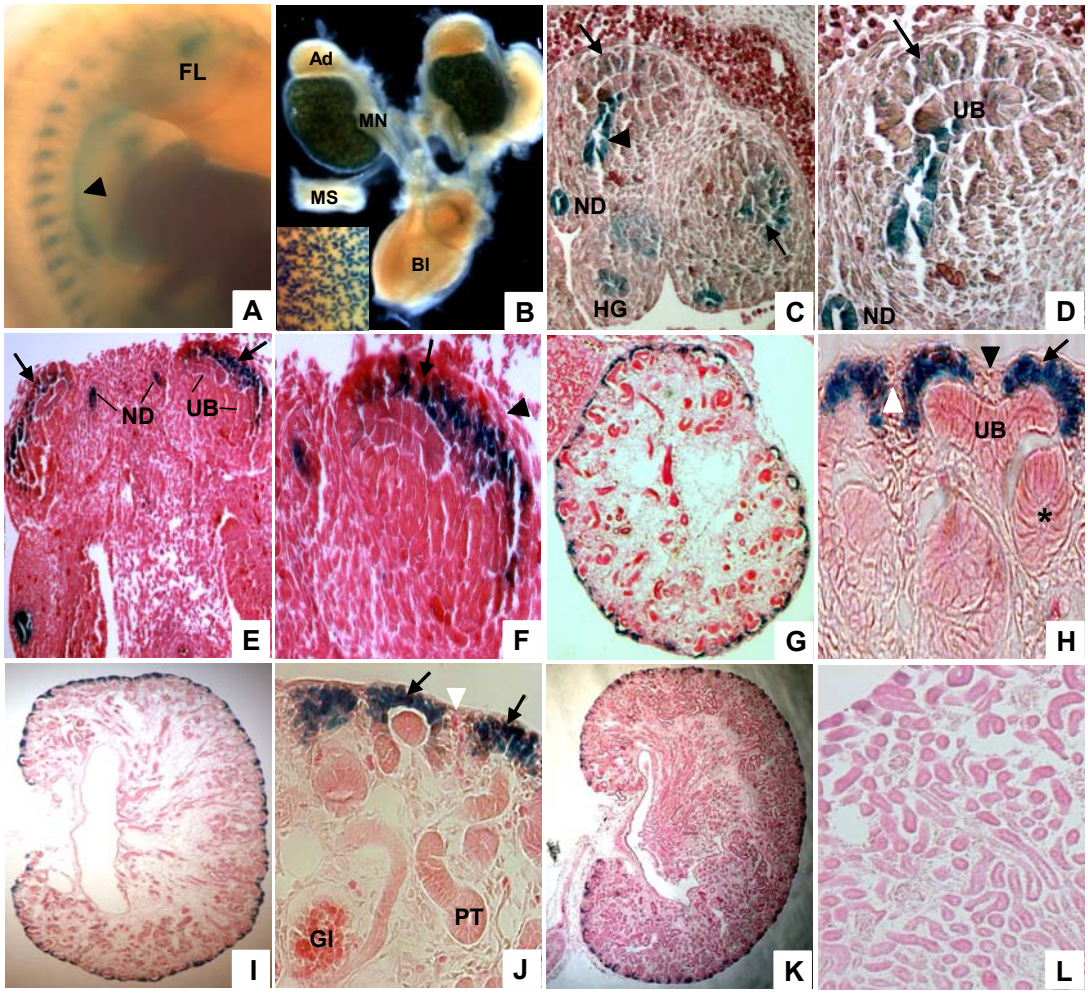
Results

Cited1 is expressed in the developing metanephric kidney

Using *Cited1*^{LacZ} animals we created an expression map for *Cited1* in the developing kidney by following β-galactosidase reporter activity (Fig. 5). Previous work has shown that *Cited1* mRNA is expressed in the ND at E10.5 (Dunwoodie *et al*., 1998). Whole mount analysis confirms expression in the ND at E11 (Fig. 5A) and indicates that as development of the UGS progresses, *Cited1* expression becomes restricted to the metanephric kidney (Fig. 5B).

During the initial stages of metanephric development *Cited1* is present in the stalk, but not the tip, of the UB (Fig. 5C,D). It is at this time that *Cited1* is first detected in the

Figure 5 – *Cited1^{LacZ} expression in the developing kidney*. Mapping of *Cited1* expression E11.0 – adult using *Cited1*^{LacZ} reporter mice. **A.** E11.0; whole mount staining. Arrowhead-nephric duct **B.** Whole mount of urogenital system from E15.5 embryo. *inset*- magnification of kidney surface in E17.5 whole mount preparation. **C,D**. E11.0; Low (C) and high (D) power images of UB invading the MM. Arrowhead-UB, Arrow - MM. **E,F.** E12.0; Low (E) and high (F) power images of metanephrogenic region; Arrow - condensing MM, Arrowhead-stromal MM. **G.** E15.5; Low power image of metanephric kidney. **H.** E15.5; High power image of branching UB and MM differentiation. Arrow - cap mesenchyme, White arrowhead - stromal mesenchyme, black arrowhead – condensed MM and stroma, Asterisk - RV. **I.** E17.5; Low power image of metanephric kidney. **J.** E17.5; Nephrogenic zone **K**. P0; metanephric kidney at birth. **L.** Adult; Low power image of cortical region. FL- forelimb, Ad- adrenal gland, MS- mesonephros, MN- metanephros, Bl- bladder, ND- nephric duct, HG- hindgut, MM- metanephric mesenchyme, UB - ureteric bud.


FL
A
Ad
MN
MS
Bl
B
ND
HG
C
UB
ND
D
ND
UB
E
F
G
UB
*
H
PT
Gl
J
I
K
L

It is at this time that *Cited1* is first detected in the MM where levels are low and its expression is patchy (Fig. 5C,D). As these cells condense around the branching UB, expression becomes more robust, and by E12 *Cited1* is expressed more widely in MM cells (Fig. 5E,F). Beginning at this early stage, its expression becomes limited to a subset of cells within the condensed MM that are most closely aggregated around UB tips, the so called 'cap' mesenchyme. The restriction of *Cited1* to the cap mesenchyme is striking, with an absence of expression in the condensed MM that lies in the cleft between UB tips (Fig. 5H) as well as the stroma. This pattern persists throughout nephrogenesis as the UB branches dichotomously and induces the formation of new nephrons (Fig. 5G-J). *Cited1* is not expressed in differentiated elements of the mature nephron or collecting system, and is absent in the adult kidney (Fig. 5I-L).

In order to verify the fidelity of the *Cited1*^{LacZ} reporter mouse and analyze Cited1 expression at the protein level, we characterized a commercially available anti-Cited1 polyclonal antibody (Neomarkers). The specificity of the antibody was confirmed by the complete lack of staining in *Cited1* null kidneys, despite persistence of cap mesenchyme cells (Fig. 6A, B). Using this antibody we mapped Cited1 protein expression over the same time course shown in the *Cited1*^{LacZ} studies. Fidelity of the reporter was confirmed and representative images are shown for E11 (Fig. 6C, compare to 5D) and E15.5 (Fig. 6D, compare to 5H). Like βgal reporter expression, Cited1 protein is restricted to the cap mesenchyme with no expression observed in the clefts between UB tips or in the surrounding stromal mesenchyme.

This antibody was also a useful tool to evaluate the subcellular localization of Cited1 in the cap mesenchyme, as well as its regulation as these cells undergo epithelial

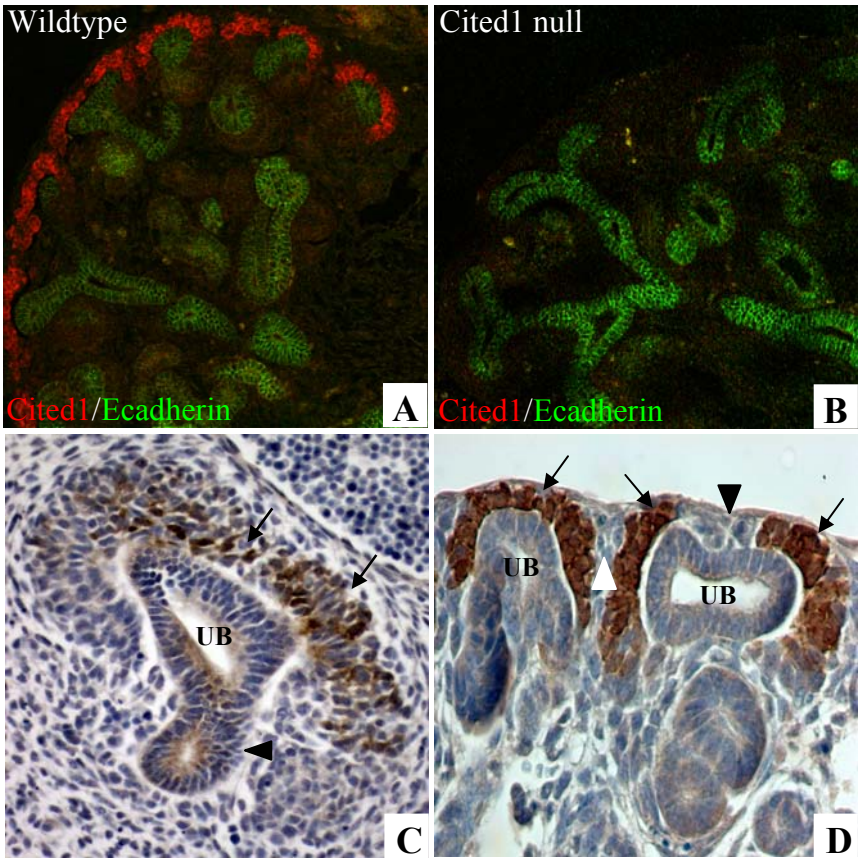


Figure 6 – *Expression of Cited1 protein in the developing kidney.* A,B. Characterization of anti-Cited1 antibody. Kidneys from E15.5 embryos colabeled with rabbit αCited1 (red) and mouse αECadherin (green). A- wildtype B- Cited1 null. **C, D**. Immnohistochemistry using rabbitαCited1 antibody; C- E11.0 Arrowhead-UB, arrow - MM. (compare to Fig 1D), D- E15.5 Arrow - cap mesenchyme, white arrowhead - stromal mesenchyme, black arrowhead – condensed MM and stroma (compare to Fig 1H).

`differentiation. In the cap mesenchyme, Cited1 is localized primarily in the cytoplasmic compartment (Fig. 7A, B). As these cells begin to differentiate they migrate around the tip of the UB as the PTA and form the renal vesicle RV, marked by expression of E-cadherin. Dual labeling demonstrates a clear gradient of expression during this transition, with Cited1 levels highest in the cap mesenchyme, reduced in the region of the PTA and completely absent in the RV (Fig. 7B; also see Fig. 5H,J; 6D). By staining sequential sections from E15.5 kidneys, we compared this expression pattern with that of two well characterized transcriptional regulators within the MM, Pax2 and WT1 (Dressler *et al*., 1990; Kreidberg *et al*., 1993; Torres *et al*., 1995). The expression patterns of Cited1 and Pax2 are largely overlapping in the condensed mesenchyme, however Pax2 is also expressed in the cleft between UB tips, as well as in the UB itself (Fig. 7C,D). While Cited1 is downregulated as cap cells begin to differentiate, Pax2 expression is robust in the forming RV. Similarly, expression of Cited1 coincides with that of WT1 in the cap mesenchyme, although WT1 expression increases as MM cells differentiate to form early epithelial structures. Furthermore, WT1 is present in glomerular podocytes, which do not express Cited1 (Fig. 7E,F).

Cited1 is not required for nephrogenesis

To evaluate the function of Cited1 in the developing kidney we analyzed *Cited1*$^{LacZ/LacZ}$ mice as this targeting strategy results in a complete loss of Cited1 protein (Howlin *et al*., 2006) (Fig. 6B). These studies were carried out using *Cited1* null mice on the 129Sv/J genetic background as deletion of *Cited1* on the C57Bl/6 background results in placental insufficiency and late gestation embryonic lethality (Rodriguez *et al*., 2004).

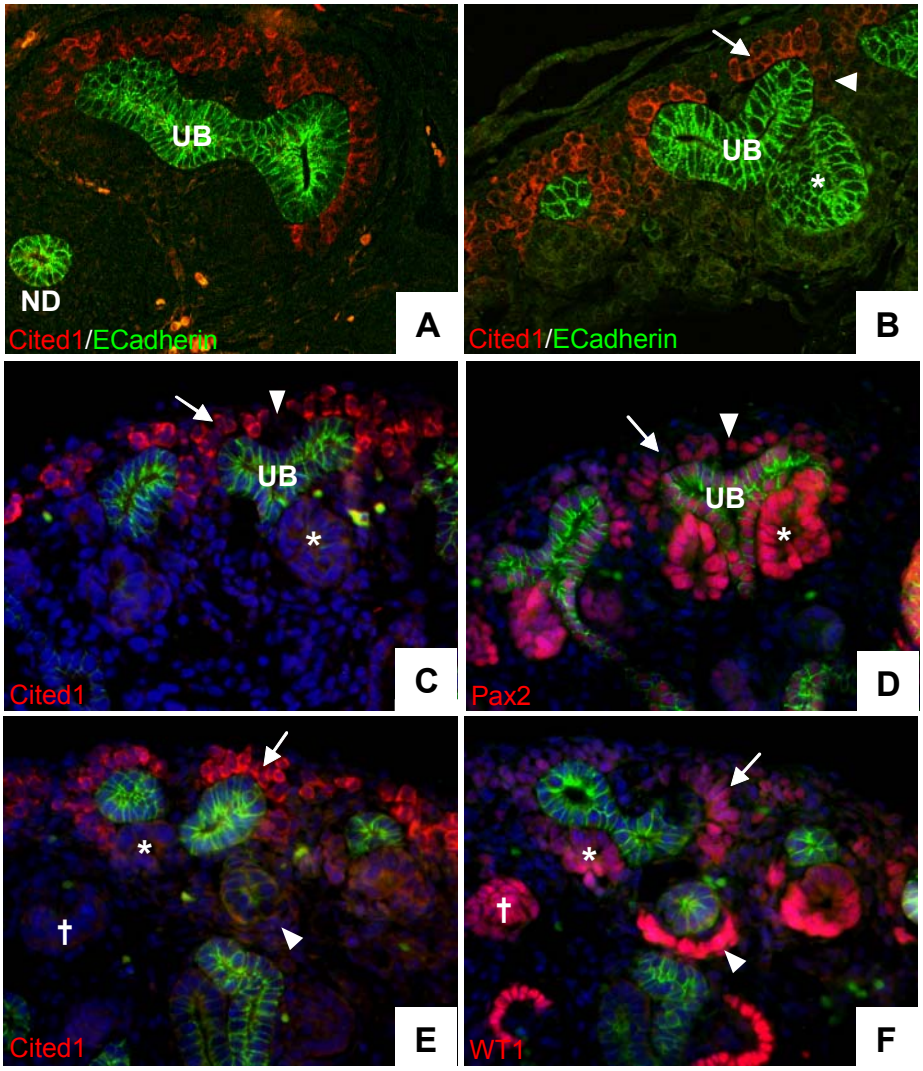


Figure 7 - *Cited1 is downregulated as cap mesenchyme cells differentiate and is has an expression pattern distinct from Pax2 and WT1.* A,B. Colabeling for Cited1 (red) and E-Cadherin (green). A- E12.0 B- E15.5; Arrow - cap MM, Arrowhead- PTA , asterisk - RV. **C-F**. E15.5; sequential sections colabeled for either Cited1 (red, C,E), Pax2 (red, D), or WT1 (red, F) and E-cadherin (green). Dapi stains nuclei blue. C,D. Cited1 and Pax 2 sequential sections. Arrow - cap MM, asterisk - RV; arrowhead - stromal MM. E,F- Cited1 and WT1 sequential sections. Arrow - cap MM, Asterisk - RV Arrowhead – glomerular podocytes, Dagger – S-shaped body.

Cited1 null animals are born in the expected proportions and display no overt physical abnormalities (data not shown). Examination of the kidney, both during development and in the adult, reveals that deletion of *Cited1* does not disrupt proper organ formation (Fig. 8). *Tetragonolobus purpureas* lectin staining demonstrates that proximal tubules form normally in *Cited1* null mice and in numbers comparable to wild type (Fig. 8C-F, data not shown). Formation of glomeruli is also unaffected in these animals. As a measure of total nephrogenic capacity, we counted glomeruli in adult wildtype and *Cited1* null animals. No reduction in total glomerular number was observed in the absence of *Cited1* (Table 2).

Cited1 and *Cited2* expression overlaps in the condensed MM

It is possible that the lack of a kidney phenotype in the *Cited1* knockout animal is the result of functional redundancy through other members of this gene family. RT-PCR using total RNA from E15.5 kidneys demonstrates that *Cited2* and *Cited4* are also expressed in the developing kidney (Fig. 9A). Preliminary results indicated that *Cited2* is expressed in the MM, while *Cited4* is expressed exclusively in the UB. As a result, we focused on Cited2 as our primary candidate for redundancy. The localization and analysis of *Cited4* during kidney development will be discussed later in this chapter. Using *in situ* hybridization and *Cited2*$^{LacZ/+}$ reporter mice, we asked to what extent Cited1 and Cited2 expression overlapped in the developing kidney. Whole mount analysis at E12.5 and E15.5 demonstrate that like *Cited1*, *Cited2* is also expressed in the condensed MM, displaying the characteristic arc shape which marks this cell population. (Fig. 9B-E). Analysis of sections from E15.5 kidneys by *in situ* hybridization and β-Gal

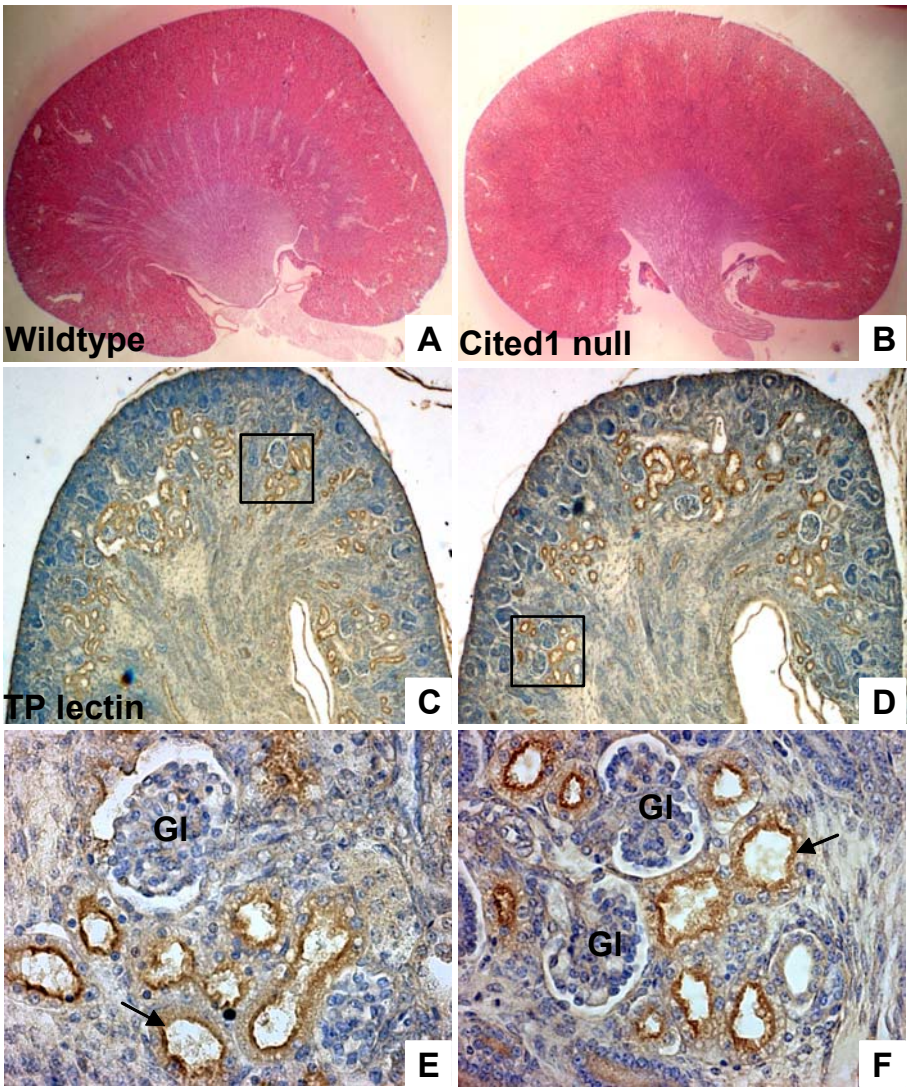
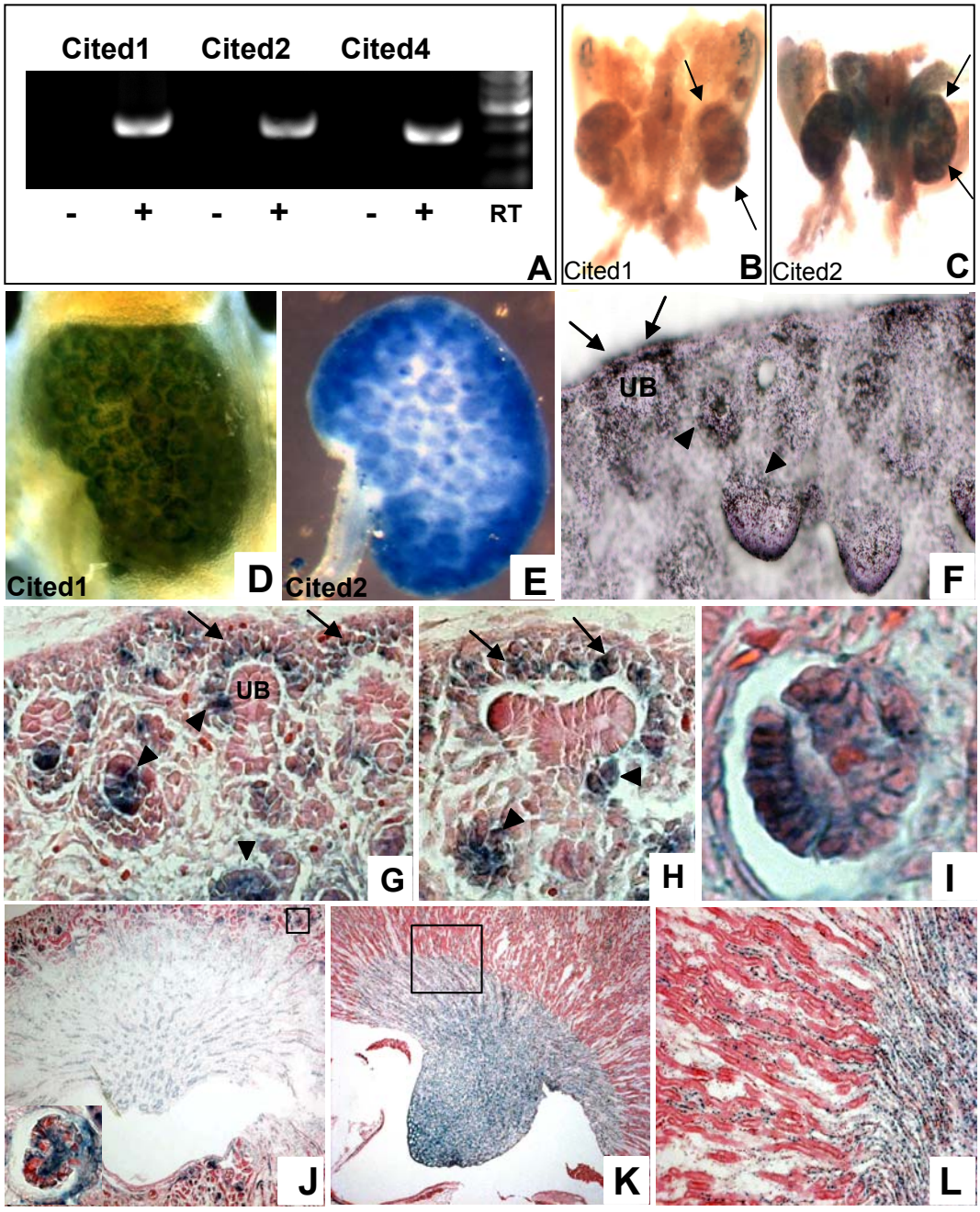


Figure 8 – ***Deletion of Cited1 does not disrupt nephrogenesis.*** **A,B.** Low power H&E stained section from adult wildtype (A) and *Cited1*$^{LacZ/LacZ}$ (B) mice. **C-F**. *Teragonoglobus purpureas* lectin staining of proximal tubules (arrow) in E18.5 wildtype (C,E) and *Cited1*$^{LacZ/LacZ}$ (D,F) kidneys. Boxes denote area of magnification. Gl- glomeruli

Figure 9. ***Cited2 expression in the developing and adult kidney.*** **A.** RT PCR using total RNA isolated from E15.5 kidneys. **B, C.** Whole mount LacZ stained E12.5 UGS from *Cited1*$^{LacZ/LacZ}$ (B) and *Cited2*$^{LacZ/+}$ mice. Arrow – condensed mesenchyme. **D.** Whole mount LacZ stained E15.5 kidney from *Cited1*$^{LacZ/LacZ}$ mouse. **E.** Whole mount *Cited2 in situ* hybridization of E15.5 kidney. **F.** *In situ* hybridization for *Cited2* on E15.5 kidney section. Arrow – condensed mesenchyme, Arrowhead – primitive nephrons. **G.** Section of E15.5 *Cited2*^{LacZ} reporter line; Arrow – condensed mesenchyme, Arrowhead – differentiating epithelial structures. **H.** High power images of individual branched UB tip in *Cited2*^{LacZ} reporter line. **I.** *Cited2*^{LacZ} expression in primitive glomeruli. **J.** *Cited2*^{LacZ} expression in the newborn papilla and glomeruli. **K.** *Cited2*^{LacZ} expression in the adult kidney. **L.** High power image of designated area in (K).


Cited1
Cited2
Cited4
- + - + - + RT
A
Cited1
B
Cited2
C
Cited1
D
Cited2
E
UB
F
UB
G
H
I
J
K
L

staining shows *Cited2* localized to the condensed mesenchyme, but also persisting in differentiated epithelial structures and glomeruli (Fig. 9 F-I).

The late stages (E17.5 onward) of kidney development are characterized by the patterning of the organ into distinct cortical and medullary elements. The most inner part of the medulla is termed the papilla and is the home to the UB derived collecting system as well as distal nephron segments, such as the loop of Henle. During this stage *Cited2* is upregulated in the forming papilla (Fig. 9J) In addition to its expression during development, Cited2 is also present in the adult kidney. In the adult, *Cited2*^{LacZ} is detected in tubular elements of nephrons in the cortical region positionally corresponding to the S3 segment of proximal tubules (data not shown). Its most striking expression, however is in the papilla where it appears to be expressed in all cell types (Fig. 9K,L).

Deletion of *Cited2* does not disrupt branching morphogenesis or induction of new nephronic epithelia

Based on its overlapping expression pattern with Cited1, we focused on Cited2 as a candidate for redundant function in the cap mesenchyme. The role of Cited2 during kidney development has not been previously evaluated. *Cited2* null mice die *in utero* beginning at e13.5 due to cardiac defects (Bamforth *et al*., 2001; Weninger *et al*., 2005), complicating embryonic studies and making analysis of adult kidneys impossible. Because of this we utilized an *ex vivo* system in which metanephric rudiments were isolated at E12 and grown in culture for a period of 5 days. Dual labeling with the UB marker calbindinD_{28K} (red) and the pan-epithelial marker E-Cadherin (green) allows us to evaluate branching of the UB and the induction of nephronic epithelia. When these images are merged, branched UB tips appear yellow, expressing both markers, while

nephronic epithelial structures express only E-Cadherin and will be green. UB tips and new epithelial structures can then be quantified (Fig 10).

