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PRKCa: Identification of a Novel Downstream Target of WT1

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A

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ABSTRACT

PRKCα: IDENTIFICATION OF A NOVEL DOWNSTREAM TARGET OF WT1

Publication No._____

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Wilms tumor is a childhood tumor of the kidney arising from the undifferentiated metanephric mesenchyme. Tumorigenesis is attributed to a number of genetic and epigenetic alterations. In 20% of Wilms tumors, Wilms tumor gene 1 (*WT1*) undergoes inactivating homozygous mutations causing loss of function of the zinc finger transcription factor it encodes. It is hypothesized that mutations in *WT1* result in dysregulation of downstream target genes, leading to aberrant kidney development and/or Wilms tumor. These downstream target genes are largely unknown, and identification is important for further understanding Wilms tumor development. Heatmap data of human Wilms tumor protein expression, generated by reverse phase protein assay analysis (RPPA), show significant correlation between *WT1* mutation status and low PRKCα expression ($p= 0.00013$); additionally, p-PRKCα (S657) also shows decreased expression in these samples ($p= 0.00373$). These data suggest that the WT1 transcription factor regulates PRKCα expression, and that PRKCα plays a potential role in Wilms tumor tumorigenesis. We hypothesize that the WT1 transcription factor directly/indirectly regulates *PRKCα* and mutations occurring in *WT1* lead to decreased expression of PRKCα. Prkcα and Wt1 have been shown to co-localize in E14.5 mesenchymal cells of the developing kidney. siRNA knockdown, in-vivo ablation, and tet-inducible expression of *Wt1* each independently confirm regulation of *Prkcα*

expression by Wt1 at both RNA and protein levels, and investigation into possible WT1 binding sites in *PRKCα* regulatory regions has identified multiple sites to be confirmed by luciferase reporter constructs. With the goal of identifying WT1 and PRKCα downstream targets, RPPA analysis of protein expression in mesenchymal cell culture, following lentiviral delivered shRNA knockdown of *Wt1* and shRNA knockdown of *Prkcα,* will be carried out.

Apart from Wilms tumor, *WT1* also plays an important role in Acute Myeloid Leukemia (AML). *WT1* mutation status has been implicated, controversially, as an independent poor-prognosis factor in leukemia, leading to decreased probability of overall survival, complete remission, and disease free survival. RPPA analysis of AML patient samples showed significant decreases in PRKCα/p-PRKCα protein expression in a subset of patients (Kornblau, personal communication); therefore, the possible role of WT1 and PRKCα in leukemia disease progression is an additional focus of this study. *WT1* mutation analysis of diploid leukemia patient samples revealed two patients with mutations predicted to affect WT1 activity; of these two samples, only one corresponded to the low PRKCα expression cohort. Further characterization of the role of *WT1* in AML, and further understanding of WT1 regulated *PRKCα* expression, will be gained following RPPA analysis of protein expression in HL60 leukemia cell lines with lentiviral delivered shRNA knockdown of *WT1* and shRNA knockdown of *PRKCα*.