After 5 days in culture, there were no gross differences in overall morphology of metanephric cultures from wildtype, *Cited1* null or *Cited2* null animals (Fig. 11). Quantitative analysis revealed no difference in total number of UB tips or nephronic epithelial structures in *Cited1* null cultures as compared to wildtype (Table 2). Cultured kidneys from *Cited2* null mice displayed an 18% reduction in nephronic epithelia compared to wildtype (Table 2; mean of 43 per kidney compared to 52 in wildtype). While this effect is statistically significant, the overall growth and UB branching of the rudiment was not significantly impaired and the MM was able to undergo epithelialization.

Compound deletion of Cited1 and Cited2 does not disrupt inductive events in the developing kidney

Having established that Cited1 and Cited2 are not individually required for branching morphogenesis or nephron induction during kidney development, we generated compound mutant mice to test our hypothesis that these two proteins could substitute for one another in the MM. During the course of these studies, we were able to generate *Cited1*$^{LacZ/LacZ}$; *Cited2*$^{+/-}$ adult animals which had normal kidneys both in terms of morphology and glomerular numbers (data not shown, table 2). This indicates that if Cited2 compensates for loss of *Cited1* in the developing kidney, one copy is sufficient.

An organ culture system was again used to evaluate early nephrogenic events owing to the embryonic lethality of *Cited2* homozygous deletion. Generation of *Cited1/2* double homozygous null embryos for organ culture however, was problematic.

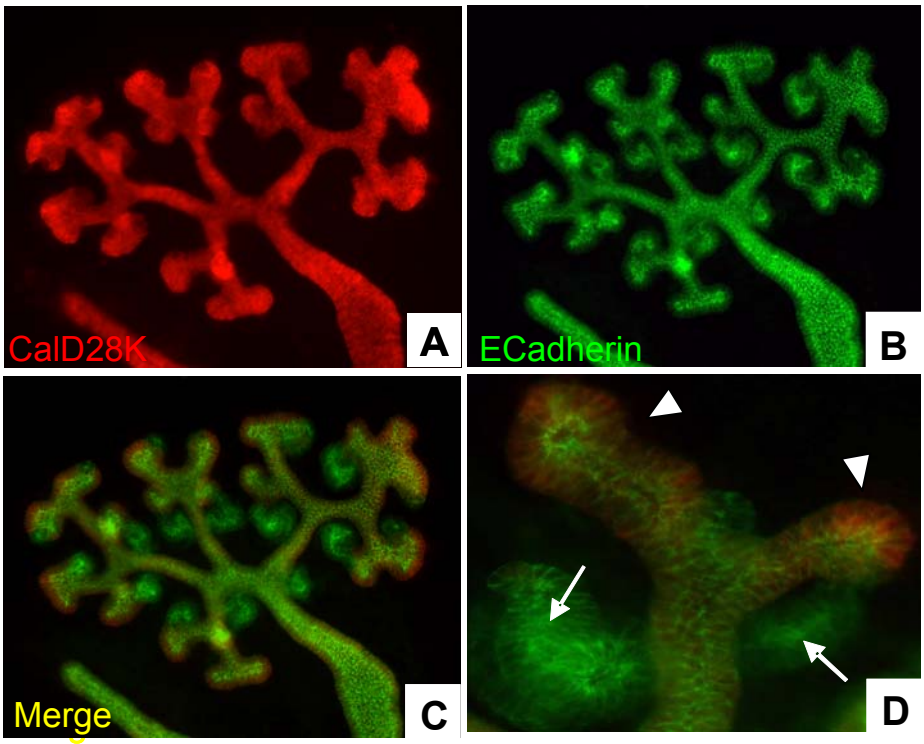


Figure 10– ***Method for analysis of UB branching and epithelial differentiation in cultured kidneys.*** **A.** CalD28K labels the ureteric tree **B**. E-Cadherin labels all epithelial cells. **C,D**. Merging images distinguishes UB tips and branches (double positive) from newly formed nephronic epithelia (E-cadheirn positive), allowing for evaluation and quantification of UB branching and formation of primitive nephrons. Arrow – nephronic epithelia; Arrowhead – UB tip.

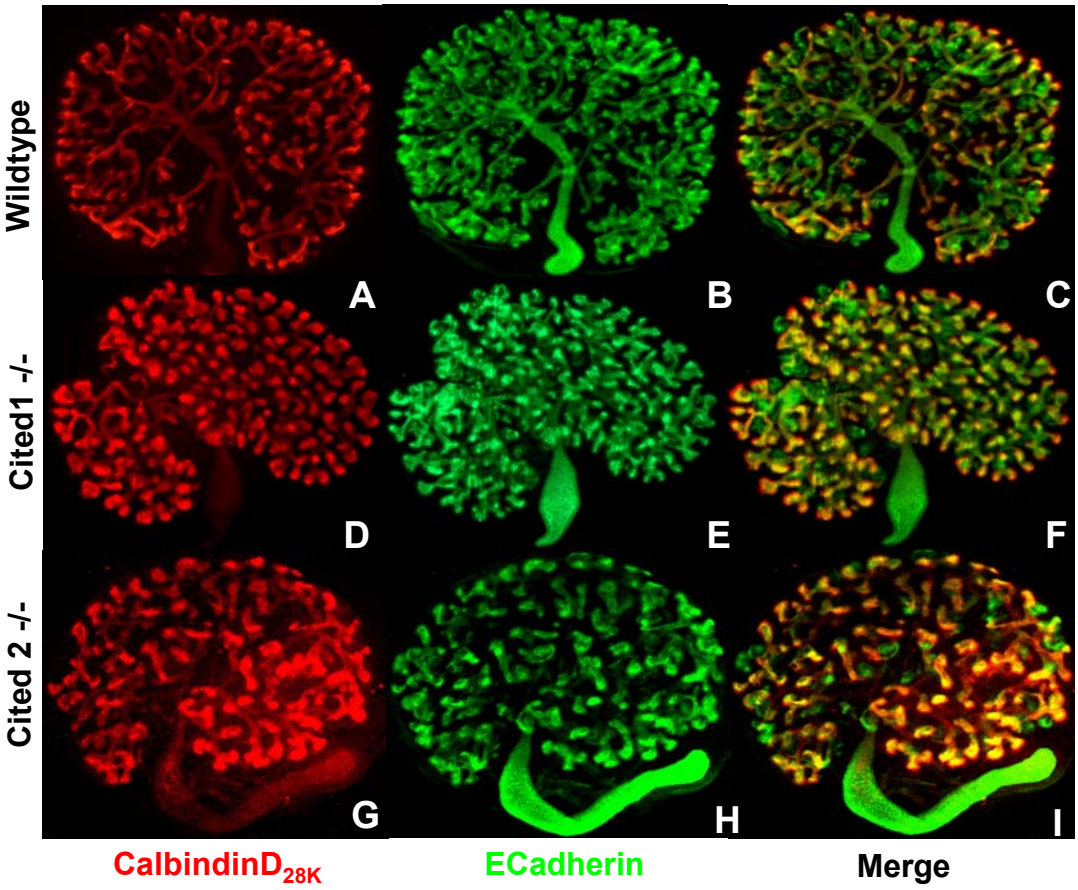


Figure 11 – ***Deletion of Cited2 does not perturb UB branching or induction of new nephronic epithelia.*** Representative images of 5 day metanephric organ cultures from WT **A-C**; *Cited1*$^{LacZ/LacZ}$ **D-F**; and *Cited2*$^{-/-}$ **G-I** mice. Left column stained with UB marker CalbindinD28K, middle column stained with pan-epithelial marker E-cadherin, right column merge of two images distinguishing UB epithelia from nephronic epithelia.

Mendelian analysis of our crosses (totaling 14 litters) dictates that of the 94 embryos recovered, 15 should be null for both *Cited1* and *Cited2*. We recovered, however, only 4 *Cited*1/2 double homozygous null animals that survived until E12 (Table 3). We were able to recover tissue from an additional 8 reabsorbed embryos, 7 of which were double null. This indicates that deleting both of these genes accelerates the embryonic lethality observed in *Cited2* null animals, increasing the proportion of animals which die prior to the onset of kidney development. Among those embryos that did reach E12 we did observe fewer branch tips and new epithelial structures in cultured kidneys (Fig. 12 G-I, table 2), though their overall morphology appears normal. We were not able to recover a sufficient number of $Cited1^{LacZ/LacZ}$//$Cited2^{-/-}$ animals to demonstrate this to be a statistically significant difference. On this basis we conclude that the key branching and inductive signaling events are intact in *Cited1/2* double null kidneys.

Table 2. ***Quantification of embryonic and adult kidney structures in wild type and CITED family mutant mice*****. * p < 0.01**

Genotype	n	UB Tips	Nephronic Epithelia	n	Adult Nephrons
WT	11	70 ± 9	52 ± 9	5	7251 ± 182
Cited1 $^{-/-}$	10	79 ± 7	51 ± 5	4	7192 ± 169
Cited2 $^{+/-}$	13	78 ± 5	52 ± 6	4	7228 ± 188
Cited2 $^{-/-}$	13	67 ± 12	43 ± 7 *	-	n/a
Cited1 $^{-/-}$; *Cited2* $^{+/-}$	12	72 ± 6	49 ± 5	4	6987 ± 141
Cited1 $^{-/-}$; *Cited2* $^{-/-}$	2	48 ± 6	38 ± 7	-	n/a

Table 3. ***Distribution of expected and observed survival to E12.5 for genotypes of interest during generation of Cited1/2 compound mutant mice***

Genotype	Expected	Recovered	Reabsorbed
WT	4-5	5	0
CITED1$^{-/-}$	15-16	17	0
CITED2$^{+/-}$	7-8	8	0
CITED2$^{-/-}$	3-4	5	1
CITED1-/- // CITED2 $^{+/-}$	31-32	37	0
CITED1-/- // CITED2$^{-/-}$	16-17	4	7

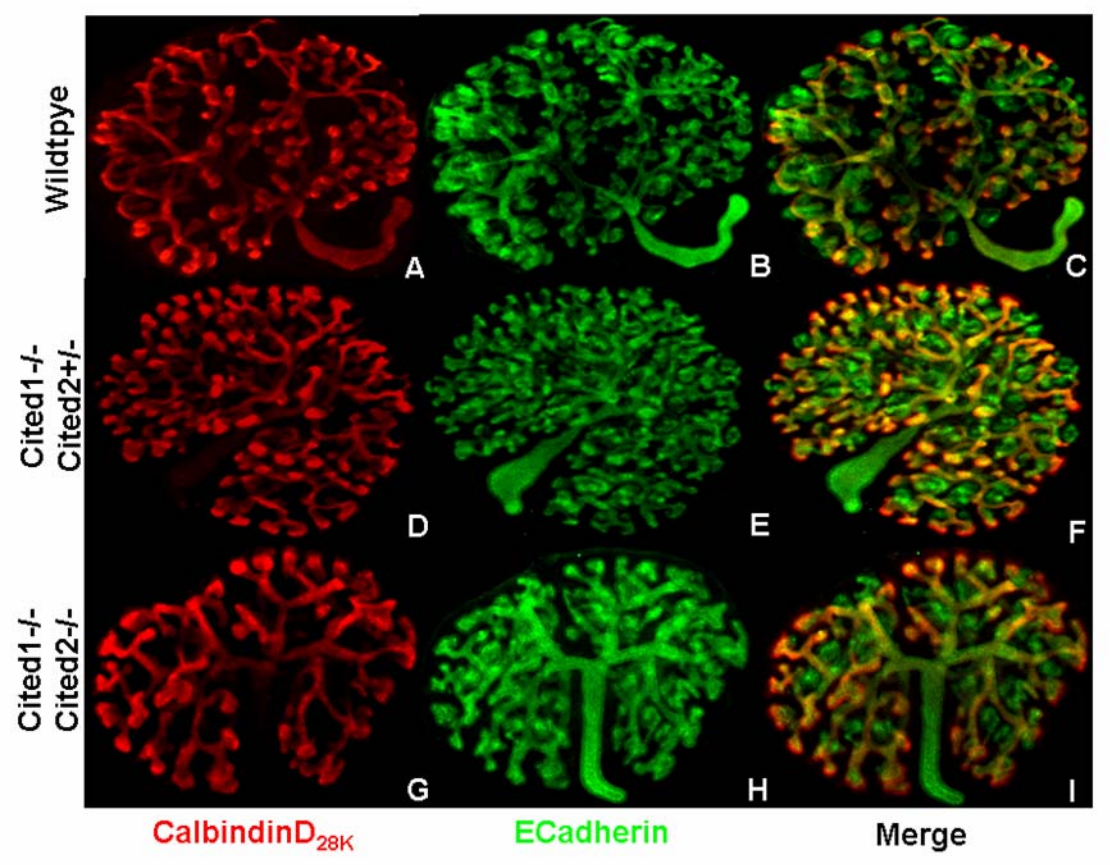


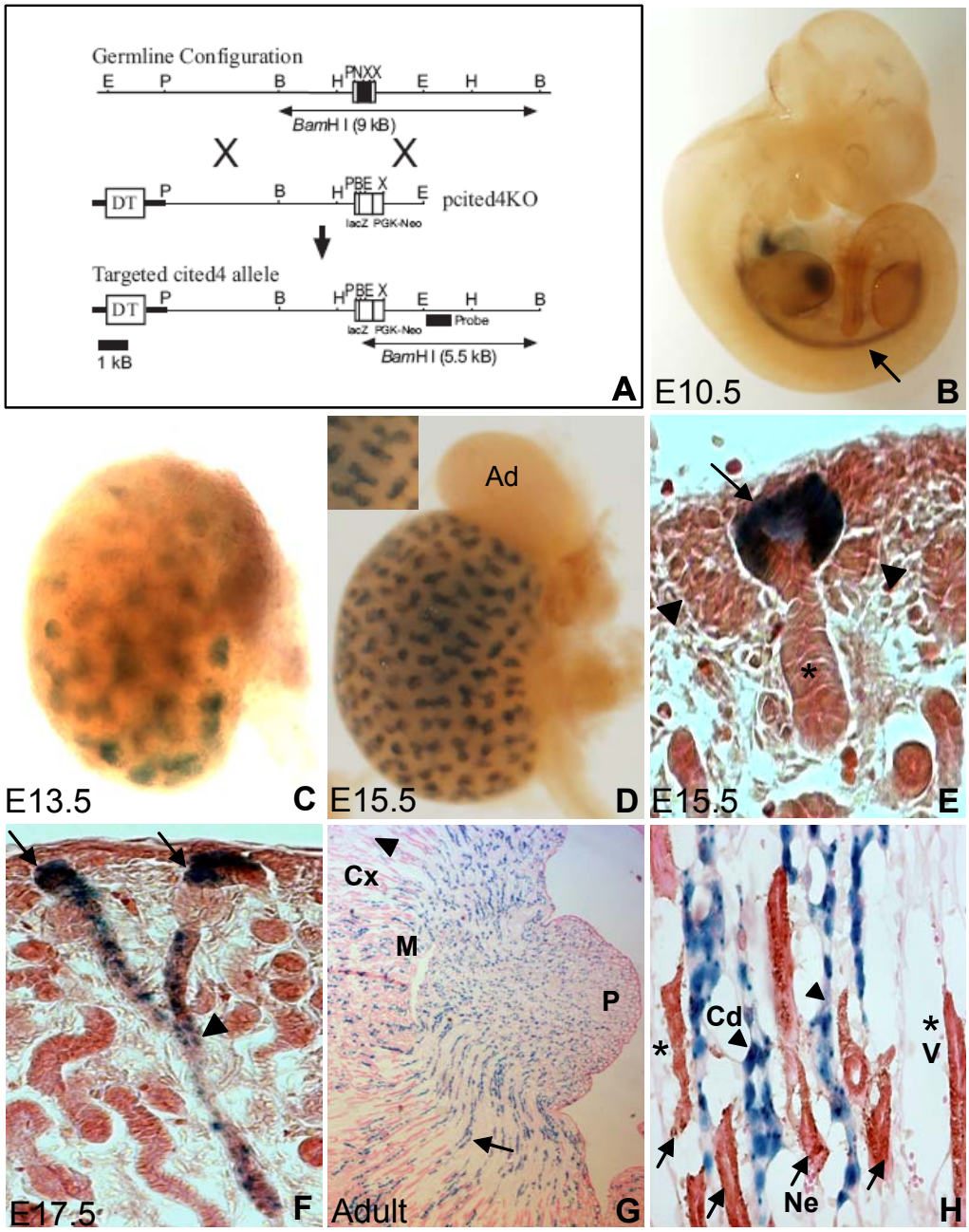
Figure 12 - ***Compound deletion of Cited1 and Cited2 does not perturb UB branching or induction of new nephronic epithelia***. Representative images of 5 day metanephric organ cultures from WT **A-C**; *Cited1*$^{LacZ/LacZ}$//*Cited2*$^{+/-}$ **D-F**; and *Cited1*^{LacZLacZ}//Cited2$^{-/-}$ **G-I** mice. Left column stained with UB marker CalbindinD28K, middle column stained with pan-epithelial marker E-cadherin, right column merge of two images differentiating UB epithelia from nephronic epithelia.

Localization and role of *Cited4* during kidney development

RT-PCR using RNA from E15.5 kidneys demonstrates that *Cited4* is also expressed during nephrogenesis (Fig. 9A). As previously discussed, preliminary results indicated that *Cited4* was not expressed in the MM, but rather in the UB (Fig. 13C). Because of this, our redundancy studies focused on Cited1 and Cited2 as they overlap in the MM. However, the expression pattern and role of Cited4 in kidney development has not been previously addressed. To evaluate Cited4 during nephrogenesis we used *Cited4*^{LacZ} reporter mice (Fig. 13A). With this line, we mapped the expression of *Cited4* in the developing and adult kidney and asked whether deletion of *Cited4* perturbed nephrogenesis. The results of these studies are presented below.

At E10.5, prior to outgrowth of the UB, *Cited4*^{LacZ} is detected in the ND (Fig 13B). It is not expressed in the initial outgrowth of the UB, but rather is upregulated in these cells after early branching events have occurred, and is clearly evident by E13.5 (data not shown, Fig 13C). Analysis of whole mount and sections from E15.5 kidneys shows that *Cited4* is localized primarily to the tips of the UB, in a pattern reciprocal to that of *Cited1* (Fig. 13D,E). By E17.5 there is more staining in UB stalks, though not in all cells, as they elongate into the medulla en route to forming the collecting system (Fig. 13F). *Cited4* expression is retained in the collecting system of the adult kidney (Fig. 13G). Using structural and marker analysis, we asked what cell type(s) is/are expressing Cited4 in the adult. *Cited4*^{LacZ} positive structures do not coincide with Na/Katpse staining nephronic tubular elements. In these sections blood vessels are identifiable by red blood cells in the lumen; we do not see *Cited4*^{LacZ} activity in these structures either (Fig. 13H).

Figure 13. ***Cited4 expression in the developing and adult kidney.*** **A.** Targeting strategy for creation of *Cited4*^{LacZ} reporter mice. **B.** E10.5 whole mount embryo; arrow- nephric duct. **C.** E13.5 whole mount kidney. **D.** E15.5 whole mount kidney. Inset- high power image showing branched UB tips. **E.** Section of E15.5 kidney; arrow- UB tip, arrowhead- condensed MM, asterisk- UB stalk. **F.** Section of E17.5 kidney; arrow- UB tip, arrowhead – UB stalk. **G.** Adult kidney. arrow- *Cited4* positive cells in medulla, entering papilla; arrowhead- Cited4 negative cells in cortex. **H.** LacZ stained adult kidney colabeld with αNa/Katpase. Arrow- Na/Katpase positive cells; arrowhead- Cited4 positive cells; asterisk- blood vessel. Ad-adrenal gland, Cd-collecting duct, Cx-cortex, M-medulla, Ne-nephron, P-papilla, V-vessel.


Germline Configuration
E P B H PNXX E H B
BamH I (9 kB)
X X
DT P B H PBE X E pcited4KO
lacZ PGK-Neo
Targeted cited4 allele
DT P B H PBE X E H B
lacZ PGK-Neo Probe
1 kB
BamH I (5.5 kB)
A
E10.5
B
E13.5
C
Ad
E15.5
D
E15.5
E
E17.5
F
Cx
M
P
Adult
G
Cd
V
Ne
H

This indicates that *Cited4* is likely confined to the collecting system, a pattern that may be expected based on its expression in the developing UB.

The *Cited4*^{LacZ} reporter insertion deletes the entire ORF of *Cited4*, and can be bred to homozygosity, producing null animals. *Cited4*$^{LacZ/LacZ}$ mice are viable and fertile, indicating that Cited4 is not required for normal development. Kidneys from these mice display no gross morphological defects and contain appropriate numbers of glomeruli (Fig 14G,H; able 3). During the course of our Cited1 and 2 organ culture studies we evaluated *Cited4* null kidneys for their ability to branch and induce nephronic epithelia *in vitro*. As expected (based on the normal morphology of kidneys in adult *Cited4* null animals) UB branching and epithelial differentiation were not perturbed in these cultures (Fig. 14A-F).

Discussion

In these studies we have evaluated the expression and role of the Cited family of transcriptional co-factors during kidney development in the mouse. We have shown that *Cited1* expression is initiated in the MM at E11 and is subsequently restricted to a subset of cells in the condensed mesenchyme closely aggregated around UB tips. Its expression is downregulated as these cells undergo epithelial conversion, and is absent in all differentiated structures. Despite its dynamic expression pattern, deletion of *Cited1* does not disrupt kidney development. We hypothesized that this was due to functional redundancy through other Cited family members. Indeed, *Cited2* and *Cited4* are also expressed in the developing kidney. *Cited2* is expressed in the MM, though unlike *Cited1* its expression persists in differentiated epithelial elements. In contrast, *Cited4* is

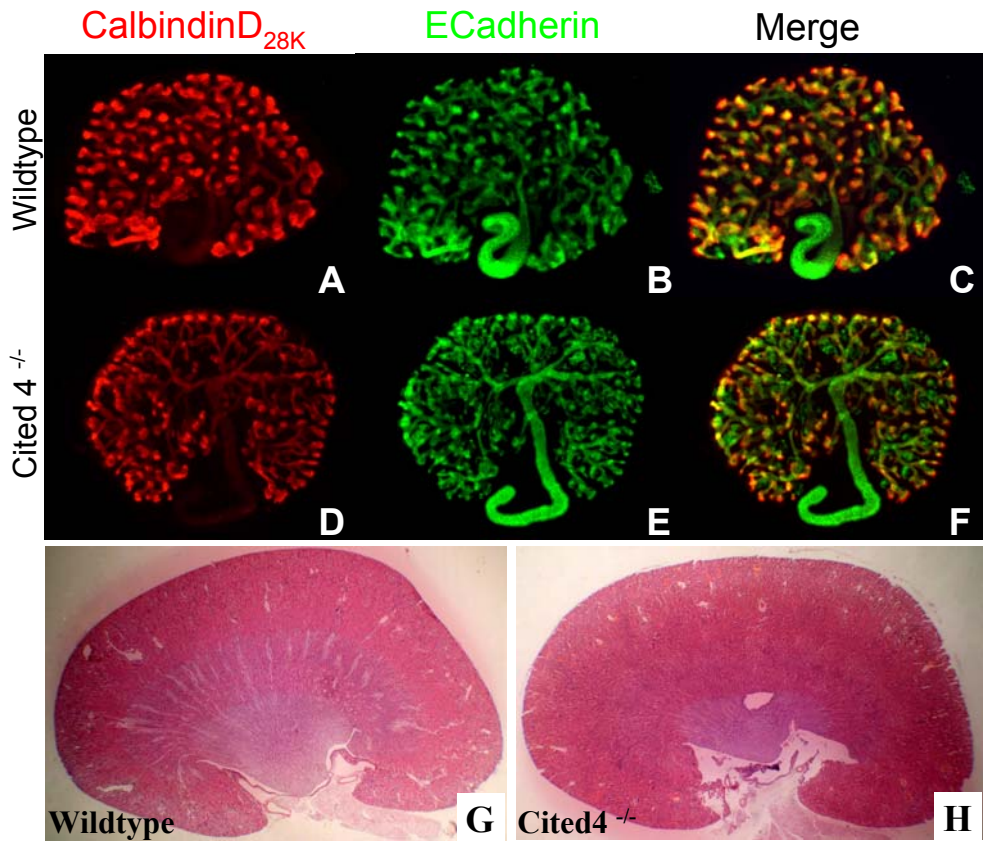


Figure 14. ***Deletion of Cited4 does not disrupt UB branching or overall nephrogenic process.*** **A-F** – Representative images of e12.5 + 5 organ cultures stained with CalbindinD28K (A,D) and ECadherin (B,E). Respective merged images (F,G). Wildtype (A-C), *Cited4*$^{-/-}$ (D-F). **G,H.** Low power H&E stained adult kidneys from wildtype (G) or *Cited4*$^{-/-}$.mice.

expressed only in the tips of the UB, in those cells directly opposed to *Cited1* expressing cells. Owing to its overlapping expression pattern we focused on Cited2 as a candidate to compensate for loss of *Cited1*. Neither deletion of *Cited2* alone, nor in combination with *Cited1* overtly disrupted branching morphogenesis or induction of new epithelia in cultured kidneys. Furthermore, *Cited4* null mice are healthy and viable and display no overt defects in kidney development.

The striking expression pattern of Cited1 is unique among other characterized transcriptional regulators of kidney development. Unlike factors such as Pax2 (Dressler *et al*., 1990), WT1 (Kreidberg *et al*., 1993), and Six1 (Xu *et al*., 2003), Cited1 is not present in the MM prior to UB invasion. After invasion, expression in the MM is patchy, but strong in the nephric duct. Cited1 is not detected in UB structures after this point. As the UB undergoes initial branching events *Cited1* is upregulated in the cap mesenchyme. These are renal progenitor cells that are directly responding to inductive cues from the UB and will soon undergo epithelial differentiation en route to becoming nephrons. It is also thought that a group of cap cells are responsible for self-renewal of the MM, though this has not been definitively shown and no markers have been identified which distinguish this niche. Expression of Cited1 within the cap mesenchyme persists throughout nephrogenesis, and overlaps with that of Six2, which is expressed in the condensed mesenchyme and is downregulated as these cells undergo differentiation (Self *et al*., 2006). However, unlike Six2, Cited1 is restricted to the cap mesenchyme and is not expressed in cells in the cleft between UB tips.

Cited1 is downregulated as cap cells form the PTA and begin to undergo epithelial differentiation. This contrasts Pax2 and WT1 which are expressed in the MM,

but are upregulated as early epithelial structures form. Furthermore, WT1 is expressed in glomerular podocytes, both in the embryo and the adult. Like Pax2, Cited1 is not expressed in the adult kidney.

Our studies show that Cited1 protein is primarily localized within the cytoplasmic compartment of cells within the cap mesenchyme (Fig. 7A,B, 15A). This is consistent with data demonstrating the existence of a strong nuclear export signal in the C-terminal domain of Cited1 (Shi *et al*., 2006). As the primary role of Cited family proteins relates to their ability to interact with and modify CBP and p300-dependent transcriptional responses (Braganca *et al*., 2003; Braganca *et al*., 2002; Shioda *et al*., 1997; Yahata *et al*., 2001; Yahata *et al*., 2002), these findings suggest that nuclear export of Cited1 may serve to limit its transcriptional effects in the cap mesenchyme. We have observed context dependent regulation of Cited subcellular localization in many settings (Fig 15). Interestingly, Cited1 expression in Wilms' tumors, a pediatric malignancy of renal origin characterized by the persistence of undifferentiated MM cells (Rivera and Haber, 2005), is primarily confined to the nucleus of these cells (Fig 15B). This difference from normal nephrogenesis raises questions about the regulation and functional impact of changes in Cited1 localization during abnormal MM differentiation, as well as the possibility that Cited1 may serve a non-transcriptional function in the cytoplasm of cap mesenchyme cells. Furthermore, we see differential localization of Cited1 in other organs during embryogenesis. In the developing liver, Cited1 is detected in the cytoplasm of hepatoblasts (Fig. 15C). Cited1 is also expressed in the developing heart in trabeculae as they delaminate from the wall of the left ventricle (Fig. 15D) Interestingly, in cells that are closest to the wall Cited1 is predominantly in found the

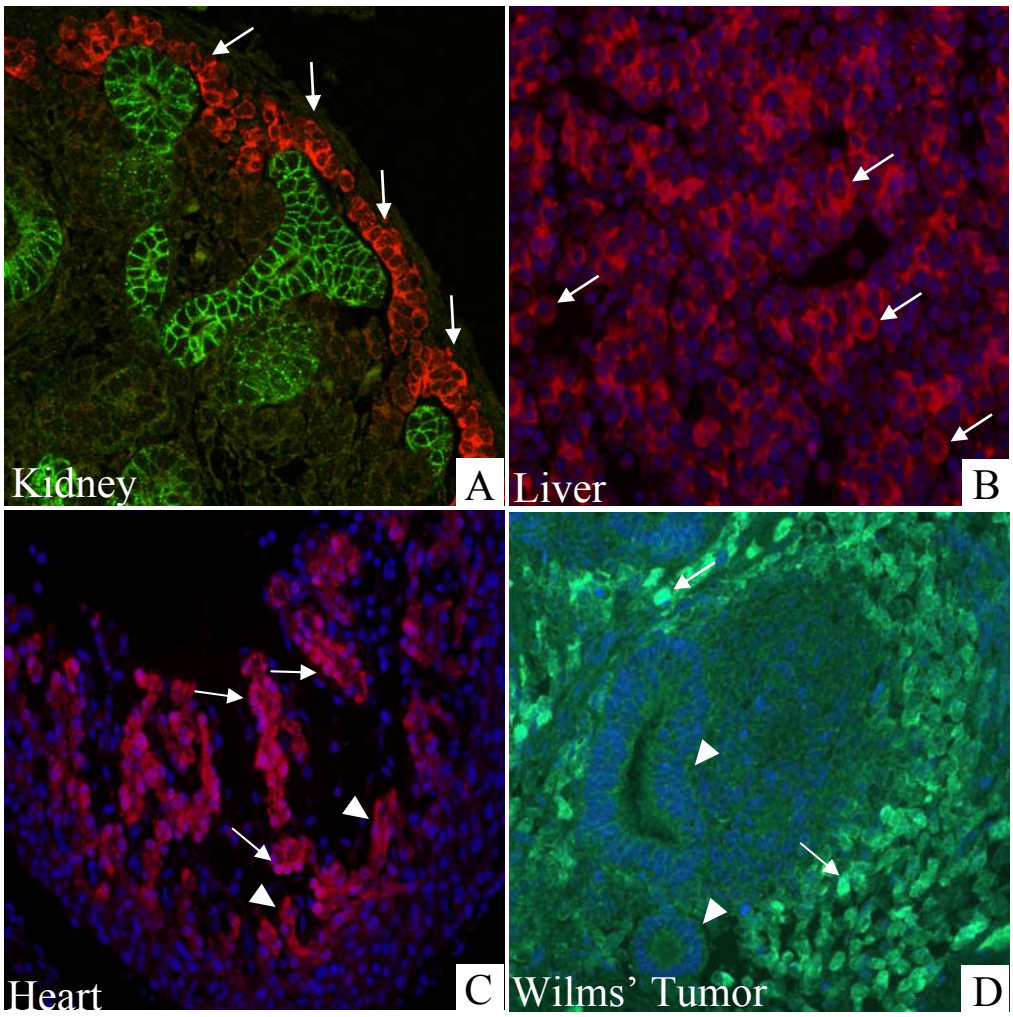


Figure 15. ***Context dependent subcellular localization of Cited1.*** Antibody staining using rabbit αCited1 antibody (red), where applicable, nuclei are labeled blue with dapi. **A.** E15.5 kidney; Arrows – cap mesenchyme cells with cytoplasmic Cited1. Epithelial cells labeled green with ECadherin. **B.** E12.5 Liver. Arrows – examples of hepatoblasts with cytoplasmic localization of Cited1. **C.** E12.5 heart; Arrow – Trabecular cell with nuclear localized Cited1, Arrowhead – cells near the muscle wall with cytoplasmic Cited1. **D.** Human Wilms' tumor; Arrow – example of blastemal cells with nuclear Cited1, Arrowhead – epithelial tubules.

cytoplasm, while in cells which have moved out into the trabeculae, Cited1 has shifted to the nucleus. Together, these data raise questions about the context dependent regulation of Cited1 localization and the role this plays in its function.

We relied on *in situ* hybridization and *Cited2* and *Cited4* LacZ reporter mice to analyze gene expression during kidney development as we were unable to detect Cited2 and Cited4 in the developing kidney using antibody staining (despite published reports using the same antibodies in different tissues; (Bhattacharya et al., 1999; Yahata et al., 2002). Results from these experiments show that *Cited1* and *Cited2* expression overlaps in condensed MM cells, but that *Cited2* persists in early epithelial structures and is present in glomeruli, as previously reported (Takemoto *et al*., 2006). *Cited2* is also expressed in the adult kidney, most prominently in the papilla. Given the well characterized role of Cited2 as a mediator of hypoxic responses, this domain is not surprising; the papilla is known to be one of the most hypoxic regions in the body.

In contrast, *Cited4* is not present in the MM, but is expressed most prominently in the UB tips. This expression pattern is reminiscent of that of the tyrosine kinase receptor Ret, which is responsible for growth of the UB in response to MM derived signals (Pachnis *et al*., 1993; Schuchardt *et al*., 1996). *Cited4* expression is also maintained in the adult, where it is restricted to collecting ducts. This is an extension of *Cited4* expression during development as the UB gives rise to the collecting system. The function of Cited4 during development is unknown, however we show that homozygous null animals are viable and fertile. These mice have morphologically normal kidneys and no impairment in UB branching or nephron induction.

Based on these findings and the observation that *Cited4* expression did not overlap with that of *Cited1*, we went on to evaluate whether Cited2 might be compensating for loss of *Cited1* in the condensed MM of *Cited1* null mice. Initial studies evaluated *Cited2* null mutant mice. In examining cultured kidneys from *Cited2* null animals we did see a decrease in the formation of new epithelial structures, though the overall morphology of the kidney was not perturbed. This modest effect is likely due to a general underdevelopment of *Cited2* null animals resulting from the associated cardiac and/or placental defects at this stage of development (Bamforth *et al*., 2001; Weninger *et al*., 2005; Withington *et al*., 2006). Having established that *Cited2* null mice did not have a major defect in nephrogenesis, we went on to ascertain whether loss of *Cited2* expression in a *Cited1* null background would unmask a defect in nephrogenesis. We were only able to recover a small number of *Cited1/2* double null embryos during the course of our studies, as deletion of both genes resulted in earlier embryonic lethality than is observed in mice lacking only *Cited2* (Bamforth *et al*., 2001; Weninger *et al*., 2005) This effect may result from exacerbation of cardiac and/or placental defects in double null animals as *Cited1* is also expressed in the developing heart and placental trophoblasts (Dunwoodie *et al*., 1998; Rodriguez *et al*., 2004). However, despite some reduction in overall growth compared to wild type kidneys at the same gestational age, we did observe induction of nephronic epithelia and relatively normal UB branching in cultured metanephric kidneys that we were able to isolate from two of the *Cited1/2* double null embryos. Furthermore, we were able to generate *Cited1* null*/Cited2* heterozygote compound mutant mice and show that these had normal kidneys. Taken

together these findings indicate that Cited2 is not required for nephrogenesis, and that its expression is not required to compensate for loss of *Cited1* in the condensed mesenchyme.

In summary, these studies show that *Cited1* is not required for kidney development and that deletion of *Cited2* does not disrupt branching of the UB or the ability of the MM to undergo epithelial differentiation. Furthermore, while compound deletion of *Cited1* and *Cited2* hastens the onset of embryonic lethality observed in *Cited2* null mice, it does not interfere with inductive events in the kidney. Based on these findings we conclude that despite their dynamic expression patterns within the MM, both Cited1 and Cited2 are dispensable for nephrogenesis. These data have now been accepted for publication (Boyle *et al*., 2007).

CHAPTER III

CITED1 REGULATES CELL ADHESION TO THE EXTRA-CELLULAR MATRIX IN MCF7 CELLS

Introduction

Given the difficulties encountered in discerning the function of Cited1 *in vivo*, I have also used *in vitro* systems as an alternative approach to address this question. Ideally, this work would be carried out in a MM cell line, but such systems are notoriously difficult to develop and are inconsistent. A good example of this problem is work I did using a rat MM cell line. RIMM (rat inducible metanephric mesenchyme) cells are a conditionally immortalized cell line designed to undergo epithelial differentiation in response to UB derived factors (Levashova *et al*., 2003). These cells express low levels of endogenous Cited1 and we were able to create stable lines overexpressing full-length Cited1 as well as two deletion mutants. Despite encouraging preliminary data, however, we were unable to reproducibly induce RIMM cell differentiation. Following several other unsuccessful efforts to use these cells to address the function of Cited1, they were discarded as a model system.

Owing to these difficulties, we elected to use MCF7 cells to investigate the function of Cited1 *in vitro*. MCF7 cells are a well characterized (Jakowlew *et al*., 1997; Lynch *et al*., 2001; Tong *et al*., 2002) ductal breast cancer line which express high levels of Cited1, primarily in the cytoplasm. We feel this line is a logical choice for functional studies given the role of Cited1 in maturation of the mammary gland during puberty (see Chapter one; (Howlin et al., 2006).

Using an siRNA based approach, we knocked down Cited1 in MCF7 cells ($MCF7^{Cited1K/D}$) and found that this manipulation inhibits cell migration. We show that this defect is related to the inability of $MCF7^{Cited1K/D}$ cells to adhere to selective components of the extra cellular matrix (ECM). Cell adhesion is mediated through interactions between the ECM and integrin receptors expressed on the surface of the effector cell. We asked whether reduced adhesion of $MCF7^{CITED1K/D}$ cells was associated with lower levels of candidate integrin subunits. We did not observe changes in cell surface expression of β1, α1, or α6 integrin in $MCF7^{Cited1K/D}$ cells. Furthermore phosphorylation of focal adhesion kinase (FAK), a critical step of integrin signaling following stimulation by ECM components, is not altered in knockdown cells.

At this point, we do not understand the mechanism by which Cited1 regulates cell adhesion; however a clue may come from observation of the actin cytoskeleton during cell attachment. At early time points we observe a reduction in filopodia formed when $MCF7^{Cited1K/D}$ cells are plated on type I collagen. Together these data suggest a role for Cited1 in regulation of cell-ECM interactions. This is an interesting candidate function for Cited1 given the role of cell movement in cap mesenchyme differentiation. As cap cells form the PTA they move around the UB tip to the ventral aspect of the branch. Upon establishing early cell-cell and cell-matrix interactions here, PTA cells must move inward together and organize to form the RV. The ability of these cells to execute this program is certainly mediated, at least in part, by the way they interact with the ECM environment in which they find themselves. It is well understood that differential expression of ECM and integrin proteins plays an important role in kidney development (for review see (Kanwar et al., 2004), most prominently during formation of glomeruli

(Kreidberg, 2003). Little is known, however about how these programs regulate cell adhesion and movement of the cap mesenchyme as it undergoes epithelial differentiation.

Experimental Procedures

Cell culture and siRNA transfection

MCF7 cells were maintained in DMEM containing 10% FBS (Gibco). For knockdown of Cited1, cells were transfected with a pool of four RNA sequences designed to optimally target *Cited1* transcripts (Dharmacon M-016022-00) or Gapdh transcripts as a control (D-001140-01-05). Unless otherwise indicated, siRNA was introduced at a final concentration of 100 nM using Effectene (Qiagen) which allows for transfection in medium containing serum. Briefly, cells were plated in 6 well dishes at $2.25x10^5$ cells/well the night before transfection. The next afternoon, medium was removed and 1.6ml of fresh DMEM/10% FBS was added. RNA was prepared according to the Quiagen protocol and added in 400µl of medium for a total volume of 2ml. Cells were transfected O/N and washed gently two times with warm PBS before replacing the medium.

Western blot analysis

For analysis of Cited1 protein levels in $MC7F^{Cited1K/D}$ and $MCF7^{GapdhK/D}$ transfected cells, lysates were collected at indicated times after transfection in cold buffer containing 25mM HEPES, 1% Triton X-100, 5mM EDTA, 150mM NaCl, 1mM sodium orthovanadate, 20mM AEBSF, and 50mM NaF. 500ng of total protein was separated on

a 12% polyacrylimide gel and transferred to PVDF membrane (Biorad #1620177). Blots were blocked for 1 hour in 10% milk in TBST (TBS + 0.1% Tween-20) and probed with 2H6, a mouse monoclonal antibody specific for Cited1 (generated in the Toshi Shioda Lab, MGH). Antibody was diluted 1:4000 in 5% milk/TBST and incubated O/N. Blots were washed 3 times 10 minutes in TBST and incubated with HRP conjugated goat α mouse secondary antibody for 1 hour, R/T. After three more washes, blots were developed with Western Lighting Plus (PerkinElmer #NEL105), exposed for 1 minute and developed. For analysis of βActin as a loading control, blots were stripped with and reprobed with a mouse monoclonal βActin antibody (Sigma clone AC-76). For phopho-FAK assays, lysates were collected from $MCF7^{Gapdh}$ and $MCF7^{Cited1}$ cells which were; Attached (plated the night before the experiment); Suspended (attached cells trypsinized, washed twice in PBS and rotated at 37°C in SFM for 30 min.); and Reattached (suspended cells plated on Type I collagen (2.5μg/ml) and lysed 1 hr later).

Migration assays

To assess cell migratory ability we used a transwell system in which cells are added to the top of a suspended filter coated with ECM and assayed for their ability to migrate through it to the other side of the membrane. The day before the assay 8 micron polycarbonate transwells (Corning Costar 3422) were coated with growth factor reduced Matrigel (BD biosciences 354230) diluted 1:10 in PBS (~0.85mg/ml) for 1 hour at 37°C. MCF7Cited1K/D and MCF7GapdhK/D cells were trypsinized, quenched with soybean trypsin inhibitor (1mg/ml) and washed 2 times in serum free medium. $1x10^5$ cells were plated in triplicate on Matrigel coated wells in serum free medium and incubated for

times indicated. After aspirating medium, filters were washed 1 time in PBS and fixed for 30 minutes in 3.7% formaldehyde in PBS at R/T. After fixation filters were washed extensively with PBS and incubated with 2% crystal violet in 10% methanol O/N. The next day wells were soaked in PBS and cells were removed from the top of the membrane using cotton swabs. Migratory cells were visualized on the underside of the membrane and five high power fields were counted for each replicate. Two-tailed t-test analysis was applied to the means of these counts to demonstrate statistical significance between groups.

Adhesion assays

Round-bottom 96 well dishes were prepared by coating with 100 µl of ECM component of interest at indicated concentrations in PBS and incubated O/N at 4°C. The next day, wells were blocked with 2% BSA in PBS for one hour at 37°C. $MCF7^{GapdhK/D}$ control and $MCF7^{Cited1K/D}$ transfected cells were collected, washed two times with PBS, and plated in triplicate onto matrix coated or BSA-only control wells at $1x10^5$ cells in 100 µl serum-free medium. Cells were allowed to attach for 1 hour at 37°C, and washed with PBS until no cells remained in the BSA control wells. Remaining cells were fixed for 20 minutes in 4% PFA, and stained for 1 hour with crystal violet staining solution (1.5% crystal violet in 20% methanol). Cells were washed 5 times with 100 µl PBS and permeabilized with 5% acetic acid, O/N. OD 595 was used to assess cell adhesion.

Fluorescence Activated Cell Sorting (FACS)

$MCF7^{GapdhK/D}$ and $MCF7^{Cited1K/D}$ siRNA cells were trypsinized and quenched with 10% FBS in DMEM, counted and allowed to recover in an excess of growth medium at

37°C for 30 minutes. Cells were then washed in PBS + 2mM EDTA, spun down and resuspended in 100 µl of serum-free medium / $5x10^5$ cells for each labeling reaction. Primary antibodies were as follows: MαH β1 integrin monoclonal- (Chemicon clone 6S6, MAB2253Z); MαH α1 integrin (Santa Cruz # SC33631); MαH α6 (Pharminigen #555734). These were added at to suspended cells at 1:50 dilution and incubated at 4°C for 45 minutes with occasional mixing. Cells were washed 2 times with PBS/EDTA and resuspended in serum-free medium containing α mouse phycoerethrin (PE) (pharmagen) at a 1:50 dilution and incubated at 4°C for 20-30 minutes. After 2 washes in PBS/EDTA cells were fixed for 2 minutes in 1% formaldehyde, spun down and resuspended in 300 µl PBS for FACS analysis, carried out at the Vanderbilt VA flow cytometry core.

Immunofluorescense

For immunofluorescent analysis cells were prepared as described for adhesion assays and plated at $1.5x10^5$ cells/well on glass coverslips coated with 2.5 µg/ml Type I collagen which had been blocked with 2% BSA in PBS. After indicated times, cells were washed 2 times with warm (37°C) serum free medium and fixed in warm 4% paraformaldehyde (buffered in 10 mM PIPES; pH 7.1, 125 mM NaCl, 5 mM KCl, 1.1 mM NaH_2PO_4, 0.4 mM KH_2PO_4, 2 mM $MgCl_2$, 1 mM EGTA, and 5.5 mM glucose) for 15 minutes at R/T. After fixation cells were washed 2 times with PBS and permeabilized in 0.4% Trition X-100 in PBS for 10 minutes. Coverslips were blocked for 1 hour with 1% BSA in PBS and incubated with rabbit α Cited1 (1:200; Neomarkers) and/or FITC conjugated Phalloidin (1:500; Sigma P5282) for 1 hour at R/T. Cells labeled with Cited1 antibody were washed 3 X 5 minutes with PBS and incubated with G α Rabbit

Rhodamine X (Jackson Immunoresearch) secondary antibody. Cells were washed again as above and mounted with Vectashield containing DAPI.

Results and Discussion

Knockdown of Cited1 in MCF7 cells

MCF7 cells are a ductal breast cancer cell line which expresses high levels of Cited1 endogenously (Fig. 16A; (Shi et al., 2006). To determine if we could modulate Cited1 protein levels in these cells we transfected them with a pool of siRNA species designed to target Cited1 transcripts or control siRNA against Gapdh. In initial experiments we used a dose range of siRNA to determine the optimal concentration for efficient knockdown (Fig. 16A). 48 hours after transfection we see Gapdh siRNA (150nM) has no effect on levels of Cited1. In contrast, cells transfected with Cited1 siRNA demonstrate a dose dependent response to increasing concentrations of siRNA. Cited1 protein is markedly reduced at the lowest concentration of 50nM and is almost completely eliminated using 100nM. Increasing the concentration of siRNA to 150nM had no additional effect on knockdown efficiency, suggesting that there is a threshold to this effect. Using a different Cited1 antibody (rabbit polyclonal, Neomarkers) we looked at Cited1 protein in transfected cells by immunofluroesense (Fig. 16B). In control cells Cited1 levels were robust compared to a virtual absence of protein in cells transfected with Cited1 siRNA. Like expression in the cap mesenchyme, we see that Cited1 is primarily localized in the cytoplasm. Interestingly, Cited1 is not expressed in all MCF7 cells indicating that there is some heterogeneity in this population. These results

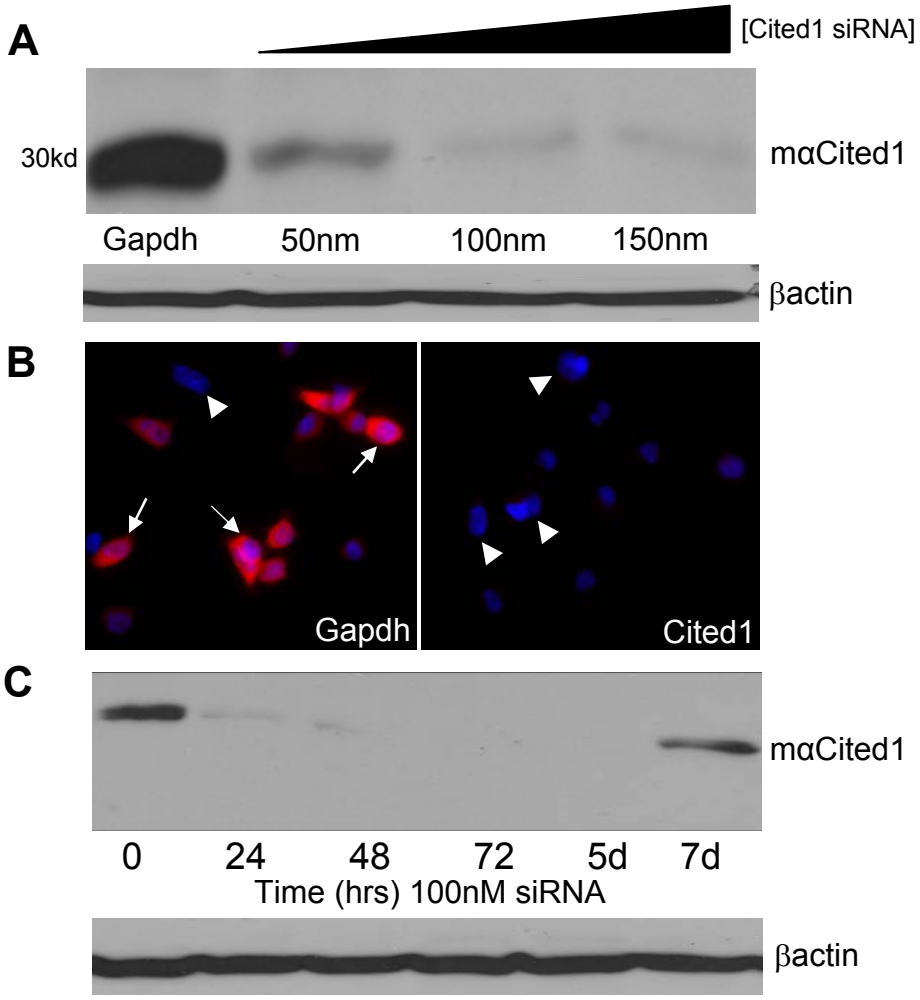


Figure 16. ***Knockdown of Cited1 in MCF7 cells.*** **A.** Western blot analysis using monoclonal αCited1 antibody (2H6) of Cited1 protein levels 48 hours after transfection of MCF7 cells with either Gapdh control siRNA or increasing concentrations of Cited1 siRNA. Membranes were stripped and reprobed with βactin antibody to verify equal protein loading. **B.** Immunofluorescense of $MCF7^{Gapdh}$ and $MCF7^{Cited1}$ cells using polyclonal αCited1 antibody. Nuclei are labeled blue with DAPI; Arrow – Cited1 positive cell, Arrowhead Cited1 negative cell. **C.** Time course of Cited1 protein levels following transfection with 100nM Cited1 siRNA.

corroborate those obtained by western blot and indicate that Cited1 protein expression can be virtually abolished in MCF7 cells using 100nM siRNA.

Because knockdown is accomplished with a transient transfection and siRNA efficacy is known to wane as cells divide, we followed Cited1 protein levels over a timecourse following transfection to determine its duration of effect (Fig. 16C). These studies indicate that maximum reduction of Cited1 is observed by 24 hours post transfection and persists for 5 days (approximately 3 doublings in our hands). After 7 days however, levels have returned to normal, suggesting that there is an absolute limit at which time the siRNA is no longer effective. Based on these results we carried out the remainder of the studies presented here 24 hours post-transfection using 100nM Cited1 or Gapdh siRNA.

Knockdown of Cited1 inhibits migration of MCF7 cells

After determining that Cited1 could be effectively knocked down in MCF7 cells we screened for changes in cell proliferation, apoptosis, and migration. These processes are known to be important for maintenance and differentiation of cap mesenchyme cells. No changes were observed in cell growth or survival (data not shown). Using a transwell system we asked weather knockdown of Cited1 altered the potential of MCF7 cells to migrate through a filter coated with Matrigel. Four hours after plating we recorded a 66% reduction in migration of $MCF7^{Cited1K/D}$ cells compared to control $MCF7^{Gapdh}$ cells Fig. 17A). Interestingly, when cells were allowed to migrate for 6 hours, control cells continued to move through the matrix while Cited1 knockdown cells did not (Fig. 17A).

This suggested to us that the reduced migration ability of MCF7$^{Cited1K/D}$ cells was due to a defect in the initial step of cells migration, cell adhesion.

MCF7$^{Cited1K/D}$ cells have a reduced capacity to bind selective ECM components

To test this hypothesis, we used adhesion assays to determine the capacity of MCF7$^{Cited1K/D}$ cells to bind different ECM substrates (Fig 17 B-F). These experiments we designed in a dose/response fashion to look at adhesion over a range of substrate concentrations and determine optimal conditions for MCF7 cell adhesion. Initial experiments were carried out using Matrigel given our observations that MCF7$^{CitedK/D}$ cells failed to migrate through this substrate. When plated on Matrigel, MCF7$^{Cited1K/D}$ cells have a reduced capacity to adhere compared to MCF7^{Gapdh} cells and this effect is observed over the entire range of Matrigel concentrations (Fig. 17B). These data support our hypothesis that MCF7$^{Cited1K/D}$ cells fail to migrate because of an inability to bind the ECM.

Matrigel is a heterogeneous mixture of ECM components, primarily composed of Laminin I and Type IV collagen. We therefore tested whether knockdown of Cited1 selectively altered the ability of MCF7 cells to bind these individual ECM substrates. Results of these studies show a marked decrease in MCF7$^{Cited1K/D}$ cell adhesion to high concentrations of Laminin I, but no effect on binding to Type IV collagen (Fig. 17C.D) We went on to test adhesion to two other substrates, Type I collagen and fibronectin, both of which are expressed in the MM. Furthermore, fibronectin is known to be important for branching morphogenesis in the developing kidney and salivary glands (Sakai *et al*., 2003; Ye *et al*., 2004). Like Matrigel, adhesion of MCF7$^{Cited1K/D}$ cells is reduced over the

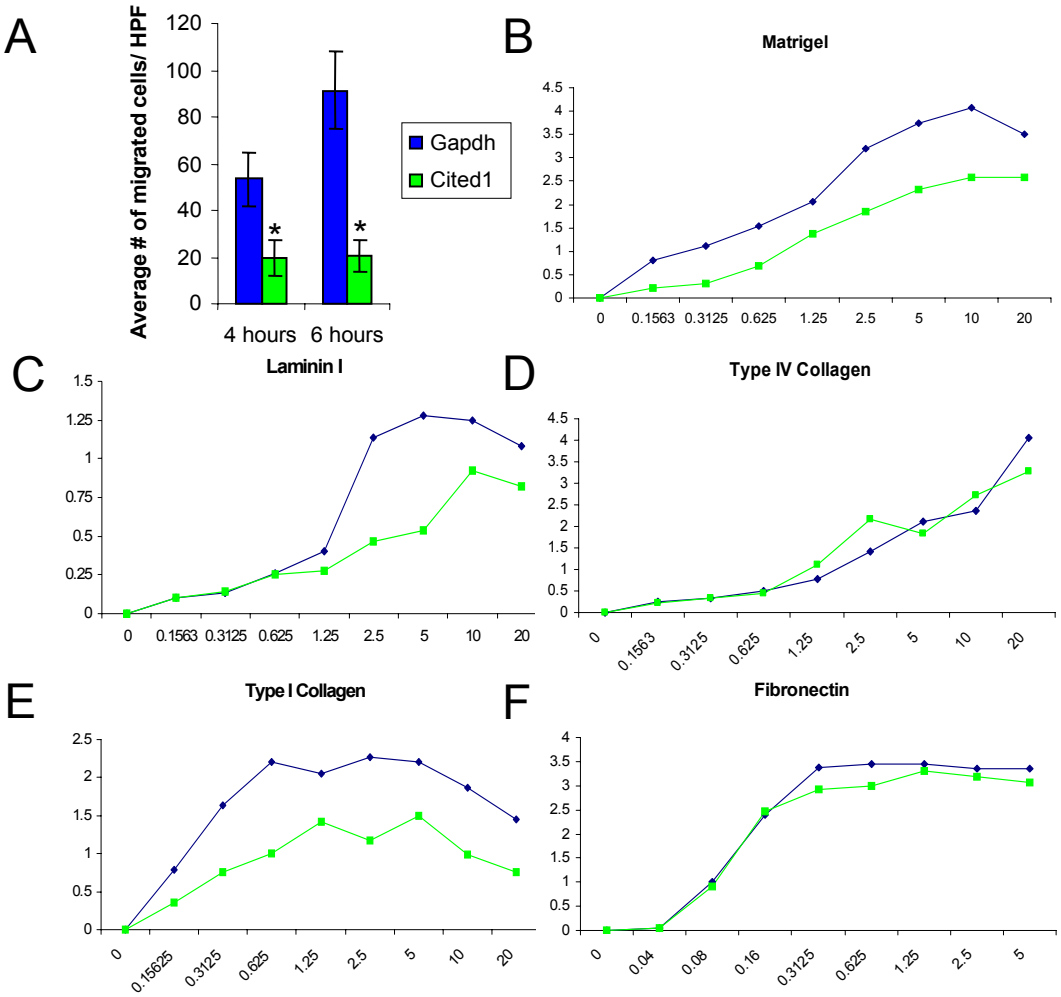


Figure 17. ***Knockdown of Cited1 in MCF7 cells inhibits cell migration and adhesion to selective ECM components*** **A.** Graphic representation of migration data comparing $MCF7^{GapdhK/D}$ (blue) and $MCF7^{Cited1K/D}$ (green) cells 4 and 6 hours after plating; * $p<0.05$. **B-F** – Dose/response curves for $MCF7^{GapdhK/D}$ vs. $MCF7^{Cited1K/D}$ cell adhesion. ECM component of interest noted above each graph. Y axis - OD595, X axis - ECM concentration (μg/ml).

entire range of Type I collagen concentrations (Fig 17E). In contrast, we see no difference in the ability of these cells to adhere to fibronectin (Fig 17F). Together these data suggest that knockdown of Cited1 in MCF7 cells results in a specific defect (as opposed to a general defect) that alters their ability to adhere to selective components of the ECM.

Attaching MCF7$^{Cited1K/D}$ cells have abnormal actin organization and impaired formation of lamelipodia and filopodia

To visualize attaching cells we compared actin organization in MCF7$^{GapdhK/D}$ and MCF7$^{Cited1K/D}$ cells one and four hours after plating. For these experiments we used wells coated with 2.5μg/ml Type I collagen, as dictated by the dose/response curve generated in our adhesion assays. At one hour MCF7$^{GapdhK/D}$ cells form numerous filopodia and lamelipodia, a feature of attaching cells (Fig. 18A). In contrast MCF7$^{Cited1K/D}$ cells have a rounded appearance and no discernable organization of actin structures (Fig 18B). At four hours control cells are well attached as indicated by the characteristic 'spread' morphology and cytoskeleton (Fig. 18C). MCF7$^{Cited1K/D}$ cells do begin to form filopodia and lamelipodia-like fronts after four hours but, have not formed the numerous focal contacts observed in MCF7$^{GapdhK/D}$ cells (Fig 18D). These data suggest that in the absence of Cited1, MCF7 cells do not form the characteristic actin structures required for cell attachment and migration, supporting a role for Cited1 in mediating cell-ECM interactions.

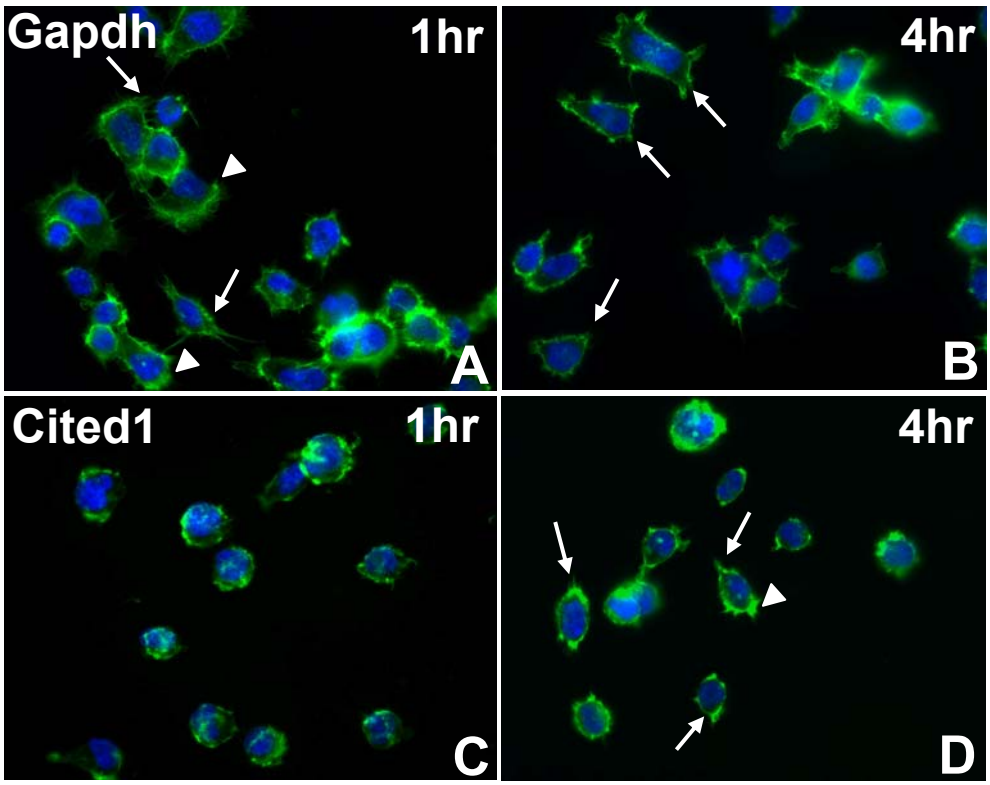


Figure 18. ***Actin organization in attaching MCF7$^{GapdhK/D}$ and MCF7$^{Cited1K/D}$ cells.*** Cells plated on 2.5µg/ml Type I collagen and visualized at indicated times. Green – FITC conjugated Phalloidin; Blue- Dapi stained nuclei. **A.** MCF7$^{GapdhK/D}$ cells at 1 hr; arrow – filopodia, arrowhead – lamelipodia. **B.** MCF7$^{GapdhK/D}$ cells at 4 hrs; arrow – attached cells with characteristic actin organization. **C.** MCF7$^{Cited1K/D}$ cells at 1hr. **D.** MCF7$^{Cited1K/D}$ cells at 4 hrs; arrow – filopodia, arrowhead lamelipodia.

Defects in MCF7$^{Cited1K/D}$ cell adhesion are not related to changes in cell surface expression of candidate integrins

Cell attachment and migration is mediated through interactions between the ECM and a diverse array of integrins expressed on the cell surface (for review see (Cox *et al*., 2006; Moissoglu and Schwartz, 2006). Integrin receptors are dimeric complexes of an α and a β subunit, the combination of which confers specificity for given ECM components. Upon binding of ECM ligands, these receptors initiate a well characterized signaling cascade which is propagated by members of the Rho/Rac family of small GTPases and culminates in reorganization of the actin cytoskeleton, allowing cell attachment or movement

To address whether knockdown of Cited1 changed cell surface expression of integrin subunits we used flow cytometry. For these studies we elected to look at three integrin subunits based on the data generated in our adhesion assays. β1 integrin is a broadly utilized subunit present in a wide variety of receptor complexes. α1 is also widely used, but shows some preference for adhesion to collagen. α6 is specific for interactions with Laminin I. These studies were carried out by binding a phycoerythrin (PE) conjugated secondary antibody to cells labeled with subunit specific primary antibodies. As a control, cells were incubated with PE secondary alone. In control cells, the population appears to the left of the multigraph, indicating that no detectable PE is present (Fig. 19A, inset) In contrast, MCF7$^{GapdhK/D}$ cells labeled with either β1, α1, or α6 antibodies display a strong rightward shift, indicating that all three receptor subunits are expressed on the cell surface (Fig. 19, left column). In MCF7$^{Cited1K/D}$ cells we do not see a change in either the fluorescence intensity (indicative of the amount of antigen a given cell expresses) or the total number of cells that are positive for a given subunit (Fig 19,

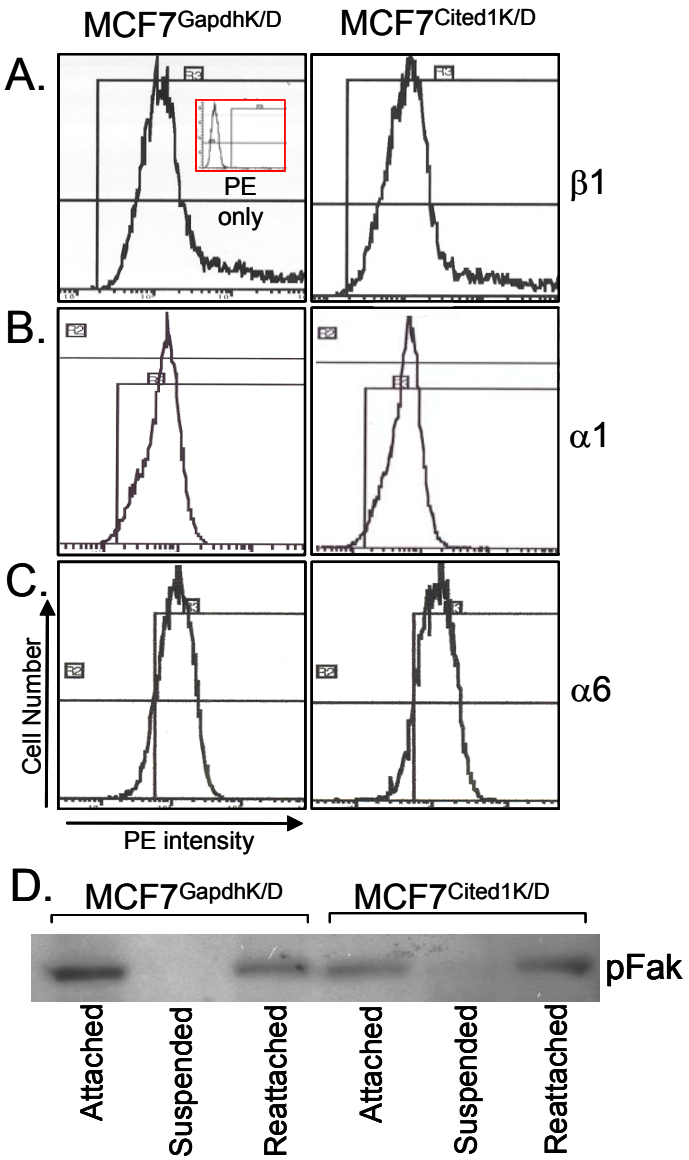


Figure 19. ***Adhesion defects in MCF7$^{Cited1K/D}$ cells are not due changes in candidate integrin expression or phosphorylation of FAK.*** **A-C**. FACS plots of MCF7$^{GapdhK/D}$ vs. MCF7$^{Cited1K/D}$ examining cell surface expression of β1 (A), α1 (B), and α6 (C) integrin. X-axis is PE intensity; Y-axis is cell number. **Inset A** – Representative mulitgraph of negative control in which cells were incubated with secondary antibody alone. **D.** Western blot analysis of FAK phosphorylation following cell attachment.

right column). From these data we can conclude that migration and adhesion defects in MCF7$^{Cited1K/D}$ cells are not caused by a reduction in cell surface expression of β1, α1, or α6 integrin.

An important early step in integrin-mediated cell attachment and migration is phosphorylation of focal adhesion kinase (FAK). As a first step to interrogating this signaling pathway in this system we examined FAK phosphorylation in MCF7$^{GapdhK/D}$ and MCF7$^{Cited1K/D}$ cells during cell attachment (Fig 19D) In control and Cited1 knockdown cells p-FAK is prominent in attached cells and disappears when cells are put into suspension. Upon plating, FAK is phosphorylated at comparable levels in both cell groups. These data indicate that if Cited1 regulates the cell adhesion/migration signaling pathway, it does so a level downstream of FAK.

Conclusion

In these studies we have used an *in vitro* system to uncover a role for Cited1 in regulation of cell adhesion and migration. We show that Cited1 can be efficiently knocked down in MCF7 cells using siRNA and that this effect persists for five days. This manipulation results in inhibition of cell migration through the ECM, and is associated with a reduced capacity of cells to adhere to Type I collagen and Laminin I. During adhesion cells which lack Cited1 have abnormal actin organization and do not form characteristic lamelipodia or filopodia. To try and understand a mechanism for the effect of Cited1 on cell adhesion we asked whether cell surface expression of candidate integrins were effected in MCF7$^{Cited1K/D}$ cells. No discernable changes were detected in

levels of β1, α1, or α6 integrin. Furthermore, FAK phosphorylation, an important early step in cell adhesion, was not effected in the absence of Cited1.

These studies demonstrate a role for Cited1 in regulation of cell migration and adhesion and provide an *in vitro* platform for the investigation of its function. Though it is unclear if these data reflect an *in vivo* role for Cited1 in cell-ECM interactions, we do know that cell migration and adhesion are important components of early MM differentiation. These processes are not well understood. This work has also yet to identify a mechanism by which Cited1 regulates cell adhesion. This is important given the insight it could offer relating to the general biology of Cited1. Future experiments should focus on identifying this mechanism and will be discussed in Chapter 5.

CHAPTER IV

GENERATION OF *Cited1-CreER*^{T2} MICE REVEALS DISTINCT PATTERNS OF CAP METANEPHRIC MESENCHYME CELL FATE

Introduction

One of the fundamental issues outstanding in the field of kidney development is the origin of the more than 20 specialized cell types required to make a functional organ. We have a general appreciation that nephronic epithelia arise from the condensed mesenchyme and that the collecting system is of UB origin, but specific cell fates and differentiation patterns have not been carefully addressed. We do not know if the cap mesenchyme gives rise to different cell types at different times during development, or if the pattern of nephron induction and growth is consistent throughout kidney development. It is unclear whether primitive nephron structures (such as the RV) are already distinct entities, containing all of the parent cells needed to make a nephron, or if certain segments are populated later by additional cells from other sources. There has also been some debate within the field regarding the possibility that condensed mesenchyme progeny participate in formation of the collecting system. Furthermore, the origin of other populations in the adult kidney, such as endothelial and interstitial cells, is unclear.

There has been a limited attempt to address some of these issues using *in vitro* lineage tracing experiments in the past. Early attempts used a retroviral system to deliver a LacZ reporter to whole kidney rudiments from rat, and showed that after a period of growth, labeled cells are found in nephronic tubules as well as collecting duct (Herzlinger

et al., 1992). However, as there is no way to verify that only MM cells (and not UB cells) were infected with the virus initially, we cannot be sure that this reflects actual lineage patterns *in vivo*. This system was improved by isolating rat MM, infecting it with the LacZ retrovirus and recombining this tissue with UB in culture (Qiao *et al*., 1995). Again, these studies showed that the MM could give rise to nephronic epithelia and some collecting duct cells. The difficulty of ensuring that no UB cells were brought along with the isolated MM again makes it unclear if this is an actual reflection of *in vivo* events.

Aside from terminal fate, the question of progenitor cell potential in the developing kidney is underscored by the issue of cap mesenchyme repopulation. As discussed above, due to the iterative nature of UB branching during kidney development, new MM must be continuously available for each round of nephron induction. It is unclear whether this in an intrinsic property of the cap mesenchyme or if this niche is repopulated by new cells migrating to each bud tip, from the stromal mesenchyme, for instance.

For these reasons we saw the expression domain of Cited1 as an unique opportunity to lineage trace a distinct group of progenitor cells in the developing kidney and address many of the questions discussed above. To do this we used a transgenic mouse strategy (described in detail below) to express a tamoxifen inducible form of Cre recombinase ($CreER^{T2}$) specifically in the Cited1 expression domain. By crossing these mice to a conditional reporter line (such as $Rosa26R^{LacZ}$) and inducing recombination with tamoxifen, we can 'tag and track' cap mesenchyme cells and ask questions about their fate, their patterns of differentiation and their capacity to renew.

Recently there have been two other published reports of mice which express Cre in the MM; *Pax3-Cre* (Grieshammer *et al*., 2005), and *Rar2b-Cre* (Kobayashi *et al*., 2005). While both of these reports make brief mention of cell lineage from this population, each of these genes is expressed in a much wider domain than Cited1, encompassing all nephrogenic mesenchyme prior to UB invasion. This breadth makes them less advantageous to address the specific fate of the condensed mesenchyme. Furthermore, both are constitutively active, lacking the temporal control of gene deletion present in our system.

Here we describe the creation and characterization of *Cited1-CreER*^{T2} transgenic mice. We have identified one founder line which expresses CreER^{T2} in the appropriate domain and demonstrates the expected pattern of recombination in response to tamoxifen. This founder carries a four copy block of transgene integrations which is stably transmitted following the F_1 generation. Using these mice, we lineage traced the overall potential of the cap mesenchyme by labeling cells at E13 and examining their fate in adults. This injection time point reveals widespread cap mesenchyme lineage in the adult kidney and demonstrates that cells in all segments of the nephron can be derived from this population. These data also demonstrate that elements of renal endothelium and collecting duct are not of cap mesenchyme origin. By tagging cells at various points during development, we have shown that at early stages cap mesenchyme gives rise to deep nephrons, those structures whose distal elements reach all the way into the papilla. In contrast, nephrons which arise after E18 become superficial nephrons, whose distal segments do not extend past the medulla. Furthermore, the extensive nephronic progeny of cap cells tagged at E13 suggests that these cells are also capable of self-renewal. By

examining embryonic timepoints, in which we can visualize the turnover of the cap mesenchyme, we have shown this to be true.

This mouse strain also allows one to circumvent a key problem in the study of kidney development in the mouse. As discussed in chapter one, many factors that are expressed at later stages of MM differentiation are also required for early inductive events (Table 1). As a result, deletion of these genes usually leads to the complete or near-complete loss of any kidney whatsoever. This makes analysis of gene function *in vivo* at later stages impossible. Utilizing *Cited1-CreER*^{T2} mice in a Cre/LoxP strategy of conditional targeting, one can control the tissue-selectivity (cap mesenchyme) and timing (tamoxifen inducible) of gene deletion.

Experimental Procedures

Generation of BAC transgene and transgenic animals

Specifics of BAC targeting vector and modification are discussed at length in the Results and Discussion section. In general, our manipulations were preformed as previously described (Lee *et al*., 2001). Briefly, a BAC (RP23-204G22, mouse 129/SvJ; Invitrogen) covering the entire *Cited1* locus, as well as 190 kb of 5' and 30 kb of 3' genomic sequence, was electroporated into the EL250 *E. coli* which carries the temperature sensitive λ phage Red recombination system and arabinose inducible Flp recombinase. A targeting vector containing a *CreER*^{T2}-IRES*eGFP*-frt*TET*^{R}frt cassette flanked by 500 bp homology arms corresponding to the *Cited1* locus at the transcriptional start site was electroporated into EL250 cells containing the *Cited1* BAC and induced to

recombine by 42°C heat shock. Positive clones were treated with arabinose to activate inducible Flp recombinase within the EL250 cell, removing the Tet^R cassette. Small scale preparation of BAC DNA was carried out using standard alkaline lysis techniques. For restriction digest DNA was cut O/N and resolved using PFGE, run for >12 hours.

For generation of transgenic animals, *Cited1-CreER*T2 BAC was purified using cesium chloride maxiprep techniques. Following removal of EtBr using butanol extraction, DNA was subjected to a series of spins using Centriprep size exclusion columns to concentrate BAC DNA in TE pH 7.4. Dilutions of purified BAC DNA were cut with Not1 and subjected to PFGE against a standard to estimate concentration. BAC DNA was diluted to 1ng/ml for pronuclear injection of zygotes derived from Fvb mice as described (Vintersten *et al*., 2004). Injected zygotes were implanted into pseudopregnant ICR females and allowed to come to term.

Mouse lines and tamoxifen injection

Transgenic mice generated using *Cited1-CreER*^{T2}-IRES*eGFP* BAC DNA are hereafter referred to as *Cited1-CreER*^{T2} mice. *Rosa26R*$^{LacZ/LacZ}$ (*R26R*^{LacZ}) mice were obtained from Jackson Laboratories (Soriano, 1999). For treatment of pregnant females, tamoxifen (Sigma T5648) was dissolved by sonication in 10% EtOH / 90% sunflower oil at a concentration of 15 mg/ml. 100 µl (1.5 mg) was injected intraperitoneally (IP) into pregnant *R26R*^{LacZ} females at times indicated. As tamoxifen injection compromises the ability of mice to have natural birth, pups were delivered by C-section at E19.5-E20 (day of vaginal plug appearance counted as E0.5). After pups were breathing independently they were transferred to foster mothers who had given birth the day before and had begun nursing. The overall success rate of fostering was >80%.

Genotyping and quantitative genomic PCR

Founders and subsequent offspring were genotyped using the following primer sets; *Cre*: 5' GGC GCG GCA ACA CCA TTT TT; 3' TCC GGG CTG CCA CGA CCAA. *Rosa26R*^{LacZ}: 5'^{A} AAA GTC GCT CTG AGT TGT TAT; 5'^{B} GCG AAG AGT TTG TCC TCA ACC; 3' GGA GCG GGA GAA ATG GAT ATG. Transgene copy number was estimated using TaqMan based real-time PCR using genomic DNA as described (Chandler et al., 2007). Briefly, the chloramphenicol cassette within the BAC vector was amplified using a custom primer set from Applied Biosystems (5': GCA CAA GTT TTA TCC GGC CTT TAT T; 3': GTC TTT CAT TGC CAT ACG GAA CTC). To estimate copy number, triplicate ΔCT values for the chloramphenicol cassette relative to a genomic DNA control, *Jun* kinase, were compared to the ΔCT values of known quantities of BAC DNA prepared to estimate copy number equivalents.

β-Gal staining

See Chapter Two.

Antibody Staining

For dual labeling, kidneys were isolated, cut bilaterally along the sagital axis (for adults) and fixed for 1 hour in 4% formaldehyde in PBS at 4°C. Following fixation, tissue was rinsed in PBS and subjected to a sucrose gradient of first 15% (4 hrs) and then 30% (O/N) in PBS at 4°C. After embedding in OCT, frozen sections were cut at 8 μm onto charged slides. For staining, slides were thawed, rinsed in PBS and blocked in 10% goat serum (Vector Labs) in PBS for 1 hour. Tissue was incubated with primary

antibody O/N at 4°C, washed 3 times in PBS, and incubated with secondary antibody for 1 hour at R/T. After washing in PBS, tissue was mounted with Vectashield mounting medium containing DAPI (Vector Labs.) When antibodies used for co-labeling were of the same species, sequential sections were used.

Antibodies

Rabbit αCited1 1:250 – Cap mesenchyme; (Neomarkers #RB-9219)

Mouse αCre 1:100 – CreER^{T2} expressing cells (Covance # MMS 106P)

Mouse αGFP – GFP expressing cells (Molecular Probes clone MAb3E6, #A11120)

Rabbit αβGal 1:5000 - Cap mesenchyme derived cells; (Cappel #55976, Lot 04993)

Mouse αNa/K ATPase (α1 subunit) 1:250 – Broad marker of nephronic tubular epithelia; (Upstate #05-369)

Rat αPCAM 1:250 - Endothelial cells; (BD Biosciences #553370)

Hamster αPodoplanin 1:500 - Glomerular epithelia (Angiobio # 11033)

WT1 1:100 – Glomerular podocytes; (Santa Cruz #SC192)

Rabbit αAquaporin 1 – 1:250 – Thin limb and proximal tubule; (Chemicon # AB3065)

Rabbit αAquaporin 2 – 1:250 – Collecting duct; (Alpha Diagnostics #AQP21-A)

Results and Discussion

Generation of targeting vector and BAC recombinant transgene

To create *Cited1-CreER*^{T2} mice we utilized BAC transgenesis. This technique allows us to insert a gene of interest into the *Cited1* locus and drive its expression using the same promoter elements that regulate endogenous transcription. BACs are

advantageous for this purpose because they can be modified in bacteria and contain large regions of genomic DNA up- and downstream of a given gene, so that we need not know precisely where promoter elements lie (for review on BAC transgenesis see (Copeland et al., 2001; Giraldo and Montoliu, 2001; Liu et al., 2003). The BAC we modified for creation of the *Cited1-CreER*^{T2} transgene (RP23-204G22) was selected based on the relative position of the *Cited1* locus within its genomic sequence (Fig. 20A). This BAC contains ~ 190 kb of upstream and ~30 kb of downstream genomic sequence, which we presume has a high probability of containing the regulatory elements to drive appropriate gene expression from the *Cited1* locus. For modification, the BAC was purified from DH10B *E. coli* (in which it is maintained) and electroporated into the EL250 *E. coli* strain (Lee *et al*., 2001). This strain is engineered to carry both a set of heat inducible genes that mediate homologous recombination and arabinose inducible FLP recombinase, which allow for the excision of unwanted Frt flanked sequences within the BAC. Following electroporation, DNA was purified from chloramphenicol resistant clones (carried by all BACs in this library) and digested with BamH1, which cuts the BAC several times. This results in a predictable banding pattern, identical to that of BAC DNA that was purified and digested from the parental DH10B strain (Fig. 21A). This confirms stable introduction of our BAC into the EL250 strain (EL250^{Cited1BAC}).

The targeting vector used to insert *CreER*^{T2} into the BAC *Cited1* locus was built on pBS-Tet^{R} (Fig 20B). This plasmid is a derivative of pBluescript SK(+) which carries a tetracycline resistance cassette flanked by Frt (FLP recombinase recognition) sites. 5' and 3' homology arms for the Cited1 locus (~500bp each) were generated by PCR from genomic DNA and inserted on either side of the Tet^{R} cassette in pBS-Tet^{R}. Homology

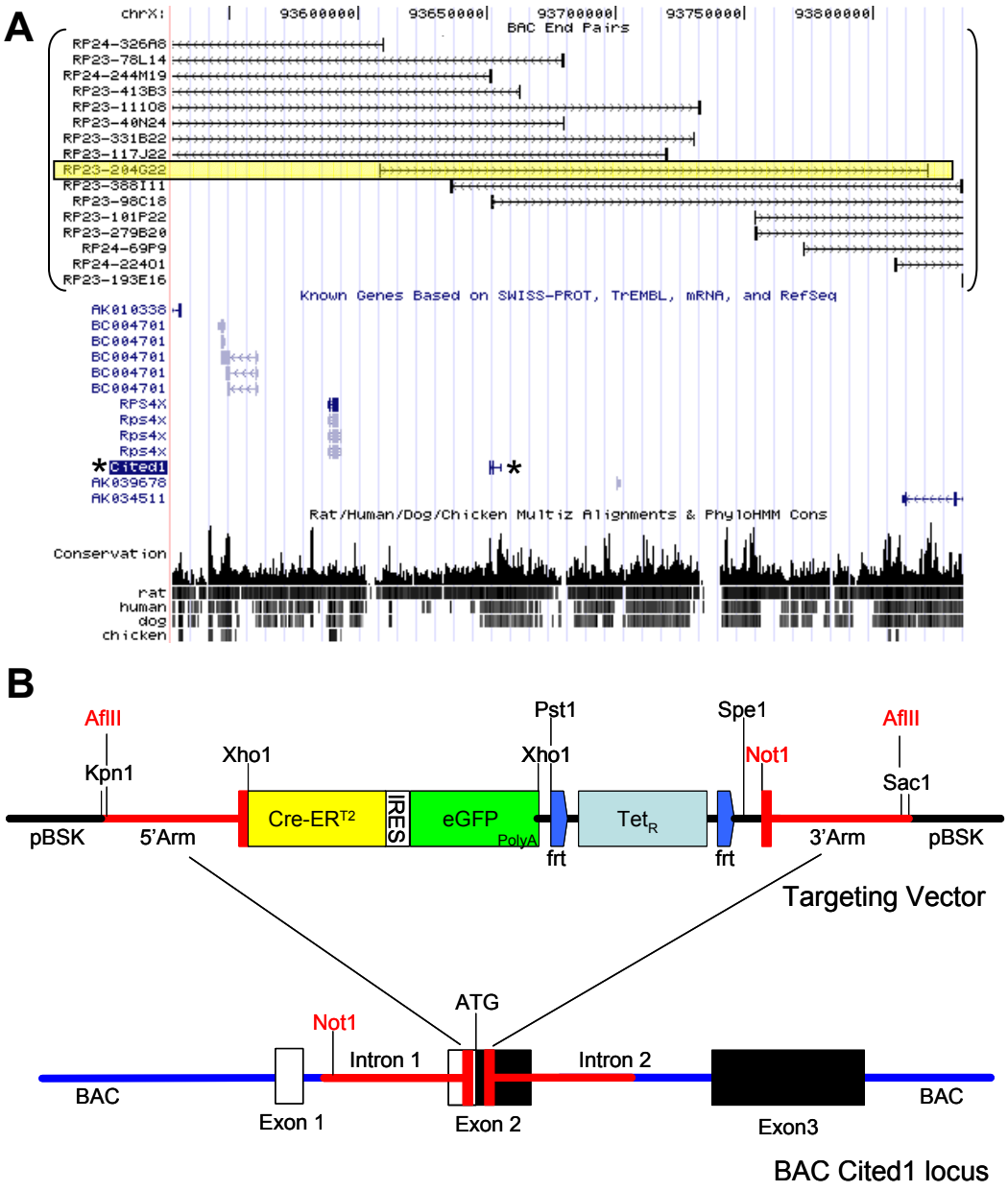


Figure 20. ***Selection and targeting of BAC for generation of Cited1-CreER^{T2} transgenic mice.*** **A.** Screenshot of UCSC genome browser showing relative position of *Cited1* locus (asterisk) on the X-chromosome and the available BAC clones (brackets) which cover this region. The BAC we selected for targeting is highlighted. **B.** Schematic of targeting vector and site of recombination within the BAC *Cited1* locus. Cut sites discussed in the text are highlighted in red. Not to scale.

arms were designed to remove the *Cited1* transcriptional start site and the subsequent 12 bp within the BAC, but do not delete any additional coding sequence (Fig 20B). We used this strategy to avoid removal of any intronic regulatory elements within the *Cited1* locus. The resulting intermediate construct (pBSTet^{R}-C1TV) can be used as a general platform for targeting *Cited1* in a BAC, as any gene of interest can be placed between the homology arms for insertion into the locus.

In these studies we have cloned a CreER^{T2}IRESeGFP^{polyA} cassette into the Xho I site of pBSTet^{R}-C1TV (pBSTet^{R}C1TV-ER^{T2}). This cassette encodes Cre recombinase fused to the ligand binding domain of the estrogen receptor, so that Cre translocates to the nucleus (and thus is active) only in the presence of the estrogen analog tamoxifen (Feil *et al*., 1996; Feil *et al*., 1997). In addition it contains an internal ribosomal entry site (IRES) allowing for independent translation of eGFP, which can then be used as a marker of transgene expression.

For BAC targeting, the AflII fragment of pBSTet^{R}C1TV-ER^{T2} was purified (Fig. 20B) and electroporated into EL250^{Cited1BAC} cells which had been grown at 42°C to induce expression of the recombination gene set. Positive clones were identified by dual tetracycline and chloramphenicol resistance and screened by PCR using primers that lie upstream in the 5' genomic region and downstream in the CreER^{T2}cassette, respectively. Restriction digest can also be used to confirm insertion. In this particular BAC, there is a Not I site ~3.5 KB upstream of the *Cited1* transcriptional start site, and recombination of the targeted locus adds a downstream Not1 site just after the Tet^{R} cassette (Fig. 20B). As a result, the correctly targeted locus gives rise to a ~9.5 kb band when digested with Not I. However, Not I digestion also releases the vector backbone for this BAC library, which is

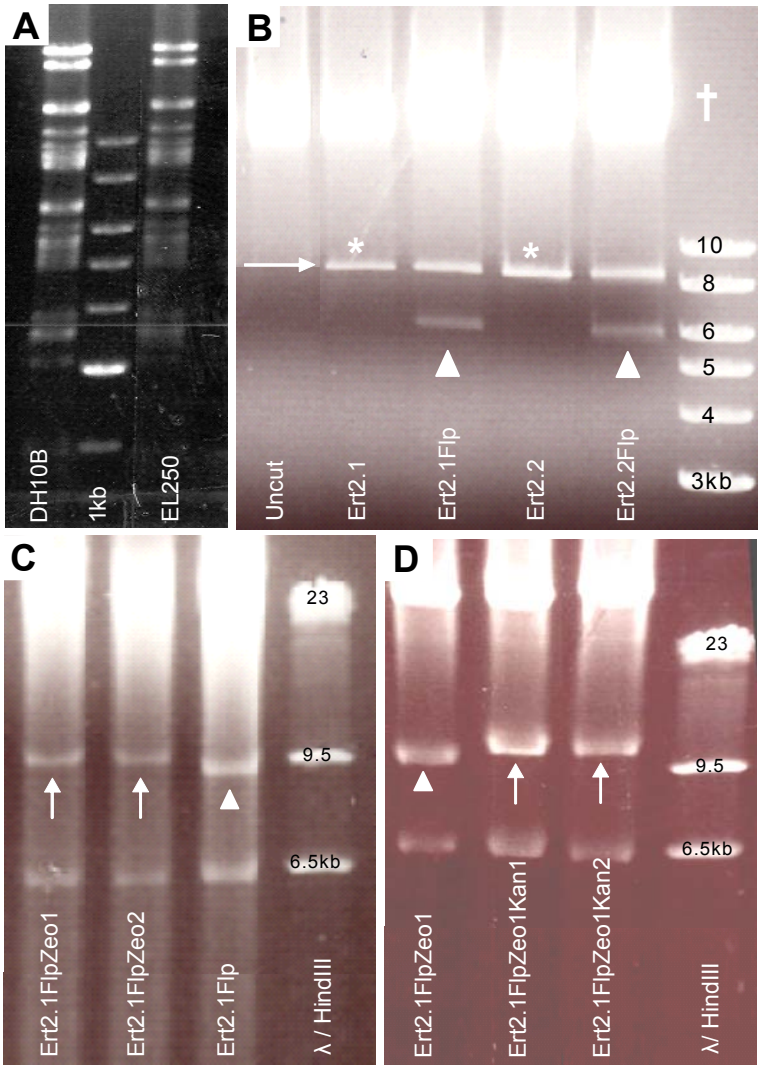


Figure 21. ***Introduction and modification of BAC into EL250 cells.*** **A.** BamH1 digest of BAC DNA purified from DH10B (parental strain) and EL250 (recombination strain) following electroporation. **B.** Not1 digest following insertion of CreER^{T2}-IRES-eGFP into BAC *Cited1* locus and removal of TetR with Flp recombinase; Arrow- vector backbone +/- insert, Asterisk- vector insert doublet not resolvable by PFGE, Arrowhead – insert following removal of Tet^{R} cassette, Dagger- genomic DNA. **C, D**. Not1 digest following sequential removal of 2 cryptic loxP sites; Arrow – vector bandshift resulting from insertion of zeocin (C) and kanamycin (D) resistance cassettes, Arrowhead – parental vector backbones. For all modifications results from two independent clones are shown.

also ~9.5 kb. These two bands are not resolvable by PFGE, and appear as a single band, even when the gel is run for >20 hours (Fig. 21B, lanes 2 and 4). The Frt sites on either side of the Tet^R cassette allow for its removal from the targeted locus in the EL250 strain (see above), preventing any unwanted effects on gene expression or function resulting from its proximity to the gene of interest. This excision reduces the size of the *CreER*^{T2} insertion by ~2.6 kb, making the vector and insert bands clearly distinguishable using PFGE (Fig. 21B).

Two additional modifications were made to the targeted BAC through homologous recombination using a system developed in the Michelle Southard-Smith lab (Vanderbilt University). These changes resulted in the removal of cryptic LoxP sites that are present in the vector backbone of the parental BAC. As BACs tend to integrate into the host genome as concatamers, these sites could inappropriately mediate self-excision of a transgene expressing Cre recombinase and should therefore be removed. Plasmids which target these sites and confer kanamycin and zeocin resistance, respectively, were introduced sequentially as described above. These modifications result in an increase in the size of the vector backbone, which can be observed as an upward band shift after Not I digestion and PFGE (Fig. 21 C,D). Unlike the Tet^R insertion, these cassettes were not removed from the BAC given that they are a considerable distance (<30kb) from the locus of interest and would not be predicted to interfere with transgene expression or activity.

The resulting BAC contains *CreER*^{T2}-IRES*eGFP* in the *Cited1* locus and is devoid of the Tet^R cassette and cryptic LoxP sites. This BAC was then used as a transgene to create *Cited1-CreER*^{T2} animals through pro-nuclear injection. Zygotes for

injection were derived from super-ovulated female mice on the FVB genetic background, which has been previously shown to support BAC integration (Auerbach *et al*., 2003). Of the ~200 zygotes injected and implanted into pseudo-pregnant ICR females, 29 live births were obtained (11 ♀/ 18 ♂). These animals were screened by PCR for the presence of CreER^{T2}, resulting in 6 potential founders (2♀/ 4♂; data not shown).

Characterization of *Cited1-CreER*^{T2} founders

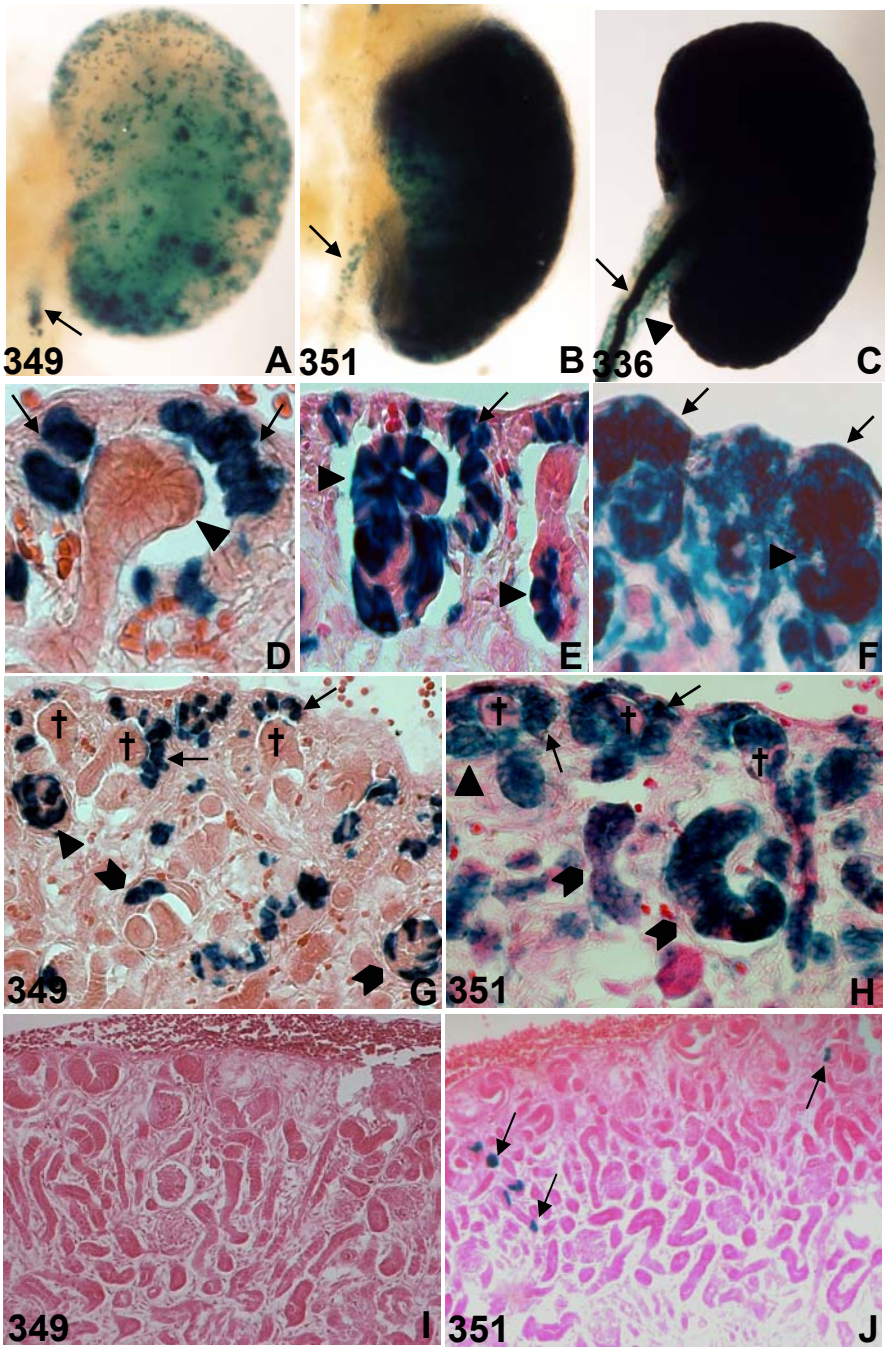
For initial analysis of transgenic recombination potential we utilized Rosa26R LacZ reporter mice (*R26R*^{LacZ}). These animals carry a floxed stop cassette between the ubiquitously expressed Rosa26 promoter and a β-Galactosidase cassette (Soriano, 1999). When exposed to active Cre recombinase the stop cassette is excised, resulting in LacZ expression in cells that underwent a recombination event and all of their progeny. For characterization of *Cited1-CreER*^{T2} transgenic animals, *R26R*^{LacZ} mice were crossed to potential founders and injected with 1 mg of tamoxifen at E12 and E13. Pregnant mice were sacrificed at E15.5 and kidneys removed from embryos for whole mount LacZ staining. Embryonic tissue was also collected to assess germline transmission of the transgene by PCR. Of the 6 potential founders, 2 did not transmit the transgene to their offspring and were discarded (data not shown). In the remaining four lines we saw three distinct patterns of recombination in the developing kidney (Fig 22A-C). In line 349 whole mount staining revealed a relatively even distribution of recombined cells throughout the kidney, which is what we would expect given that we intended to label a subset of cells which would then only have ~72 hours to divide. Line 351 showed much more intense LacZ staining throughout the kidney, while in line 336 it appeared as if all

cells had been labeled. In all three lines we observed recombination in the ureter (Fig 22A-C), likely a result of early *Cited1* expression in the ND and primary stalk of the UB, which gives rise to the ureter (Fig. 5 C,D). In addition line 336 shows recombination in vascular structures surrounding the ureter (Fig 22C). This suggests that this line expresses $CreEr^{T2}$ outside of the *Cited1* expression domain as there is no evidence these cells arise from cap mesenchyme.

To determine in which cell types recombination was occurring in these lines we sectioned our whole mount preparations. In line 349 recombination has occurred in the condensed MM, but not in the UB (Fig. 22D). In contrast, widespread recombination in the condensed MM and the UB was observed in lines 351 and 336 (Fig. 22E,F). At this point line 336 was discarded as it seemed to induce recombination during early kidney development clearly outside of the Cited1 expression domain, lacking the specificity needed for careful lineage tracing. In addition the fourth line which transmitted the transgene in the germline, 346, also demonstrated the same broad pattern of recombination (data not shown).

There are a number of possible explanations for recombination occurring in the UB at this stage in line 351. One of these is that at this early injection timepoint (E12) there is residual Cited1 expression in UB stalks resulting in recombination in a large percentage of UB structures, distinct from the expected recombination observed in the ureter. This effect may not be observed in line 349 due to differences in levels of transgene expression. If this were the case, we would expect that by injecting tamoxifen at E15, we would extinguish recombination in the UB in line 351, given that Cited1 is not expressed in any UB structures at this point (Fig. 5G, 6A).

Figure 22. ***Characterization of Cited1-CreER^{T2} founder lines.*** Potential founders were crossed to *R26R*^{LacZ} reporter mice. **A-C.** Whole mount staining of E15.5 kidneys following tamoxifen injections at E12 and E13. Line designation listed bottom left; Arrow- ureter, Arrowhead – vasculature surrounding ureter. **D-F**. Sections of A-C, respectively; Arrow- condensed MM, Arrowhead – UB. **G, H.** Sections of E18.5 kidneys following E15 tamoxifen injection. Line designation listed bottom left; Arrow- condensed MM, Arrowhead- RV, Dagger- UB, Chevron- Differentiating epithelial elements (tubular epithelium and glomeruli). **I, J**. Cre (+) newborn animals whose mothers were not injected with tamoxifen in line 349 (I) and 351 (J). Arrow- recombined cells.


349
A
351
B
336
C
D
E
F
349
G
351
H
349
I
351
J

To test this hypothesis, we again crossed our potential founders (349 and 351) with *R26R*^{LacZ} mice, injected tamoxifen at E15, and observed the pattern of recombination at E18 (Fig. 22G,H). In line 349 we observed recombination in the condensed MM, RVs, early epithelial structures, and primitive glomeruli (Fig. 22G). This pattern reflects the cell types we would expect to arise from the cap mesenchyme. In contrast, line 351 again showed widespread recombination in the UB, as well as robust recombination in the condensed MM and primitive nephrons (Fig. 22H). This shows that recombination in the UB in line 351 is not a consequence of early expression of Cited1 in the nephric duct or UB stalk but results from either ectopic expression of the transgene or loss of transgene regulation by tamoxifen, rendering it constitutively active. Both of these possibilities can be readily addressed.

By analyzing NB *Cited1-CreER*^{T2} / *R26R*^{LacZ} embryos whose mothers have not been injected with tamoxifen, we can determine if CreER^{T2} is active at any time during kidney development in the absence of tamoxifen. This would include activity in the nephric duct and UB stalk which could explain the UB recombination observed in line 351. In line 349 we did not observe any labeled cells in Cre positive NB offspring of mothers who had not been injected with tamoxifen (Fig. 22I). In line 351 there were a few labeled cells in the absence of tamoxifen, but it does not appear that this level would be sufficient to produce the widespread pattern of recombination we observed in these mice (Fig. 22J).

The other possibility is that in line 351 CreER^{T2} is ectopically expressed in UB structures at later stages of kidney development. To test this we looked at Cre expression in both lines using antibody staining in E15.5 kidneys. The specificity of this antibody

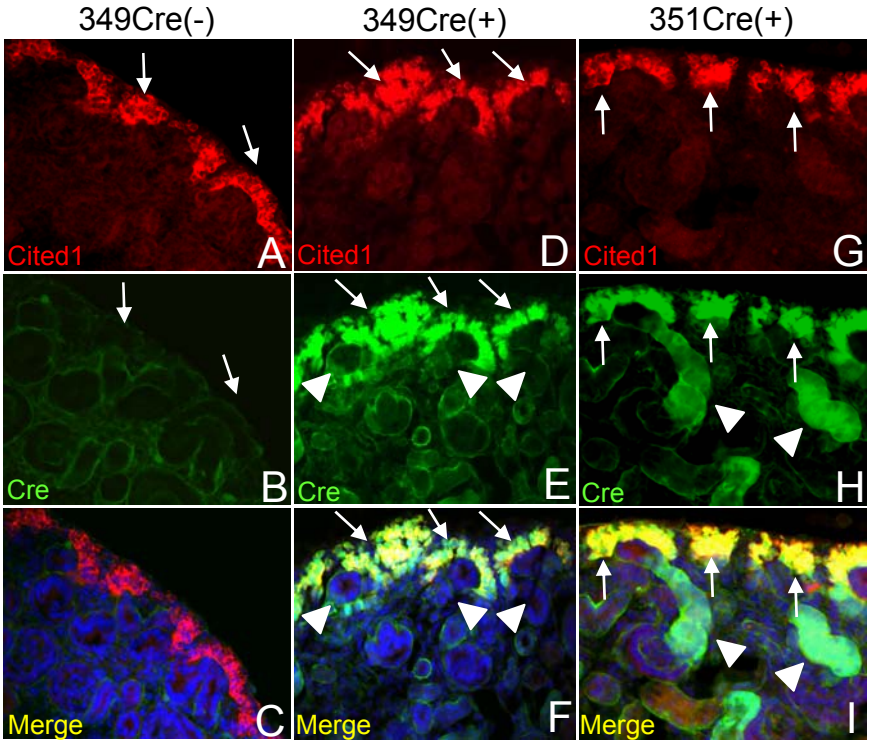


Figure 23. *CreER*^{T2} and Cited1 expression at E15.5 in offspring from two potential founder lines. A,D,G – Rabbit α Cited1; B,E,H – Mouse α Cre; C,F,I - Overlays of respective images. **A-C** – Transgenic line 349, Cre (-) animal; Arrow- cap mesenchyme. **D-F** – Transgenic line 349, Cre (+) animal. Arrow – cap mesenchyme, Arrowhead – UB. **G-I** – Transgenic line 351, Cre (+) animal. Arrow – cap mesenchyme, Arrowhead – UB.

was confirmed by staining kidneys which were Cre negative (Fig 23A-C). In line 349 we observed that expression of *CreER*^{T2} was restricted to the cap mesenchyme in a pattern that exactly overlaps that of *Cited1* (Fig. 23D-F). By contrast, we detect CreER^{T2} in both the cap mesenchyme and the UB in line 351 (Fig. 23G-I). This demonstrates that recombination in the UB at multiple injection timepoints in line 351 most likely resulted from ectopic expression of CreER^{T2}. Because this pattern is not reflective of endogenous *Cited1* expression, line 351 was discarded. All subsequent studies werecarried out in mice derived from founder 349 (hereafter referred to as *Cited1-CreER*^{T2} mice).

Propagation and copy number transmission of *Cited1-CreER*^{T2}

Because BACs contain large segments of genomic DNA (on the order of 200kb) and tend to integrate into the genome as concatamers, transmission of an equal number of copies of the transgene from parent to offspring can be variable and is often not established until after the F1 generation (Doug Mortlock, personal communication). To characterize copy number transmission in *Cited1-CreER*^{T2} mice we utilized a genomic quantitative PCR method developed in the Doug Mortlock laboratory. This assay compares the copy number of the CAM cassette, contained in all BACs from this library, to that of a housekeeping gene. By normalizing samples in this way we can infer transgene copy number in a given animal and track its transmission to offspring (Fig. 24).

Using this method we determined that our founder (349, F_0) carried five copies of the CreER^{T2} transgene, and by crossing this mouse to a wildtype female, we examined the transmission pattern of these elements (Fig. 24, F_0-F_1). Analysis of the F_1 generation showed that among Cre positive animals there were mice that received one,

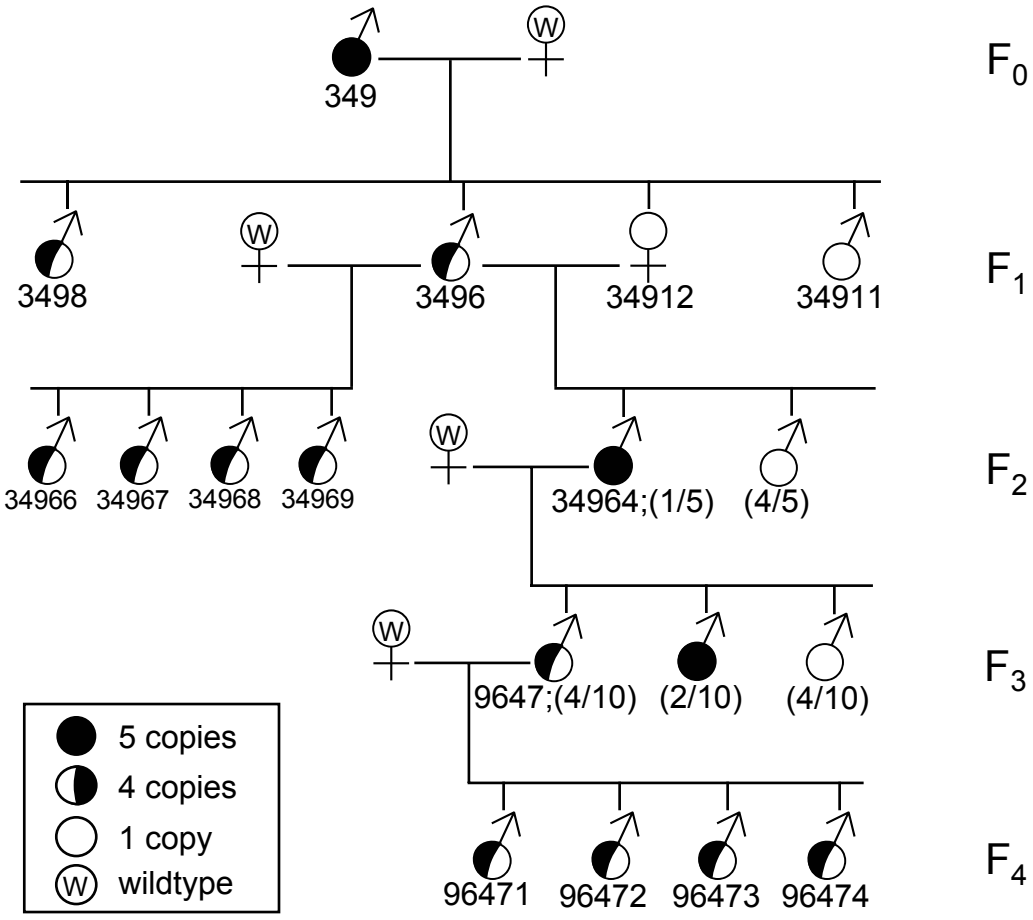


Figure 24. ***Schematic of Cited1-CreER^{T2} transgene copy number transmission from founder (F_0) through generation four (F_4).*** Legend indicates copy number per mouse determined by genomic quantitative PCR. Every mouse resulting from each cross was not evaluated, only those males pre-determined to be Cre (+). Numbers in parenthesis indicate distribution of mouse type resulting from each cross. Where this is not noted, all mice are listed indicating stable transmission of transgene (34966-9; 96471-4)

four, or five copies of the transgene. This suggests that there is a block of four copies of *CreER*^{T2} which segregate together and a single copy that segregates individually. Indeed, in crosses of F_1 males carrying four copies to wildtype females, all Cre positive progeny carried four copies of the transgene (Fig. 24 F_1-F_2, left column). Furthermore, in intercrosses using an F_1 four copy male with a single copy F_1 female, the resulting Cre positive mice carried either one or five copies. By crossing a five copy F_2 male with a wildtype female we observed the four copy block again segregating independently from the single copy. This block was then transmitted intact from the F3 to the F4 generation (Fig. 24 F_2-F_4, right column). Based on this analysis we conclude that our founder carries a four copy block of the *Cited1-CreER*^{T2} BAC which is stably transmitted following the F_1 generation and can be propagated through multiple generations of mice. Interestingly, analysis of transgenic line 351, in which we saw ectopic expression of Cre and inappropriate recombination in the UB, revealed that these animals carried 17 copies of the transgene (data not shown). It is possible that high integration levels in this line have resulted in inappropriate transgene expression.

eGFP expression in *Cited1-CreER*^{T2} transgenic kidneys

As discussed above, the BAC transgene we constructed to make these mice gives rise to a bicistronic message with IRES-*eGFP* fused downstream of *CreER*^{T2}. This strategy results in independent translation of eGFP, which can be used as an acute marker of transgene expression. Our initial attempts to detect eGFP signal during founder analysis were unsuccessful. It is possible, given our copy number and transmission analysis, that we were using mice for these studies that contained only one copy of

CreER^{T2}. Perhaps the level of transgene expression achieved with one copy does not result in detectable levels of eGFP. This problem could be compounded by the fact that translation from an IRES is often reduced when compared to a standard ribosomal recognition site.

Having established stable transmission of a four copy block of *CreER*^{T2} we asked whether eGFP was detectable in these animals. Using formaldehyde-fixed kidneys from E15.5 transgenic mice we carried out two experiments to detect eGFP. By simply looking for fluorescence in these sections, we saw that eGFP expression was clearly evident in the cap mesenchyme (Fig 25A). eGFP signal was also detected on sections which had been stained with an α GFP antibody, confirming translation of eGFP (Fig. 25 B-D). However, the strength of the eGFP signal was markedly reduced following antibody staining, and required long exposures to detect (4 seconds compared to 0.75 seconds in tissue which was not subjected to antibody staining). This suggests that some component of the staining process quenches eGFP signal. From these studies we conclude that eGFP can be used in *Cited1-CreER*^{T2} animals as an acute marker of transgene expression, and that this is best accomplished on tissue which has been fixed but otherwise untreated.

The cap mesenchyme gives rise to cells in all segments of the mature nephron

To test the total potential of cap mesenchyme cells, we crossed *Cited1-CreER*^{T2} mice to the *R26R*^{LacZ} reporter line and injected pregnant females at E13. At this time we know that *Cited1* is strongly expressed in the cap mesenchyme which should lead to widespread recombination. Waiting until 6 weeks of age to examine cell lineage (by

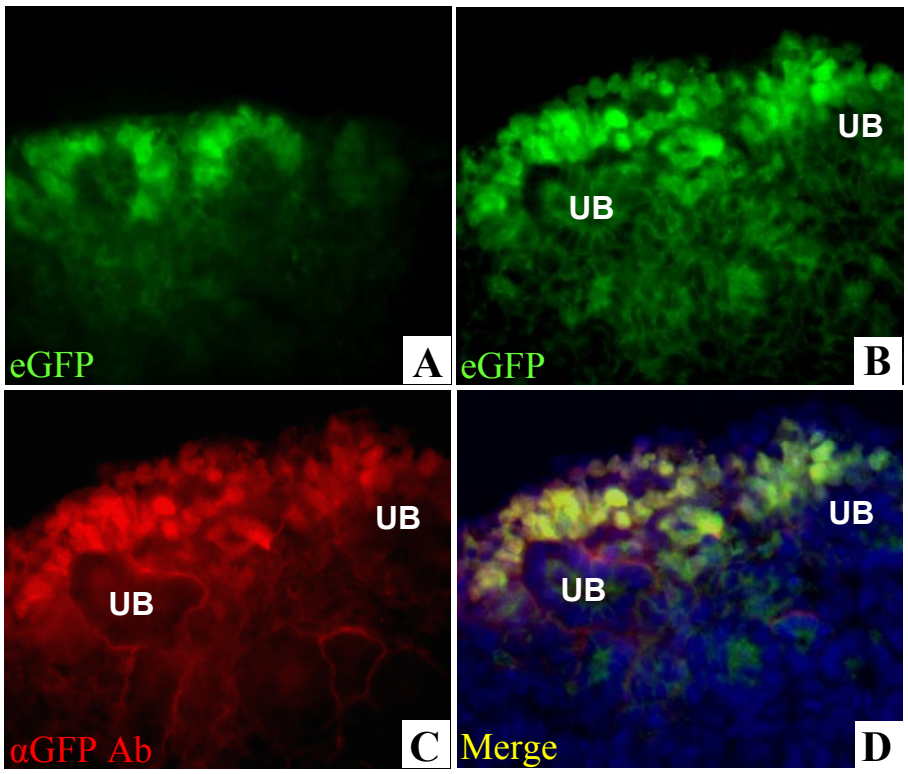
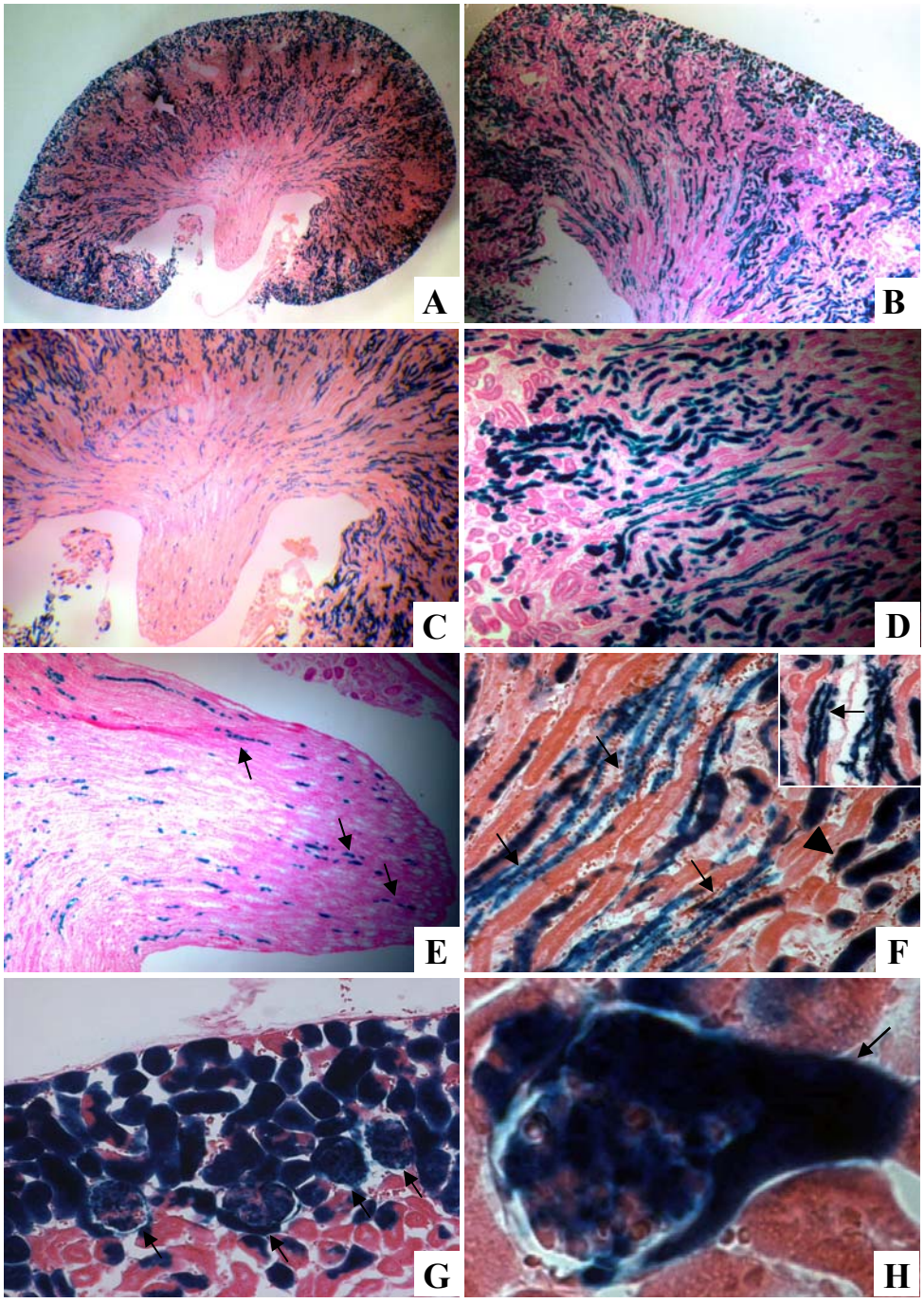


Figure 25. ***eGFP expression in Cited1-CreER^{T2} transgenic kidneys.*** E15.5 kidneys fixed in formalin for 1 hour. **A.** eGFP signal on section which has been formalin fixed only. Approx. exposure 0.750 sec. **B.** eGFP signal on section colabled with mouse αGFP antibody. Approx. exposure 4 secs. **C.** αGFP antibody stain. **D.** Merged image of B & C, plus Dapi marking nuclei. UB – ureteric bud

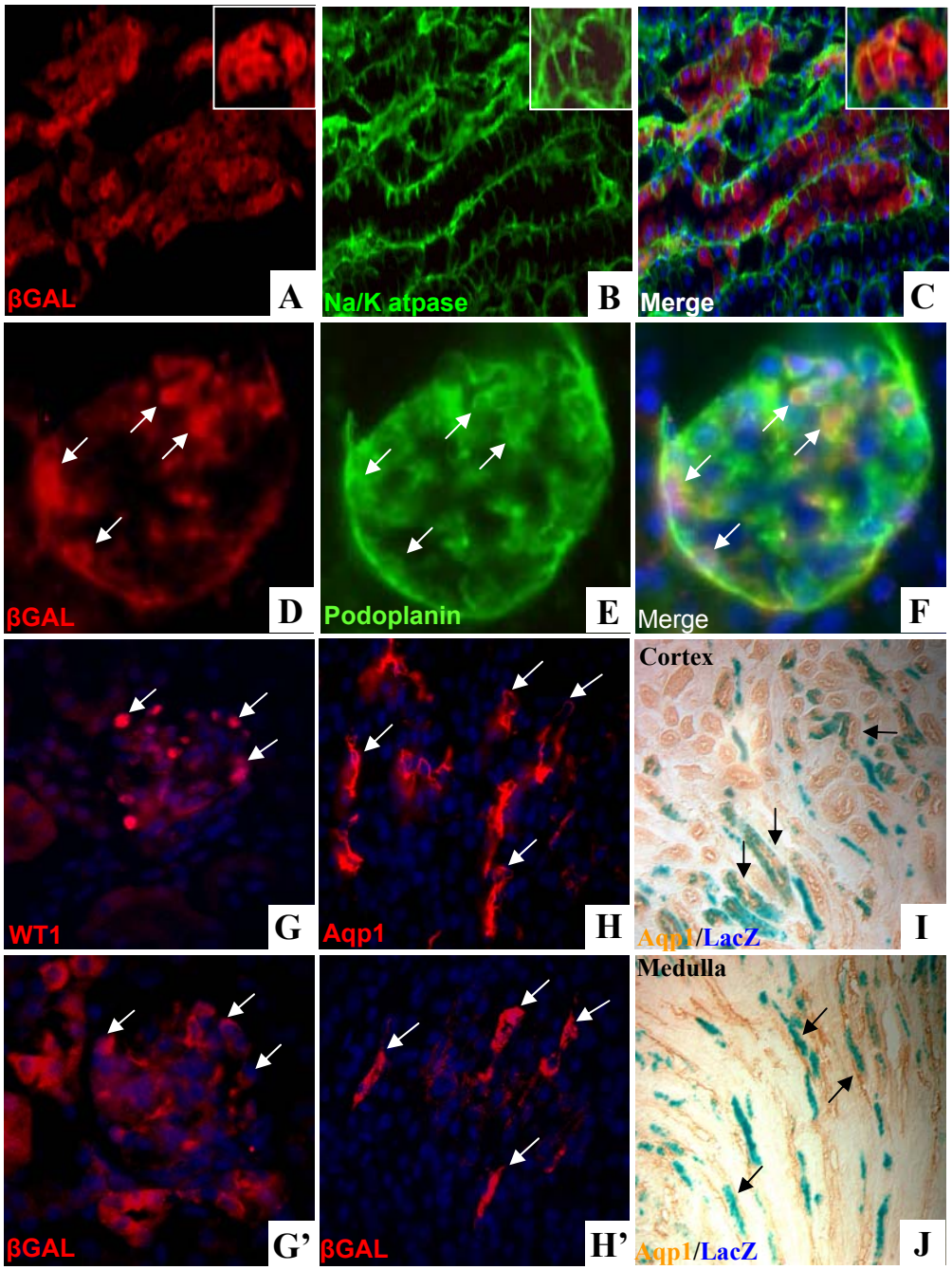
Figure 26. ***Lineage tracing of cap metanephric mesenchyme labeled at E13.*** Pregnant Cited1CreER^{T2}/R26R^{LZ} mice were injected with tamoxifen at E13. Resulting Cre positive mice were analyzed by LacZ staining at 6 weeks of age. **A.** Low power sagital image of kidney. All subsequent pictures are higher power images of this kidney. **B.** Cortex and cortico-medullary junction. **C.** Medulla and papilla. **D.** Cortico-medullary junction. E. High power image of papilla, Arrow – cap mesenchyme derived cells which have reached the papilla. **F.** High power image of cortico-medullary region; Arrow – cap mesenchyme derived cells with elongated morphology, Arrowhead – cap mesenchyme derived cells with with proximal tubular morphology. **G.** High power image of cortex; Arrow – glomeruli. **H.** Individual glomeruli and associated proximal tubule; Arrow – proximal tubule.


A
B
C
D
E
F
G
H

which time nephrogenesis is well completed), allows us to ascertain the complete potential of *Cited1* expressing cap cells. Analysis of X-Gal stained sections reveals that by injecting at E13 we achieved widespread recombination in a large percentage of nephrons, and that the cap mesenchyme gave rise to multiple cell lineages in the adult kidney, including cortical and medullary cell types (Fig. 26). Generally speaking, some of these lineages were immediately evident based on morphology and position, including proximal tubules and cells within glomeruli (Fig. 26G, H). In the medulla however, cell types were more difficult to distinguish morphologically. Here, descending nephronic elements and collecting ducts bundle with their associated vasculature as they move through the medulla into the renal papilla (Fig. 26B-D) This region contains LacZ positive cells which have a thinner, more elongated phenotype (Fig. 26D-F). This morphology and position is consistent with multiple cell types in the adult kidney including loop of Henle and vascular endothelium. Furthermore, it was difficult to distinguish nephronic epithelia from collecting duct in deeper parts of the kidney by simply evaluating cell morphology. In order to determine exactly which cell types do or do not arise from the cap mesenchyme we utilized an antibody against β-Gal and cell type specific markers for dual immunofluoresence.

Previous to these studies, there has been a general appreciation that progenitor cells within the cap mesenchyme give rise to multiple cell types within the mature nephron, based on observations using labeled cells in rat tissue recombination experiments. It is not surprising then, that β-Gal colocalized with Na/K ATPase expressing cells, a broadly expressed marker of nephronic epithelia (Fig. 27A-C). Cap mesenchyme derived cells within glomeruli coincide with podoplanin-expressing cells,

Figure 27. ***Cap cell lineage in specialized nephronic epithelia.*** Pregnant Cited1CreER^{T2}/R26R^{LZ} mice were injected with tamoxifen at E13 and lineage was analyzed in the adult. **A-C.** Cortical region stained with αβgal (A) or αNa/K atpase (B) antibody and respective merged image (C). Insets – identical staining demonstrating a tubular structure in cross section. **D-F.** High power image of glomeruli stained with αβgal (D) or αPodoplanin (E) antibody and respective merged image (F); Arrow- glomerular epithelial cells of cap mesenchyme origin. **G.** Glomeruli stained with αWT1 antibody. **G'**. Sequential section to (G) stained with αβGal antibody. Arrow – cells in which βgal and WT1 are colocalized (podocytes). **H.** Papilla stained with αAqp1 antibody. **H'.** Sequential section to (H) stained with αβgal antibody; Arrow - cells in the loop of Henle in which are of cap mesenchyme origin. **I**. Cortex stained with X-Gal and αAqp1. Arrow – proximal tubule cells of cap mesenchyme origin. **L.** Papilla stained with X-Gal and αAqp1. Arrow – thin ascending limb of cap mesenchyme origin.


βGAL
A
Na/K atpase
B
Merge
C
βGAL
D
Podoplanin
E
Merge
F
WT1
G
Aqp1
H
Cortex
Aqp1/LacZ
I
βGAL
G'
βGAL
H'
Medulla
Aqp1/LacZ
J

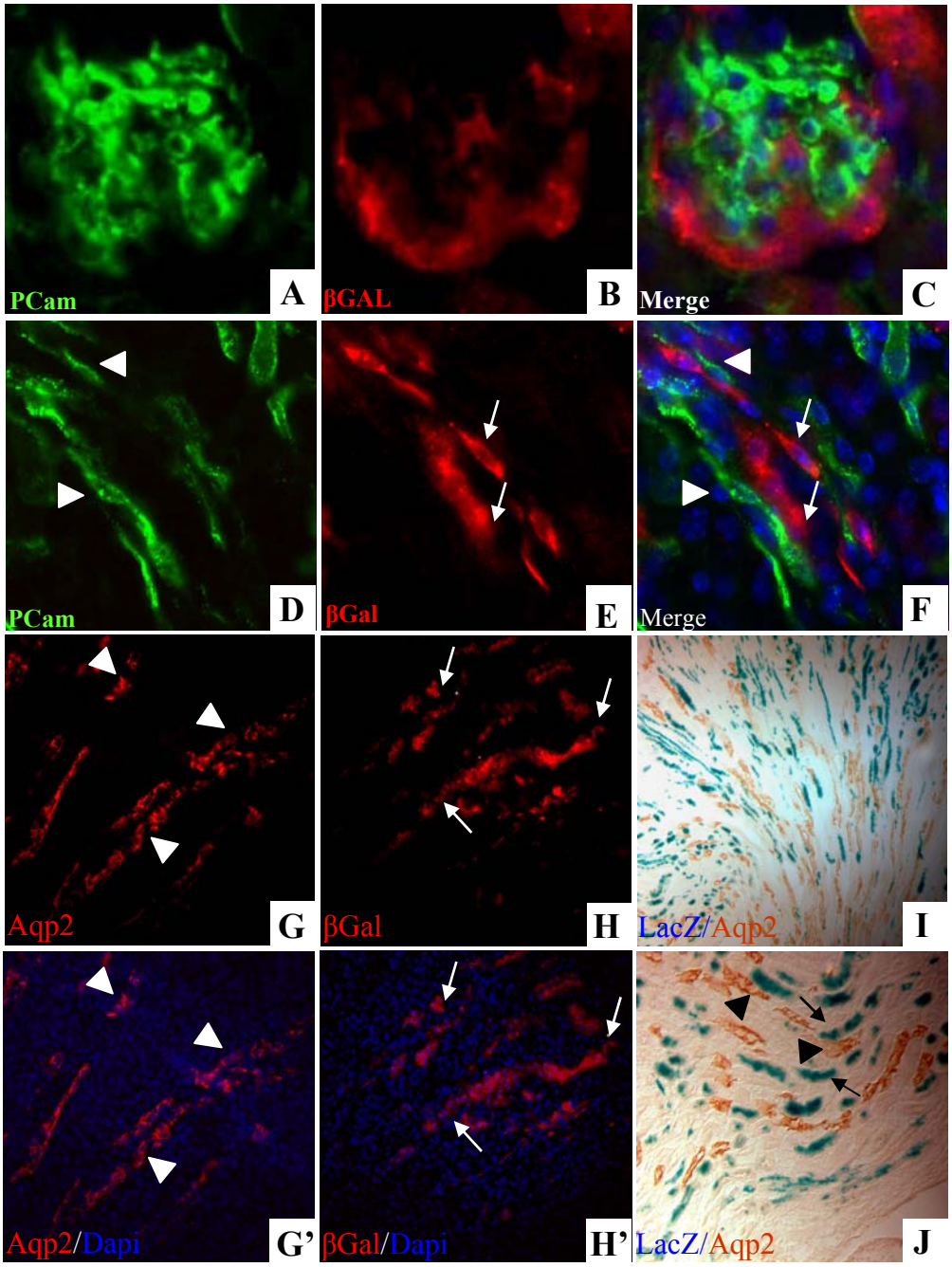
indicating lineage in a broad segment of the glomerular epithelial compartment (Fig. 27D-F). Furthermore, staining on sequential sections using a podocyte-specific antibody detecting WT1 shows that these highly specialized cells within the glomerulus are also of cap mesenchyme origin (Fig. 27G, J).

Aquaporin 1 marks both cortical proximal tubule elements as well as thin limb. Staining of sequential sections with an αAqp1 antibody and β-Gal reveals that the thin limb within the papilla is derived from the cap mesenchyme (Fig. 27H, K) This lineage connection can be demonstrated with greater resolution by performing immunoperoxidase staining on sections which have been X-Gal stained. Using this method, we detected cap derived loop cells in the medulla that also expressed Aqp1 on their cell surface (Fig. 27J). In the β-Gal positive cells that express Aqp1 were also observed in the cortex, demonstrating cap mesenchyme lineage in the proximal tubule (Fig. 27I). Together, these studies show that the cap mesenchyme gives rise to a wide variety of epithelial cell types in the nephron including proximal, distal and glomerular elements. This is the first time these fate connections have been explicitly defined using a genetic tagging method.

Cap mesenchyme does not give rise to renal endothelium or contribute to collecting duct

To determine if the cap mesenchyme gives rise to cell types outside of the nephron proper, I again used a β-Gal antibody and cell specific markers for dual immunofluorescent labeling. Based on morphology and envelopment of RBCs, we speculated that some of the elongated cells we see in our X-Gal stained kidneys could be

Figure 28. ***The cap metanephric mesenchyme does not give rise to renal endothelial cells or contribute to collecting duct.*** Pregnant Cited1CreER^{T2}/R26R^{LZ} mice were injected with tamoxifen at E13 and lineage was analyzed in the adult. **A-C.** Glomerulus stained with αPCAM (A) or αβgal (B) antibody and respective merged image (C). **D-F.** High power image of medulla stained with αPCAM (D) or αβgal (E) antibody and respective merged image (F); Arrowhead- PCAM positive blood vessel. Arrow - βgal positive cells of cap mesenchyme origin. **G.** Medulla stained with αAqp2 antibody. **G'**. Image (G) merged with dapi to identify nuclei. **H.** Sequential section to (G) stained with αβGal antibody. **H'.** Image (H) merged with dapi to identify nuclei; Arrowhead – Aqp2 positive collecting duct. Arrow- βgal positive cells of cap mesenchyme origin. **I**. Medullary/ papillary junction stained with X-Gal and αAqp2. **J.** Magnification of (I) Arrow – LacZ positive cells of cap mesenchyme origin; Arrowhead – Aqp2 positive collecting duct.


PCam
A
βGAL
B
Merge
C
PCam
D
βGal
E
Merge
F
Aqp2
G
βGal
H
LacZ/Aqp2
I
Aqp2/Dapi
G'
βGal/Dapi
H'
LacZ/Aqp2
J

glomerular endothelial cells. Furthermore, the origin of renal endothelium is not well understood. A lineage connection between the cap mesenchyme and the renal endothelium would be unprecedented, and our system allows us a unique opportunity to directly address this question. In these studies we asked whether there were cap derived β-Gal positive cells that also expressed the endothelial specific marker PCAM. Using this analysis we saw no evidence of overlap between these cell populations, in either glomeruli (Fig. 28A-C) or in medullary vessels (Fig 28D-F). We conclude that the elongated cells present in our X-Gal stained lineage traced kidneys are in fact nephronic elements (i.e. thin limb) that are running alongside their closely associated vasculature (Fig. 28F).

As discussed in the Introduction, there has been speculation that the cap mesenchyme can contribute to the collecting duct, though results from previous lineage tracing studies are inconclusive and none were carried out using a genetic system *in vivo*. The system we have developed offers a direct opportunity to address this question carefully. By using the collecting duct specific marker Aquaporin 2 and asking whether it was expressed β-Gal-positive cells we determined whether the cap mesenchyme can indeed contribute to the collecting system. Staining for Aqp2 and β-Gal on sequential sections showed no overlap between these two markers (Fig 28G/G', H/H'). We can examined this in greater resolution by using the Aqp2 antibody on top of sections which have been X-Gal stained (Fig 28I, J). This analysis again demonstrated that while nephrons and collecting ducts are closely opposed as they move through the medulla into the papilla, β-Gal marked cells and collecting ducts are mutually exclusive (Fig. 28J).

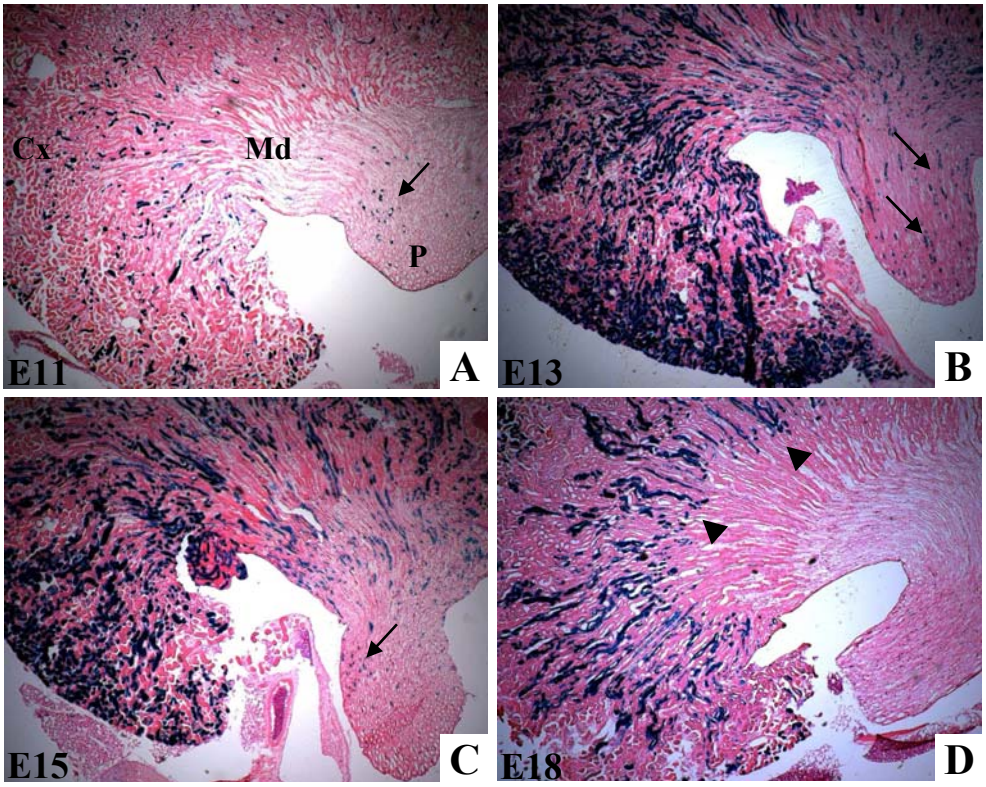


Fig 29. ***Progressive fate of cap mesenchyme cells during the course of kidney development.*** *Cited1-CreER*^{T2} mice were crossed to *R26R*^{LacZ} reporter animals and injected with 1.5mg of tamoxifen at times indicated in lower left. Mice sacrificed at 6 weeks of age. Arrows - deep nephronic elements in papilla. Arrowhead – cortio-medullary junction where superficial nephrons stop. Cx- cortex, Md- medulla, P- papilla

From these studies we conclude that the cap mesenchyme does not give rise to renal endothelial cells or cells within the collecting system.

Patterns of nephron formation during development

A fundamental question that was addressed using this lineage-tracing system was whether cap mesenchyme cells have different fate patterns at different times during development. To test this we crossed *Cited1-CreER*^{T2} mice with *R26R*^{LacZ} reporter mice, injected tamoxifen at varying timepoints during embryogenesis, and examined lineage connections patterns at six weeks of age. At the earliest injection timepoint, E11, we saw that the cap mesenchyme gave rise to cells in all parts of the kidney, including cortical and medullary regions (Fig 29A). Furthermore, the relatively low number of nephrons labeled by injecting at this point is consistent with the fact that only a few cells in the condensing MM express *Cited1* at E11 (Fig. 5C,D). As previously discussed, injecting at E13 labeled a large percentage of total nephrons, with cap derived cells again present from the cortex to the papilla (Fig 29B). An E15 injection gave a similar result, although fewer total nephrons were labeled (Fig. 29C). This is to be expected, as it is well known that many nephron precursors arise from the cap mesenchyme prior to E15. When tamoxifen was injected at E18, however, we observed a different pattern of labeling in which cap- derived cells were present in the cortex and the medulla, but did not extend into the papilla (Fig. 29D). This likely represents a distinction between so called 'deep' and 'superficial' nephrons. Deep nephrons extend all the way into the papilla where their loops of Henle turn and ascend to the collecting duct junction. Superficial nephrons contain all of the same cell types as deep nephrons, but do not extend past the boundary

between the medulla and the papilla. These studies indicate that deep nephrons arise only during the early phase of nephrogenesis and that later stages are marked by the formation of superficial nephrons. These data do not tell us if both types arise during early stages of nephron induction, while only superficial structures are generated from the cap later, but are consistent with the prevailing model of two distinct phases of nephrogenesis.

The cap metanephric mesenchyme is a self-renewing progenitor cell population

One of the striking things uncovered in the studies described above is the high percentage of total nephrons in the adult that were labeled with an E13 injection. The adult mouse has (depending on strain) on the order of 8-10,000 nephrons, yet at E13 there are perhaps only 16 cap condensations containing renal progenitor cells. As discussed above, this speaks to the need for a mechanism to either maintain or recruit new cap cells for induction by the UB. If new cap mesenchyme was generated by recruiting cells from an outside population, then labeling at E13 would only tag the cap mesenchyme present at that time, resulting in a correspondingly low number of nephrons labeled in the adult kidney. Instead, an E13 injection resulted in a large percentage of labeled nephrons in the adult (Fig 26A). This lineage connection strongly suggests that cells in the cap mesenchyme not only give rise to nephronic epithelia, but also divide to produce new renal progenitor cells.

To address this question, we again crossed *Cited1-CreER*^{T2} mice with the *R26R*^{LacZ} reporter and injected tamoxifen at E13. This time, however, we examined cell lineage at E19.5. This timepoint is advantageous because it contains well differentiated

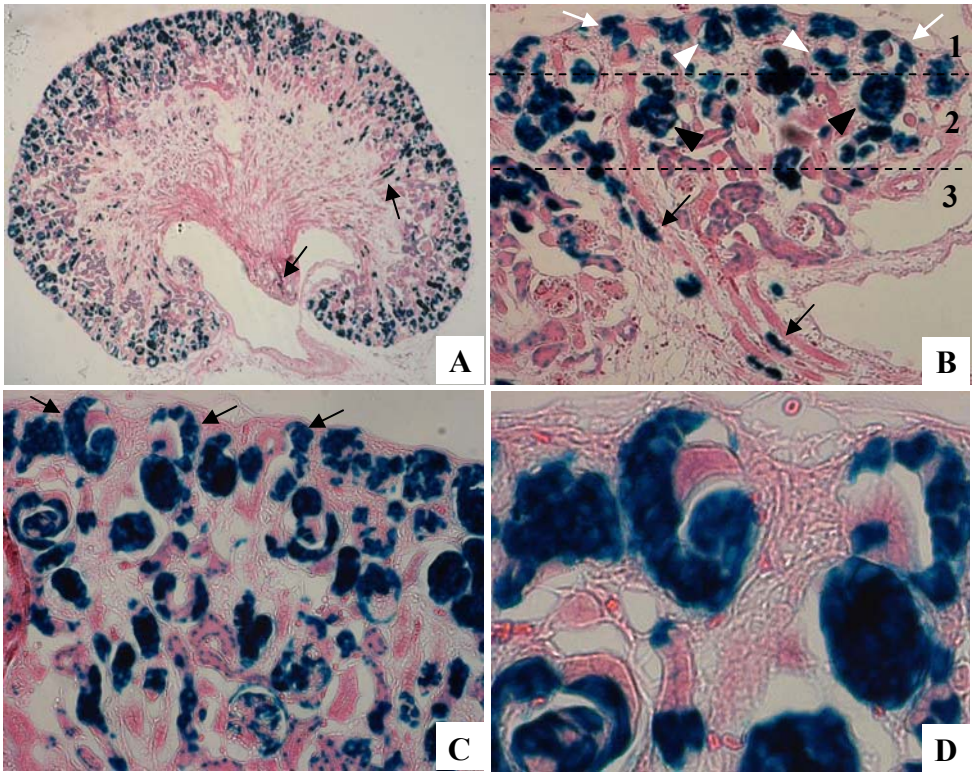


Figure 30. ***The cap metanephric mesenchyme is a self-renewing progenitor cell population***. Pregnant *Cited1-CreER*^{T2}/*R26R*^{LZ} mice were injected with tamoxifen at E13 and lineage was examined at E19.5. **A.** Low power image of entire kidney. Arrow – cells of cap mesenchyme origin which are moving into distal locations. **B.** Image of nephrogenic zone and primitive cortico-medullary junction; Black arrow – cap derived cells which have reached the medulla, black arrowhead – s-shaped body, white arrowhead – RVs and comma-shaped bodies. White arrow – cap metanephric mesenchyme. **C.** Nephrogenic zone. Arrows – cap mesenchyme. **D.** High power image of branched UB.

nephronic epithelia as well as immature structures and cap mesenchyme. In these mice we see that most of the cap mesenchyme progeny are located in the cortex, with only a few distal cells which have reached the forming papilla (Fig. 30A). This is consistent with a proposed model in which nephrons are born and build up in the cortex during embryonic development, while later stages are characterized by the growth and extension of these primitive structures (Cebrian *et al*., 2004). Looking closely at the nephrogenic zone and early cortico-medullary boundary we see three general zones in which cap mesenchyme progeny lie (Fig. 30B). In the deepest zone are the cells that arose from the cap first, as they have gone through the stepwise process of nephron induction and are already extending into the medulla. The next zone is characterized by primitive nephrons in the later stages of induction, most notably s-shaped bodies. In the most outer zone we see cap derived cells in the early stages of differentiation including RVs and comma-shaped bodies. This pattern demonstrates that since the injection at E13, at least 3 generations of nephrons have arisen from the cap mesenchyme. If outside cells were being recruited to repopulate this niche then the cap cells present at this time (E19.5, 6.5 days after injection) would not be labeled, as the originally tagged cells would have been depleted by the induction process and replaced with unlabeled cells. Instead, we see that almost all of the cap mesenchyme in these kidneys is labeled, strongly supporting the hypothesis that they are progeny of the cap cells labeled at E13 (Fig. 30C,D).

To rule out the possibility that labeling of cap mesenchyme at E19.5 in these mice was due to inappropriate, persistent nuclear translocation of Cre, we examined its subcellular localization following tamoxifen injection (Fig. 31). For these studies mice carrying transgenic litters were injected between E14 and E15 and sacrificed at indicated

Figure 31. ***Kinetics of Cre subcellular localization following tamoxifen injection in Cited1-CreER^{T2} kidneys.*** **A-D**. Cre localization using antibody staining in E16.5 kidneys; **A'–D'** corresponding images overlaid with the nuclear stain Dapi. **A.** Uninjected animal; Arrow- example of cell with cytoplasmic Cre localization. **B.** Cre localization 8 hours after injection; Arrow – cell with nuclear and cytoplasmic Cre, Arrowhead – cell with nuclear localization of Cre. **C.** Cre localization 24 hours after injection; Arrowhead – cell with nuclear localization of Cre. **D.** Cre localization 4 days after injection. Arrow – cell with cytoplasmic Cre

0 hours
8 hours
24 hours
96 hours
A
B
C
D
A'
B'
C'
D'
UB
UB
UB
UB
mαCre
mαCre / Dapi

times. In uninjected mice, antibody staining detects cytoplasmic localization of Cre (Fig. 31A, A'). 8 hours after injection, we begin to see nuclear translocation of Cre, although some cells still have predominantly cytoplasmic expression (Fig. 31B,B') By 24 hours, most cells have Cre strongly localized to the nucleus (Fig. 31C, C') Importantly, by 96 hours Cre has left the nucleus and is thus no longer active (Fig. 31D, D') These results are consistent with previously published data in using this system (Hayashi and McMahon, 2002). These data rule out the possibility that cap cells are labeled at E19.5 due to persistent recombination, and strongly support a model of the cap metanephric mesenchyme as a self-renewing progenitor cell population.

Conclusion

Here we have described the creation of *Cited1-CreER*^{T2} transgenic mice. These animals express tamoxifen inducible Cre recombinase in the Cited1 expression domain, which is the cap metanephric mesenchyme in the developing kidney. This line represents a novel tool in the field and allows for lineage tracing of renal progenitor cells as well as temporally and spatially controlled gene deletion. Using these mice we have investigated the fate of the cap mesenchyme at varying points during development. We show that this population is capable of giving rise to cells in all segments of the adult nephron, and that by tagging cells at E13 we see lineage in most mature nephrons. Furthermore, we show that nephrons which arise prior to E18 are capable of becoming deep nephrons, extending well into the papilla, while after E18 new tubules adopt the superficial fate. Lastly, we show for the first time that the cap metanephric mesenchyme

is capable of self-renewal, meaning that all specialized epithelia in the mature nephron likely arise from a common progenitor pool.

These findings are important as this is the first study to completely trace the lineage of cap mesenchyme in mice and demonstrate *in vivo* that specialized epithelia in all segments of the nephron can be derived from this cell population. Our observation that after E18 the cap mesenchyme gives rise to only superficial nephrons is striking, and supports a model of distinct phases of nephrogenesis. Most importantly, we provide strong evidence that during development cells within the cap mesenchyme give rise to new rounds of renal progenitor cells. Aside from the studies presented here, the *Cited1-CreER*^{T2} mouse can now be used as a powerful tool to examine cap cell behavior real-time in organ culture, and as a platform for temporally controlled gene deletion in the cap metanephric mesenchyme.

CHAPTER V

DISCUSSION and FUTURE DIRECTIONS

The primary focus of my research has been to investigate the function of the Cited family of transcriptional co-factors in the developing kidney. Though this question remains largely unanswered, I think the work presented here adds important information and tools to the field of kidney development. It also offers ample future opportunities to further understand the normal and abnormal regulation of this process. Below, I will discuss the key findings of my research and their implications, while interspersing interesting future experiments which they suggest.

Cited1 in the developing kidney

We show that *Cited1*, *Cited2*, and *Cited4* are expressed dynamically in the developing kidney, in cell types which are of high interest to the field at large. The expression domain of Cited1 is particularly striking, as it is upregulated in the MM response to UB invasion and is restricted to a subset of the condensing mesenchyme called the cap mesenchyme. These are the cells that aggregate most tightly around the branched ends of the UB and undergo a characteristic pattern of differentiation in response to factors secreted by UB tips. This expression domain is unique among other characterized factors present in the MM. The closest pattern is that of Six2 (Self *et al*., 2006), which is also expressed in the cap structure. *Six2*, however, is expressed in the uninduced mesenchyme and in the clefts between the UB tips. Based on this expression

domain, an interesting experiment would be to fate map Six2 expressing cells as we did with Cited1 expressing cells (chapter 4) and compare the two lineage patterns (in fact, tools to do such an experiment are currently being developed in the Andy McMahon laboratory at Harvard). It is possible that the cells in the clefts between UB tips have a distinct lineage, such as interstitum, which would be absent in our lineage studies.

In the cap mesenchyme, Cited1 is primarily localized in the cytoplasmic compartment and is downregulated during the earliest phases of progenitor cell differentiation. During this stage, cells move to the ventral aspect of the UB tip and form first the PTA and then the RV. By the time cells reach this initial stage of polarization, Cited1 is completely absent. This pattern of subcellular localization and dynamic expression is interesting in general, but may have broader implications when considering data generated in our laboratory regarding the pediatric malignancy Wilms' tumor. This lesion is characterized by the inappropriate persistence of undifferentiated MM which becomes the aggressive component of the tumor. Cited1 expression is retained in these cells in nearly all Wilms' tumors examined. Together with our data in the developing kidney, this strongly suggests that Cited1 expression is associated with early, undifferentiated renal progenitor cells. Furthermore, in Wilms' tumor, Cited1 is primarily found in the nucleus. This raises questions about the regulation of Cited1 expression and localization and the impact it has on normal and abnormal kidney development.

As no mouse model of Wilms' tumor exists, it would be interesting to see if persistent Cited1 expression in the cap mesenchyme was sufficient to create nephrogenic rests and predispose mice to this lesion. Using our *Cited1-CreER*^{T2} system we could

address this question. To do this we would create a transgenic or knock-in mouse which had a floxed stop cassette in between a ubiquitously expressed promoter (such as *R26R*) and full-length mouse *Cited1*. By crossing this mouse to the *Cited1-CreER*^{T2} animal we could 'turn on' persistent expression of Cited1 in cells that would normally downregulate it in response to differentiation cues. This is essentially an *in vivo* recapitulation of what we have done using a TAT fusion protein to overexpress Cited1 in cultured kidneys *in vitro*. In these experiments Cited1 blocked the formation of new epithelial structures and expanded the condensed MM. Based on this, these types of experiments seem likely to yield important information about the role of Cited1 in the MM, regardless of whether they result in mice predisposed to Wilms' tumor. If these mice did yield an interesting phenotype, one could imagine several modifications we could make to the expression construct to test different questions regarding the function of Cited1. For instance, an epitope tagged version of the construct could be introduced to facilitate ChIp on CHIP assays in the search for MM specific Cited1 target genes and/or protein binding partners. Furthermore, deletion mutants could be expressed to ask *in vivo* questions about structure/function relationships. Several good candidates have already been characterized including ΔCR2 (no transactivation domain), ΔSID (no effect downstream of TGFβ signals) and ΔNES (point mutated NES which renders protein constitutively nuclear).

Despite its unique expression pattern, we have shown that Cited1 is dispensable for normal kidney development. This was of course disappointing, but does not rule out the possibility that Cited1 plays other important roles in this context. Perhaps defects in *Cited1* null mice could be uncovered using one of the many injury models available for study of the kidney. In fact, preliminary data from our lab suggests that Cited1 is

upregulated in the proximal tubule in response to mercury induced tubular injury and dedifferentiation. Furthermore, as discussed above, Cited1 may prove to be an important player in the pathogenesis of Wilms' tumor.

Cited2 and Cited4 in the developing and adult kidney

Knowing that deletion of *Cited1* does not result in defects in kidney development, we hypothesized that other members of this gene family could be substituting for Cited1 in the context of the MM. We show that *Cited2* and *Cited4* also have interesting expression domains in the developing kidney, though these are distinct from that of *Cited1*. *Cited2* is expressed in the condensed mesenchyme but persists in more differentiated epithelial structures, including striking expression in glomeruli. *Cited4,* on the other hand, is expressed in the UB, most prominently in the tips. Neither of these proteins has been previously investigated in the context of kidney development.

Owing to their overlapping expression pattern we focused on Cited2 as a candidate for functional redundancy with Cited1 in the MM. To test this hypothesis we examined kidney development in *Cited2*$^{-/-}$ and *Cited1*$^{-/-}$/*Cited2*$^{-/-}$ compound mutant mice. These studies required the use of an organ culture system as deletion of *Cited2* results in embryonic lethality near the onset of kidney development. In the absence of *Cited2* we did not observe major perturbation of UB branching or total number of new nephrons induced. While this leads us to conclude that Cited2 is not required in the MM during early induction and differentiation events, it does not rule out a role later for Cited2 in the developing kidney. The organ culture system we employed is limited to the study of early events as nephron maturation and kidney patterning do not take place *in vitro*. The

expression domain of *Cited2* in glomeruli and the forming papilla suggest that it could play a role in both of these processes. This could be addressed in two ways. Isolated kidney rudiments from *Cited2*$^{-/-}$ mice could be grown under the kidney capsule of an adult host mouse, which allows for maturation and vascularization of the glomerulus as well as some cortico-medullary patterning. Alternatively, *Cited2* could be conditionally deleted in the MM (and thus in all its derivatives) using our *Cited1-CreER*^{T2} animal. In this way we would circumvent the requirement for Cited2 in the heart and placenta and have the opportunity to study its function later in the context of nephrogenesis. In fact, our collaborator Sallie Dunwoodie has recently published the creation of a *Cited2* conditional allele which could be used for these studies (Preis *et al*., 2006).

Our attempt to generate *Cited1*$^{-/-}$ / *Cited2* $^{-/-}$ double mutant mice revealed that in the absence of both of these factors the embryonic lethality observed in *Cited2* null mice is hastened. In our studies (totaling 14 litters) only four double null embryos survived until E12 (the time of kidney dissection for organ culture), compared just one *Cited2* null mouse which did not survive. Deletion of both genes likely exacerbates cardiac defects observed in *Cited2*$^{-/-}$ mice, as Cited1 is also expressed in the developing heart (Fig 15D; (Dunwoodie et al., 1998). In those embryos from which we were able to culture kidneys (2 total) we saw a reduction in UB branching and induction of new nephronic epithelia, though the overall processes of branching and induction do seem to be intact. We were not, however, able to recover a sufficient quantity of double null kidneys to demonstrate statistical significance or examine this possible defect in detail. This is, again, an excellent application for conditional targeting. By crossing the *Cited1-CreER*^{T2} transgene (on a *Cited*1 null background) to the conditional *Cited2* allele, we could create double

mutants in which *Cited2* is deleted only in the condensed MM. By temporally regulating control of gene deletion in this system we would hopefully be able to avoid early, lethal defects in the heart which may be caused by expression of the transgene, (and subsequent deletion of Cited2), in that tissue.

Cited4 is also expressed in the developing kidney but in a pattern completely distinct from *Cited1* and *Cited2*. It is localized exclusively to the UB, and is expressed most prominently in the tips. This expression pattern is reminiscent of the receptor tyrosine kinase Ret, which is required for UB growth in response to Gdnf. Furthermore, both Wnt9b and Wnt11 are expressed in this domain, raising the possibility that Cited4 modulates transcriptional responses downstream in this pathway (based on the ability of Cited1 to do so). Furthermore, *Cited4* is upregulated in cells within the stalk of the UB at later stages of kidney development. It is at this time that UB is elongating and growing towards the medulla en route to forming the collecting system. This presumably requires programs which drive proliferation within stalk cells. Given its expression in tip cells, which are highly proliferative, it is possible that Cited4 acts to regulate cell division in the UB. If this is the case, its function is not required *in vivo*, as we have shown that deletion of *Cited4* does not perturb UB branching or growth and results in adult mice with morphologically normal kidneys.

Unlike *Cited1*, *Cited2* and *Cited4* are both expressed in the adult kidney. *Cited2* is most prominently expressed in the papilla, and appears to be present in all cell types there. *Cited4* expression is restricted to the collecting duct, which along with interstitial cells and loops of Henle makes up much of the papilla. These domains are quite intriguing as the papilla is know to be highly hypoxic and the ability of Cited2 and Cited4

to regulate hypoxic signaling is well documented (Bhattacharya *et al*., 1999; Bhattacharya and Ratcliffe, 2003; Fox *et al*., 2004; Freedman *et al*., 2003). This raises questions about the role of these factors in development and maintenance of cells within the papilla through modulation of the hypoxic environment. In terms of Cited2, this is again an excellent opportunity for conditional gene deletion to study its role in papillary development, as the strategies we have employed thus far do not allow us to examine the effects of *Cited2* deletion at later stages of development. These genes may also serve an important role in papillary function in the adult, which can only be uncovered by experimentally stressing the system and investigating the physiological consequences of *Cited2/4* deletion in this context. Either way, the interesting expression pattern of these proteins in the adult offers an additional opportunity to address the function of Cited family genes *in vivo*.

Regulation of cell-ECM interactions by Cited1

As an adjunct to the work I have done *in vivo*, I have also used cell culture systems to address the function of Cited1. Initial attempts at this highlighted the well known difficulties associated with working with MM derived cell lines. The data I generated using a rat MM cell line were inconsistent and difficult to reproduce and left us in search of another platform on which to address the function of Cited1. Using MCF7 cells, a ductal breast cancer line which express high levels of Cited1 endogenously, we modulated Cited1 protein levels using siRNA. This approach uncovered a role for Cited1 in regulation of cell-ECM interactions as knockdown of Cited1 compromised the ability of MCF7 cells to migrate through a matrigel matrix. This effect was associated with a

defect in the ability of these cells to adhere to selective components of the ECM including Laminin I and Type 1 collagen and a failure of characteristic actin organization during attachment. From a kidney development standpoint this is interesting because the way cells within the cap mesenchyme interact with the environment in which they find themselves almost certainly determines their ability to move around the UB tip and form early epithelial structures.

It is unclear by what mechanism Cited1 exerts its effect on cell migration and adhesion, but we have ruled out changes in cell surface expression of β1, α1, or α6 integrin as well as impaired phosphorylation of FAK. There are several issues that can be addressed in an attempt to understand the function of Cited1 in this setting. The first step in this process would be to show that mouse Cited1 expressed in MCF7$^{Cited1K/D}$ cells could elude siRNA and rescue defects in cell adhesion. I have developed several of the tools needed for this system and work in the lab is ongoing.

Provided we could rescue this phenotype with transfected m*Cited1*, we could test different parameters of its function in this system. For instance, one issue is whether Cited1 mediates MCF7 adhesion through transcriptional or a non-transcriptional mechanisms. The fact that we so often detect Cited1 predominantly in the cytoplasm raises the possibility that it may have non-nuclear functions. These types of experiments would be best accomplished using a system in which we could regulate the trafficking of Cited1 to the nucleus through fusion to a steroid receptor. In this way we could express full length Cited1 and deletion mutants and assay for their ability to rescue the adhesion defect and the ask questions about the role of subcellular localization in this process. Having this information would allow us to focus on a potential mechanism en route to

understanding how Cited1 regulates adhesion. For instance, if we determined that cytoplasmic localization was necessary for this action we could further interrogate the integrin signaling pathway downstream of FAK by looking at activation of other important players in cell adhesion and migration, such as the Rho and Rac family of small GTPases. If we saw that nuclear translocation and transcriptional activity were required to rescue the defect, we could use microarray or ChIP on chip to look for Cited1 target genes which regulate cell adhesion. Interesting findings from these studies could then be applied to *in vivo* investigation in the developing kidney.

Cited1-CreER^{T2} mice and patterns of cap mesenchyme differentiation

Despite the fact that Cited1 is not required for normal nephrogenesis, we saw its expression domain as a unique opportunity to fate map a distinct population of renal progenitor cells. To do this we utilized a BAC strategy to create a transgenic mouse which expresses an inducible form of Cre recombinase under the control of Cited1 promoter elements. By crossing this mouse with a conditional reporter line, such as *R26R*^{LZ}, we can acutely tag Cited1 expressing cells in the cap mesenchyme. Because the 'tag' in this system occurs at the genomic level, all progeny of cells that were tagged in the cap will express the reporter. In this way we can ask question about the fate and differentiation patterns of these progenitor cells.

My work has shown that cells within the cap mesenchyme are capable of giving rise to specialized epithelial cells in all segments of the mature nephron. Furthermore, nephrons which arise prior to E18 can become so called 'deep' nephrons, whose distal elements extend all the way to the papilla. By contrast, nephrons which are specified

after E18 only occupy the superficial compartment, with their deepest elements reaching only the into the medulla. This distinct pattern of timing of nephron differentiation has not been previously shown.

We have also used this tool to address the question of cap mesenchyme repopulation. Because successive generations of new nephrons are induced throughout kidney development, there must be a mechanism that provides a continual pool of progenitor cells at each UB tip. This process is not well understood. The two models most often discussed are 1) new cap mesenchyme is recruited from surrounding cell populations, such as the stroma or 2) the cap mesenchyme has programs which hold cells in an undifferentiated state and allow for their self-renewal. Using *Cited1-CreER*^{T2} mice in our lineage tracing system, we have shown that much of the cap mesenchyme remains labeled more that 6 days after tamoxifen administration, this despite the fact that 3 to 4 generations of nephrons have arisen from the cap since injection. If new progenitor cells were migrating in from outside the cap structure we would expect that labeled cells in the cap would be exhausted by formation of primitive nephrons and progressively replaced by unlabeled cells. Instead we see that nearly all cap cells are labeled, indicating that they are progeny of the cells originally tagged. This phenomena has been suspected, but not previously shown. Importantly, a recent paper has demonstrated that the transcription factor Six2 is required to oppose MM differentiation and promote renewal of the cap mesenchyme (Self *et al*., 2006). In the absence of *Six2*, ectopic renal vesicles form dorsal to the UB, and there is a progressive loss of condensed mesenchyme. Combined with our data, these results strongly support a model in which renal progenitor cells are capable of self-renewal.

As detailed above, we envision the *Cited1-CreER*^{T2} transgenic mouse as a broadly applicable tool for the study of kidney development. Though many experiments using this line can be imagined, I would like to highlight two that are of particular interest.

The possibility that Cited1 regulates cell adhesion and migration brings to light how little we know about this process in the context of the cap mesenchyme. For instance, we do not understand the dynamics of cell movement out of the cap mesenchyme en route to forming the PTA and RV or how these cells change their ability to interact with one another and the ECM. Such issues have been exquisitely addressed in the UB using time-lapse live cell imaging in cultured kidney explants (Shakya *et al*., 2005). In these studies the UB is fluorescently labeled allowing for detailed analysis of how the UB grows and branches. By crossing the *Cited1-CreER*^{T2} mouse with a fluorescent conditional reporter, such as *R26R*^{YFP}, we can ask similar questions about the cap mesenchyme. Though not shown here, we have demonstrated efficient recombination using soluble tamoxifen in kidneys cultured from *Cited1-CreER*^{T2}/*R26R*^{LZ} mice. Using this system we could image real-time events of renal progenitor cell differentiation including early movements and fusion with the UB. Results of these studies could then be extended to investigate factors important for these processes, both in organ culture through the addition of transgenes or soluble growth factors, and *in vivo* using this platform for conditional gene targeting.

Another important experiment which comes to mind is the question of cell fate specification within cap mesenchyme cells. We have shown that when you label a large number of these cells, lineage is observable in all segments of the mature nephron. This does not address, however, the potential of an individual cell within the cap mesenchyme.

For instance is cell fate pre-determined at this early stage? Does a given cap cell which enters the PTA give rise to say, only podocytes, or can it produce cells in multiple parts of the mature nephron? Furthermore, is there a subset of cells within the cap whose only job is to self renew, or are all cap cells capable of this and positional cues determine differentiation state? To address this question properly, we would need to devise a way to label a single cell in each cap and then follow its lineage throughout development. A first approach to this would be to identify a dose of tamoxifen which was sufficiently low to tag only one cell per cap. This could be confirmed acutely, by looking at labeled cells shortly after injection. For instance, if a single cell has been labeled and divided once we would expect to see these two cells in close proximity to one another, whereas if many cells in the cap had been labeled we would see clusters of cells within the MM. This early lineage could be confirmed by co-administering BrdU which would be retained in daughters of originally labeled cap cells. Once this dose has been established we could repeat our long term lineage studies to ask questions about the potential of individual cap cells. This could also be carried out in organ culture by using *R26R*^{YFP} mice and live cell imaging. After determining an appropriate dose of soluble tamoxifen to label single cells, we could track the lineage of these cell in real-time. This method has limitations however, as epithelial structures formed in these cultured kidneys do not undergo later patterning events.

If we could not identify a dose of tamoxifen which has this effect, an alternative approach would be to utilize the LaacZ system of clonal analysis (Bonnerot and Nicolas, 1993; Nicolas *et al*., 1996). In this system a reporter construct which contains a duplication of the LacZ ORF is inserted downstream of an appropriate promoter, and

expressed as a transgene in mice. This duplication results in a non-functional LacZ transcript. In a very small percentage of cells, however, this duplication is corrected by an intergenic recombination event, restoring proper LacZ expression. The likelihood that this correction would happen in more than one cells in a given population is extremely low. Using this method, we could administer a normal dose of tamoxifen yet only label single cells within the cap. The progeny of these cells could then be examined in the embryo and the adult, addressing questions of differentiation patterns and fate potential of clonal derivatives of individual progenitor cells.

Concluding remarks

The studies presented here demonstrate that the Cited family of transcriptional co-factors are dynamically expressed in the developing kidney, but are not required for nephrogenesis. While we do not yet understand the function of these proteins in this setting, this work has opened other avenues worthy of investigation which should shed light broader aspects of kidney development. Most notably, we have described the generation of a *Cited1-CreER*^{T2} transgenic animal which has allowed us to uncover previously unknown patterns of cell fate in the developing and adult kidney. Aside from these studies, the spatial restriction and inducible nature of this transgene makes it a novel, powerful tool which can be widely applied in the field of kidney development to study gene function in the cap mesenchyme.

BIBLIOGRAPHY

Auerbach, A. B., Norinsky, R., Ho, W., Losos, K., Guo, Q., Chatterjee, S. and Joyner, A. L. (2003). Strain-dependent differences in the efficiency of transgenic mouse production. *Transgenic Res* **12**, 59-69.

Ayres, J. A., Shum, L., Akarsu, A. N., Dashner, R., Takahashi, K., Ikura, T., Slavkin, H. C. and Nuckolls, G. H. (2001). DACH: genomic characterization, evaluation as a candidate for postaxial polydactyly type A2, and developmental expression pattern of the mouse homologue. *Genomics* **77**, 18-26.

Bamforth, S. D., Braganca, J., Eloranta, J. J., Murdoch, J. N., Marques, F. I., Kranc, K. R., Farza, H., Henderson, D. J., Hurst, H. C. and Bhattacharya, S. (2001). Cardiac malformations, adrenal agenesis, neural crest defects and exencephaly in mice lacking Cited2, a new Tfap2 co-activator. *Nat Genet* **29**, 469-74.

Bamforth, S. D., Braganca, J., Farthing, C. R., Schneider, J. E., Broadbent, C., Michell, A. C., Clarke, K., Neubauer, S., Norris, D., Brown, N. A. et al. (2004). Cited2 controls left-right patterning and heart development through a Nodal-Pitx2c pathway. *Nat Genet* **36**, 1189-96.

Barasch, J., Yang, J., Ware, C. B., Taga, T., Yoshida, K., Erdjument-Bromage, H., Tempst, P., Parravicini, E., Malach, S., Aranoff, T. et al. (1999). Mesenchymal to epithelial conversion in rat metanephros is induced by LIF. *Cell* **99**, 377-86.

Barbera, J. P., Rodriguez, T. A., Greene, N. D., Weninger, W. J., Simeone, A., Copp, A. J., Beddington, R. S. and Dunwoodie, S. (2002). Folic acid prevents exencephaly in Cited2 deficient mice. *Hum Mol Genet* **11**, 283-93.

Bhattacharya, S., Michels, C. L., Leung, M. K., Arany, Z. P., Kung, A. L. and Livingston, D. M. (1999). Functional role of p35srj, a novel p300/CBP binding protein, during transactivation by HIF-1. *Genes Dev* **13**, 64-75.

Bhattacharya, S. and Ratcliffe, P. J. (2003). ExCITED about HIF. *Nat Struct Biol* **10**, 501-3.

Bonnerot, C. and Nicolas, J. F. (1993). Application of LacZ gene fusions to postimplantation development. *Methods Enzymol* **225**, 451-69.

Bouchard, M., Souabni, A., Mandler, M., Neubuser, A. and Busslinger, M. (2002). Nephric lineage specification by Pax2 and Pax8. *Genes Dev* **16**, 2958-70.

Boyle, S. and de Caestecker, M. (2006). Role of transcriptional networks in coordinating early events during kidney development. *Am J Physiol Renal Physiol* **291**, F1-8.

Braganca, J., Eloranta, J. J., Bamforth, S. D., Ibbitt, J. C., Hurst, H. C. and Bhattacharya, S. (2003). Physical and functional interactions among AP-2 transcription factors, p300/CREB-binding protein, and CITED2. *J Biol Chem* **278**, 16021-9.

Braganca, J., Swingler, T., Marques, F. I., Jones, T., Eloranta, J. J., Hurst, H. C., Shioda, T. and Bhattacharya, S. (2002). Human CREB-binding protein/p300-interacting transactivator with ED-rich tail (CITED) 4, a new member of the CITED family, functions as a co-activator for transcription factor AP-2. *J Biol Chem* **277**, 8559-65.

Brodbeck, S. and Englert, C. (2004). Genetic determination of nephrogenesis: the Pax/Eya/Six gene network. *Pediatr Nephrol* **19**, 249-55.
Brophy, P. D., Ostrom, L., Lang, K. M. and Dressler, G. R. (2001). Regulation of ureteric bud outgrowth by Pax2-dependent activation of the glial derived neurotrophic factor gene. *Development* **128**, 4747-56.
Cacalano, G., Farinas, I., Wang, L. C., Hagler, K., Forgie, A., Moore, M., Armanini, M., Phillips, H., Ryan, A. M., Reichardt, L. F. et al. (1998). GFRalpha1 is an essential receptor component for GDNF in the developing nervous system and kidney. *Neuron* **21**, 53-62.
Carroll, T. J., Park, J. S., Hayashi, S., Majumdar, A. and McMahon, A. P. (2005). Wnt9b plays a central role in the regulation of mesenchymal to epithelial transitions underlying organogenesis of the mammalian urogenital system. *Dev Cell* **9**, 283-92.
Cebrian, C., Borodo, K., Charles, N. and Herzlinger, D. A. (2004). Morphometric index of the developing murine kidney. *Dev Dyn* **231**, 601-8.
Chandler, R. L., Chandler, K. J., McFarland, K. A. and Mortlock, D. P. (2007). Bmp2 transcription in osteoblast progenitors is regulated by a distant 3' enhancer located 156.3 kilobases from the promoter. *Mol Cell Biol* **27**, 2934-51.
Cheng, H. T., Kim, M., Valerius, M. T., Surendran, K., Schuster-Gossler, K., Gossler, A., McMahon, A. P. and Kopan, R. (2007). Notch2, but not Notch1, is required for proximal fate acquisition in the mammalian nephron. *Development* **134**, 801-11.
Copeland, N. G., Jenkins, N. A. and Court, D. L. (2001). Recombineering: a powerful new tool for mouse functional genomics. *Nat Rev Genet* **2**, 769-79.
Costantini, F. and Shakya, R. (2006). GDNF/Ret signaling and the development of the kidney. *Bioessays* **28**, 117-27.
Cox, B. D., Natarajan, M., Stettner, M. R. and Gladson, C. L. (2006). New concepts regarding focal adhesion kinase promotion of cell migration and proliferation. *J Cell Biochem* **99**, 35-52.
Donovan, M. J., Natoli, T. A., Sainio, K., Amstutz, A., Jaenisch, R., Sariola, H. and Kreidberg, J. A. (1999). Initial differentiation of the metanephric mesenchyme is independent of WT1 and the ureteric bud. *Dev Genet* **24**, 252-62.
Dressler, G. R. (2006). The cellular basis of kidney development. *Annu Rev Cell Dev Biol* **22**, 509-29.
Dressler, G. R., Deutsch, U., Chowdhury, K., Nornes, H. O. and Gruss, P. (1990). Pax2, a new murine paired-box-containing gene and its expression in the developing excretory system. *Development* **109**, 787-95.
Dudley, A. T., Godin, R. E. and Robertson, E. J. (1999). Interaction between FGF and BMP signaling pathways regulates development of metanephric mesenchyme. *Genes Dev* **13**, 1601-13.
Dudley, A. T., Lyons, K. M. and Robertson, E. J. (1995). A requirement for bone morphogenetic protein-7 during development of the mammalian kidney and eye. *Genes Dev* **9**, 2795-807.
Dunn, N. R., Winnier, G. E., Hargett, L. K., Schrick, J. J., Fogo, A. B. and Hogan, B. L. (1997). Haploinsufficient phenotypes in Bmp4 heterozygous null mice and modification by mutations in Gli3 and Alx4. *Dev Biol* **188**, 235-47.

Dunwoodie, S. L., Rodriguez, T. A. and Beddington, R. S. (1998). Msg1 and Mrg1, founding members of a gene family, show distinct patterns of gene expression during mouse embryogenesis. *Mech Dev* **72**, 27-40.
Feil, R., Brocard, J., Mascrez, B., LeMeur, M., Metzger, D. and Chambon, P. (1996). Ligand-activated site-specific recombination in mice. *Proc Natl Acad Sci U S A* **93**, 10887-90.
Feil, R., Wagner, J., Metzger, D. and Chambon, P. (1997). Regulation of Cre recombinase activity by mutated estrogen receptor ligand-binding domains. *Biochem Biophys Res Commun* **237**, 752-7.
Fluge, O., Bruland, O., Akslen, L. A., Lillehaug, J. R. and Varhaug, J. E. (2006). Gene expression in poorly differentiated papillary thyroid carcinomas. *Thyroid* **16**, 161-75.
Fox, S. B., Braganca, J., Turley, H., Campo, L., Han, C., Gatter, K. C., Bhattacharya, S. and Harris, A. L. (2004). CITED4 inhibits hypoxia-activated transcription in cancer cells, and its cytoplasmic location in breast cancer is associated with elevated expression of tumor cell hypoxia-inducible factor 1alpha. *Cancer Res* **64**, 6075-81.
Freedman, S. J., Sun, Z. Y., Kung, A. L., France, D. S., Wagner, G. and Eck, M. J. (2003). Structural basis for negative regulation of hypoxia-inducible factor-1alpha by CITED2. *Nat Struct Biol* **10**, 504-12.
Fryknas, M., Wickenberg-Bolin, U., Goransson, H., Gustafsson, M. G., Foukakis, T., Lee, J. J., Landegren, U., Hoog, A., Larsson, C., Grimelius, L. et al. (2006). Molecular markers for discrimination of benign and malignant follicular thyroid tumors. *Tumour Biol* **27**, 211-20.
Giraldo, P. and Montoliu, L. (2001). Size matters: use of YACs, BACs and PACs in transgenic animals. *Transgenic Res* **10**, 83-103.
Godley, L. A., Kopp, J. B., Eckhaus, M., Paglino, J. J., Owens, J. and Varmus, H. E. (1996). Wild-type p53 transgenic mice exhibit altered differentiation of the ureteric bud and possess small kidneys. *Genes Dev* **10**, 836-50.
Grieshammer, U., Cebrian, C., Ilagan, R., Meyers, E., Herzlinger, D. and Martin, G. R. (2005). FGF8 is required for cell survival at distinct stages of nephrogenesis and for regulation of gene expression in nascent nephrons. *Development* **132**, 3847-57.
Grobstein, C. (1953). Inductive epitheliomesenchymal interaction in cultured organ rudiments of the mouse. *Science* **118**, 52-5.
Hayashi, S. and McMahon, A. P. (2002). Efficient recombination in diverse tissues by a tamoxifen-inducible form of Cre: a tool for temporally regulated gene activation/inactivation in the mouse. *Dev Biol* **244**, 305-18.
Herzlinger, D., Koseki, C., Mikawa, T. and al-Awqati, Q. (1992). Metanephric mesenchyme contains multipotent stem cells whose fate is restricted after induction. *Development* **114**, 565-72.
Hogan, B. (1994). Manipulating the mouse embryo : a laboratory manual. Plainview, N.Y.: Cold Spring Harbor Laboratory Press.
Howlin, J., McBryan, J., Napoletano, S., Lambe, T., McArdle, E., Shioda, T. and Martin, F. (2006). CITED1 homozygous null mice display aberrant pubertal mammary ductal morphogenesis. *Oncogene* **25**, 1532-42.

Huang, Y., Prasad, M., Lemon, W. J., Hampel, H., Wright, F. A., Kornacker, K., LiVolsi, V., Frankel, W., Kloos, R. T., Eng, C. et al. (2001). Gene expression in papillary thyroid carcinoma reveals highly consistent profiles. *Proc Natl Acad Sci U S A* **98**, 15044-9.

Jakowlew, S. B., Moody, T. W. and Mariano, J. M. (1997). Transforming growth factor-beta receptors in human cancer cell lines: analysis of transcript, protein and proliferation. *Anticancer Res* **17**, 1849-60.

Jena, N., Martin-Seisdedos, C., McCue, P. and Croce, C. M. (1997). BMP7 null mutation in mice: developmental defects in skeleton, kidney, and eye. *Exp Cell Res* **230**, 28-37.

Kanwar, Y. S., Wada, J., Lin, S., Danesh, F. R., Chugh, S. S., Yang, Q., Banerjee, T. and Lomasney, J. W. (2004). Update of extracellular matrix, its receptors, and cell adhesion molecules in mammalian nephrogenesis. *Am J Physiol Renal Physiol* **286**, F202-15.

Karavanov, A. A., Karavanova, I., Perantoni, A. and Dawid, I. B. (1998). Expression pattern of the rat Lim-1 homeobox gene suggests a dual role during kidney development. *Int J Dev Biol* **42**, 61-6.

Kispert, A., Vainio, S. and McMahon, A. P. (1998). Wnt-4 is a mesenchymal signal for epithelial transformation of metanephric mesenchyme in the developing kidney. *Development* **125**, 4225-34.

Kobayashi, A., Kwan, K. M., Carroll, T. J., McMahon, A. P., Mendelsohn, C. L. and Behringer, R. R. (2005). Distinct and sequential tissue-specific activities of the LIM-class homeobox gene Lim1 for tubular morphogenesis during kidney development. *Development* **132**, 2809-23.

Kreidberg, J. A. (2003). Podocyte differentiation and glomerulogenesis. *J Am Soc Nephrol* **14**, 806-14.

Kreidberg, J. A., Sariola, H., Loring, J. M., Maeda, M., Pelletier, J., Housman, D. and Jaenisch, R. (1993). WT-1 is required for early kidney development. *Cell* **74**, 679-91.

Lee, E. C., Yu, D., Martinez de Velasco, J., Tessarollo, L., Swing, D. A., Court, D. L., Jenkins, N. A. and Copeland, N. G. (2001). A highly efficient Escherichia coli-based chromosome engineering system adapted for recombinogenic targeting and subcloning of BAC DNA. *Genomics* **73**, 56-65.

Levashova, Z. B., Plisov, S. Y. and Perantoni, A. O. (2003). Conditionally immortalized cell line of inducible metanephric mesenchyme. *Kidney Int* **63**, 2075-87.

Levinson, R. and Mendelsohn, C. (2003). Stromal progenitors are important for patterning epithelial and mesenchymal cell types in the embryonic kidney. *Semin Cell Dev Biol* **14**, 225-31.

Li, X., Oghi, K. A., Zhang, J., Krones, A., Bush, K. T., Glass, C. K., Nigam, S. K., Aggarwal, A. K., Maas, R., Rose, D. W. et al. (2003). Eya protein phosphatase activity regulates Six1-Dach-Eya transcriptional effects in mammalian organogenesis. *Nature* **426**, 247-54.

Liu, P., Jenkins, N. A. and Copeland, N. G. (2003). A highly efficient recombineering-based method for generating conditional knockout mutations. *Genome Res* **13**, 476-84.

Luo, G., Hofmann, C., Bronckers, A. L., Sohocki, M., Bradley, A. and Karsenty, G. (1995). BMP-7 is an inducer of nephrogenesis, and is also required for eye development and skeletal patterning. *Genes Dev* **9**, 2808-20.

Lynch, M. A., Petrel, T. A., Song, H., Knobloch, T. J., Casto, B. C., Ramljak, D., Anderson, L. M., DeGroff, V., Stoner, G. D., Brueggemeier, R. W. et al. (2001). Responsiveness to transforming growth factor-beta (TGF-beta)-mediated growth inhibition is a function of membrane-bound TGF-beta type II receptor in human breast cancer cells. *Gene Expr* **9**, 157-71.

Mansouri, A., Chowdhury, K. and Gruss, P. (1998). Follicular cells of the thyroid gland require Pax8 gene function. *Nat Genet* **19**, 87-90.

Miyazaki, Y., Oshima, K., Fogo, A., Hogan, B. L. and Ichikawa, I. (2000). Bone morphogenetic protein 4 regulates the budding site and elongation of the mouse ureter. *J Clin Invest* **105**, 863-73.

Moissoglu, K. and Schwartz, M. A. (2006). Integrin signalling in directed cell migration. *Biol Cell* **98**, 547-55.

Moore, M. W., Klein, R. D., Farinas, I., Sauer, H., Armanini, M., Phillips, H., Reichardt, L. F., Ryan, A. M., Carver-Moore, K. and Rosenthal, A. (1996). Renal and neuronal abnormalities in mice lacking GDNF. *Nature* **382**, 76-9.

Nair, S. S., Chaubal, V. A., Shioda, T., Coser, K. R. and Mojamdar, M. (2001). Over-expression of MSG1 transcriptional co-activator increases melanin in B16 melanoma cells: a possible role for MSG1 in melanogenesis. *Pigment Cell Res* **14**, 206-9.

Nicolas, J. F., Mathis, L., Bonnerot, C. and Saurin, W. (1996). Evidence in the mouse for self-renewing stem cells in the formation of a segmented longitudinal structure, the myotome. *Development* **122**, 2933-46.

Obara-Ishihara, T., Kuhlman, J., Niswander, L. and Herzlinger, D. (1999). The surface ectoderm is essential for nephric duct formation in intermediate mesoderm. *Development* **126**, 1103-8.

Ohto, H., Kamada, S., Tago, K., Tominaga, S. I., Ozaki, H., Sato, S. and Kawakami, K. (1999). Cooperation of six and eya in activation of their target genes through nuclear translocation of Eya. *Mol Cell Biol* **19**, 6815-24.

Oxburgh, L., Dudley, A. T., Godin, R. E., Koonce, C. H., Islam, A., Anderson, D. C., Bikoff, E. K. and Robertson, E. J. (2005). BMP4 substitutes for loss of BMP7 during kidney development. *Dev Biol* **286**, 637-46.

Pachnis, V., Mankoo, B. and Costantini, F. (1993). Expression of the c-ret proto-oncogene during mouse embryogenesis. *Development* **119**, 1005-17.

Perantoni, A. O., Dove, L. F. and Williams, C. L. (1991). Induction of tubules in rat metanephrogenic mesenchyme in the absence of an inductive tissue. *Differentiation* **48**, 25-31.

Pichel, J. G., Shen, L., Sheng, H. Z., Granholm, A. C., Drago, J., Grinberg, A., Lee, E. J., Huang, S. P., Saarma, M., Hoffer, B. J. et al. (1996). Defects in enteric innervation and kidney development in mice lacking GDNF. *Nature* **382**, 73-6.

Plaza, S., Prince, F., Jaeger, J., Kloter, U., Flister, S., Benassayag, C., Cribbs, D. and Gehring, W. J. (2001). Molecular basis for the inhibition of Drosophila eye development by Antennapedia. *Embo J* **20**, 802-11.

Plisov, S., Tsang, M., Shi, G., Boyle, S., Yoshino, K., Dunwoodie, S. L., Dawid, I. B., Shioda, T., Perantoni, A. O. and de Caestecker, M. P. (2005). Cited1 is a bifunctional

transcriptional cofactor that regulates early nephronic patterning. *J Am Soc Nephrol* **16**, 1632-44.

Plisov, S. Y., Ivanov, S. V., Yoshino, K., Dove, L. F., Plisova, T. M., Higinbotham, K. G., Karavanova, I., Lerman, M. and Perantoni, A. O. (2000). Mesenchymal-epithelial transition in the developing metanephric kidney: gene expression study by differential display. *Genesis* **27**, 22-31.

Plisov, S. Y., Yoshino, K., Dove, L. F., Higinbotham, K. G., Rubin, J. S. and Perantoni, A. O. (2001). TGF beta 2, LIF and FGF2 cooperate to induce nephrogenesis. *Development* **128**, 1045-57.

Prasad, M. L., Pellegata, N. S., Kloos, R. T., Barbacioru, C., Huang, Y. and de la Chapelle, A. (2004). CITED1 protein expression suggests Papillary Thyroid Carcinoma in high throughput tissue microarray-based study. *Thyroid* **14**, 169-75.

Preis, J. I., Wise, N., Solloway, M. J., Harvey, R. P., Sparrow, D. B. and Dunwoodie, S. L. (2006). Generation of conditional Cited2 null alleles. *Genesis* **44**, 579-83.

Qiao, J., Cohen, D. and Herzlinger, D. (1995). The metanephric blastema differentiates into collecting system and nephron epithelia in vitro. *Development* **121**, 3207-14.

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

Rodriguez, T. A., Sparrow, D. B., Scott, A. N., Withington, S. L., Preis, J. I., Michalicek, J., Clements, M., Tsang, T. E., Shioda, T., Beddington, R. S. et al. (2004). Cited1 is required in trophoblasts for placental development and for embryo growth and survival. *Mol Cell Biol* **24**, 228-44.

Rogers, S. A., Ryan, G., Purchio, A. F. and Hammerman, M. R. (1993). Metanephric transforming growth factor-beta 1 regulates nephrogenesis in vitro. *Am J Physiol* **264**, F996-1002.

Sado, T., Fenner, M. H., Tan, S. S., Tam, P., Shioda, T. and Li, E. (2000). X inactivation in the mouse embryo deficient for Dnmt1: distinct effect of hypomethylation on imprinted and random X inactivation. *Dev Biol* **225**, 294-303.

Sainio, K., Suvanto, P., Davies, J., Wartiovaara, J., Wartiovaara, K., Saarma, M., Arumae, U., Meng, X., Lindahl, M., Pachnis, V. et al. (1997). Glial-cell-line-derived neurotrophic factor is required for bud initiation from ureteric epithelium. *Development* **124**, 4077-87.

Sajithlal, G., Zou, D., Silvius, D. and Xu, P. X. (2005). Eya 1 acts as a critical regulator for specifying the metanephric mesenchyme. *Dev Biol* **284**, 323-36.

Sakai, T., Larsen, M. and Yamada, K. M. (2003). Fibronectin requirement in branching morphogenesis. *Nature* **423**, 876-81.

Sanchez, M. P., Silos-Santiago, I., Frisen, J., He, B., Lira, S. A. and Barbacid, M. (1996). Renal agenesis and the absence of enteric neurons in mice lacking GDNF. *Nature* **382**, 70-3.

Sariola, H. (2002). Nephron induction revisited: from caps to condensates. *Curr Opin Nephrol Hypertens* **11**, 17-21.

Schmidt-Ott, K. M., Lan, D., Hirsh, B. J. and Barasch, J. (2006). Dissecting stages of mesenchymal-to-epithelial conversion during kidney development. *Nephron Physiol* **104**, p56-60.

Schuchardt, A., D'Agati, V., Pachnis, V. and Costantini, F. (1996). Renal agenesis and hypodysplasia in ret-k- mutant mice result from defects in ureteric bud development. *Development* **122**, 1919-29.
Sedghizadeh, P. P., Williams, J. D., Allen, C. M. and Prasad, M. L. (2005). MSG-1 expression in benign and malignant melanocytic lesions of cutaneous and mucosal epithelium. *Med Sci Monit* **11**, BR189-94.
Self, M., Lagutin, O. V., Bowling, B., Hendrix, J., Cai, Y., Dressler, G. R. and Oliver, G. (2006). Six2 is required for suppression of nephrogenesis and progenitor renewal in the developing kidney. *Embo J* **25**, 5214-28.
Shakya, R., Watanabe, T. and Costantini, F. (2005). The role of GDNF/Ret signaling in ureteric bud cell fate and branching morphogenesis. *Dev Cell* **8**, 65-74.
Shawlot, W. and Behringer, R. R. (1995). Requirement for Lim1 in head-organizer function. *Nature* **374**, 425-30.
Shi, G., Boyle, S. C., Sparrow, D. B., Dunwoodie, S. L., Shioda, T. and de Caestecker, M. P. (2006). The transcriptional activity of CITED1 is regulated by phosphorylation in a cell cycle-dependent manner. *J Biol Chem* **281**, 27426-35.
Shioda, T., Fenner, M. H. and Isselbacher, K. J. (1996). msg1, a novel melanocyte-specific gene, encodes a nuclear protein and is associated with pigmentation. *Proc Natl Acad Sci U S A* **93**, 12298-303.
Shioda, T., Fenner, M. H. and Isselbacher, K. J. (1997). MSG1 and its related protein MRG1 share a transcription activating domain. *Gene* **204**, 235-41.
Shioda, T., Lechleider, R. J., Dunwoodie, S. L., Li, H., Yahata, T., de Caestecker, M. P., Fenner, M. H., Roberts, A. B. and Isselbacher, K. J. (1998). Transcriptional activating activity of Smad4: roles of SMAD hetero-oligomerization and enhancement by an associating transactivator. *Proc Natl Acad Sci U S A* **95**, 9785-90.
Soriano, P. (1999). Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat Genet* **21**, 70-1.
Stark, K., Vainio, S., Vassileva, G. and McMahon, A. P. (1994). Epithelial transformation of metanephric mesenchyme in the developing kidney regulated by Wnt-4. *Nature* **372**, 679-83.
Takemoto, M., He, L., Norlin, J., Patrakka, J., Xiao, Z., Petrova, T., Bondjers, C., Asp, J., Wallgard, E., Sun, Y. et al. (2006). Large-scale identification of genes implicated in kidney glomerulus development and function. *Embo J* **25**, 1160-74.
Tong, G. M., Rajah, T. T., Zang, X. P. and Pento, J. T. (2002). The effect of antiestrogens on TGF-beta-mediated chemotaxis of human breast cancer cells. *Anticancer Res* **22**, 103-6.
Torres, M., Gomez-Pardo, E., Dressler, G. R. and Gruss, P. (1995). Pax-2 controls multiple steps of urogenital development. *Development* **121**, 4057-65.
Treisman, J. E. (1999). A conserved blueprint for the eye? *Bioessays* **21**, 843-50.
Tsang, T. E., Shawlot, W., Kinder, S. J., Kobayashi, A., Kwan, K. M., Schughart, K., Kania, A., Jessell, T. M., Behringer, R. R. and Tam, P. P. (2000). Lim1 activity is required for intermediate mesoderm differentiation in the mouse embryo. *Dev Biol* **223**, 77-90.
Vintersten, K., Testa, G. and Stewart, A. F. (2004). Microinjection of BAC DNA into the pronuclei of fertilized mouse oocytes. *Methods Mol Biol* **256**, 141-58.

Vize, P. D., Seufert, D. W., Carroll, T. J. and Wallingford, J. B. (1997). Model systems for the study of kidney development: use of the pronephros in the analysis of organ induction and patterning. *Dev Biol* **188**, 189-204.
Vize, P. D., Woolf, A. S. and Bard, J. B. L. (2002). The kidney : from normal development to congenital diseases. Amsterdam ; Boston: Academic Press.
Wellik, D. M., Hawkes, P. J. and Capecchi, M. R. (2002). Hox11 paralogous genes are essential for metanephric kidney induction. *Genes Dev* **16**, 1423-32.
Weninger, W. J., Floro, K. L., Bennett, M. B., Withington, S. L., Preis, J. I., Barbera, J. P., Mohun, T. J. and Dunwoodie, S. L. (2005). Cited2 is required both for heart morphogenesis and establishment of the left-right axis in mouse development. *Development* **132**, 1337-48.
Withington, S. L., Scott, A. N., Saunders, D. N., Lopes Floro, K., Preis, J. I., Michalicek, J., Maclean, K., Sparrow, D. B., Barbera, J. P. and Dunwoodie, S. L. (2006). Loss of Cited2 affects trophoblast formation and vascularization of the mouse placenta. *Dev Biol* **294**, 67-82.
Xu, P. X., Adams, J., Peters, H., Brown, M. C., Heaney, S. and Maas, R. (1999). Eya1-deficient mice lack ears and kidneys and show abnormal apoptosis of organ primordia. *Nat Genet* **23**, 113-7.
Xu, P. X., Zheng, W., Huang, L., Maire, P., Laclef, C. and Silvius, D. (2003). Six1 is required for the early organogenesis of mammalian kidney. *Development* **130**, 3085-94.
Xu, P. X., Zheng, W., Laclef, C., Maire, P., Maas, R. L., Peters, H. and Xu, X. (2002). Eya1 is required for the morphogenesis of mammalian thymus, parathyroid and thyroid. *Development* **129**, 3033-44.
Yahata, T., de Caestecker, M. P., Lechleider, R. J., Andriole, S., Roberts, A. B., Isselbacher, K. J. and Shioda, T. (2000). The MSG1 non-DNA-binding transactivator binds to the p300/CBP coactivators, enhancing their functional link to the Smad transcription factors. *J Biol Chem* **275**, 8825-34.
Yahata, T., Shao, W., Endoh, H., Hur, J., Coser, K. R., Sun, H., Ueda, Y., Kato, S., Isselbacher, K. J., Brown, M. et al. (2001). Selective coactivation of estrogen-dependent transcription by CITED1 CBP/p300-binding protein. *Genes Dev* **15**, 2598-612.
Yahata, T., Takedatsu, H., Dunwoodie, S. L., Braganca, J., Swingler, T., Withington, S. L., Hur, J., Coser, K. R., Isselbacher, K. J., Bhattacharya, S. et al. (2002). Cloning of mouse Cited4, a member of the CITED family p300/CBP-binding transcriptional coactivators: induced expression in mammary epithelial cells. *Genomics* **80**, 601-13.
Ye, P., Habib, S. L., Ricono, J. M., Kim, N. H., Choudhury, G. G., Barnes, J. L., Abboud, H. E. and Arar, M. Y. (2004). Fibronectin induces ureteric bud cells branching and cellular cord and tubule formation. *Kidney Int* **66**, 1356-64.
Yin, Z., Haynie, J., Yang, X., Han, B., Kiatchoosakun, S., Restivo, J., Yuan, S., Prabhakar, N. R., Herrup, K., Conlon, R. A. et al. (2002). The essential role of Cited2, a negative regulator for HIF-1alpha, in heart development and neurulation. *Proc Natl Acad Sci U S A* **99**, 10488-93.
Yu, J., McMahon, A. P. and Valerius, M. T. (2004). Recent genetic studies of mouse kidney development. *Curr Opin Genet Dev* **14**, 550-7.