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LIST OF ABBREVIATIONS

AML – Acute Myeloid Leukemia

AP-2 – Activator protein 2

BSA – bovine serum albumin

cDNA – complimentary deoxyribonucleic acid

DAG – Diacyglycerol

DFS – disease free survival

DNA – deoxyribonucleic acid

E14.5 – embryonic day 14.5

EGR-1 – Early growth response protein 1

EYA1 – Eyes absent homolog 1

HEK 293 – Human embryonic kidney cell line 293

IF – immunofluorescence

IGF2 – Insulin-like growth factor 2

IHC – immunohistochemistry

IPTG – Isopropyl β-D-1-thiogalactopyranoside

ITGA2 – Integrin alpha 2

KTS – three amino acid insert lysine/threonine/serine

LoxP – Locus of exchange P1

M15 – mouse mesenchymal cell line 15

MOI – multiplicity of infection

NGS – normal goat serum

OS – overall survival

PAX2 – Paired box gene 2

PBS – phosphate buffer system

PCR – Polymerase chain reaction

PDK-1 – Pyruvate dehydrogenase kinase 1

PFA – Paraformaldehyde

PRKCα – Protein Kinase C alpha

p-PRKCα – phosphorylated Protein Kinase C alpha

qPCR – quantitative polymerase chain reaction

RPPA – reverse phase protein array

S657 – Serine 657

shRNA – small hairpin ribonucleic acid

siRNA – small interfering ribonucleic acid

SIX1 – Sine oculis homeobox homolog 1

SP1 – Specificity protein 1

T497 – Threonine 497

T638 – Threonine 638

TGFb – Transforming growth factor beta

THBS1 – Thrombospondin 1

VEGF – Vascular endothelial growth factor

WT1 – Wilms Tumor gene 1

ZNF – Zinc Finger

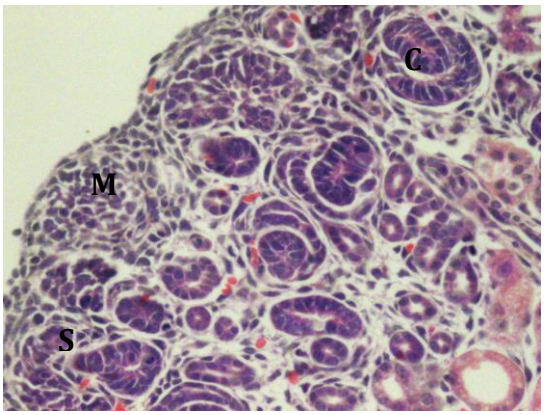
Chapter 1 – Introduction

Wilms tumor and Wilms Tumor Gene 1:

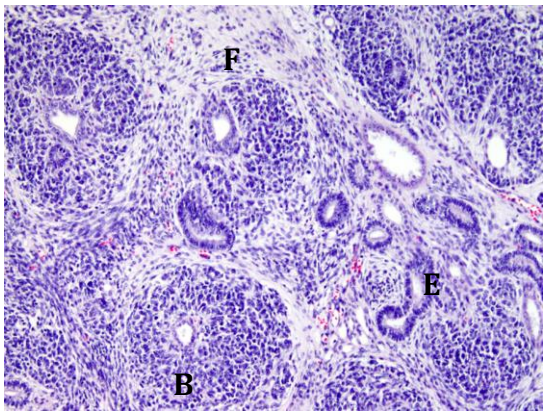
Wilms tumor is the most common childhood kidney tumor, occurring in 1 of every 10,000 live births. Tumors arise from the undifferentiated metanephric mesenchyme and can occur both unilaterally and bilaterally; sporadic and familial forms of the disease exist, with the majority of tumors being unilateral and sporadic. Cases of Wilms tumor can be accompanied by congenital anomalies such as sporadic aniridia, hemihypertrophy, and genitourinary anomalies. The co-occurrence of Wilms tumor and aniridia in disease patients allowed cytogenetic localization of *WT1* tumor suppressor gene at chromosome 11p13. *WT1* encodes a protein with four c-terminal zinc fingers shown to bind DNA and is inactivated in 15-20% of Wilms tumor cases (Background Figure 1) (Bickmore et al., 1992; Huff, 1998).

WT1 is important during kidney development for correct formation of cellular structures; interactions between the Wolffian duct and metanephric mesenchyme drive the process of kidney formation as outgrowth of the Wolffian duct derived uteric bud invades nearby nephrogenic mesenchyme and key molecules such as WT1 drive downstream signaling cascade (Kuure, Vuolteenaho, & Vainio, 2000). As the uteric bud comes into contact with uncondensed nephrogenic mesenchyme, the mesenchymal cells begin to condense around the uteric bud in cap formations and undergo mesenchymal-to-epithelial transition. In the mouse, this process of reciprocal signaling begins around embryonic day 10.5-11 (E10.5-11) post conception. (Saxen, 1987). Asynchronous mesenchymal-to-epithelial transition results in formation of early epithelial structures

NB mouse kidney



Wilms tumor

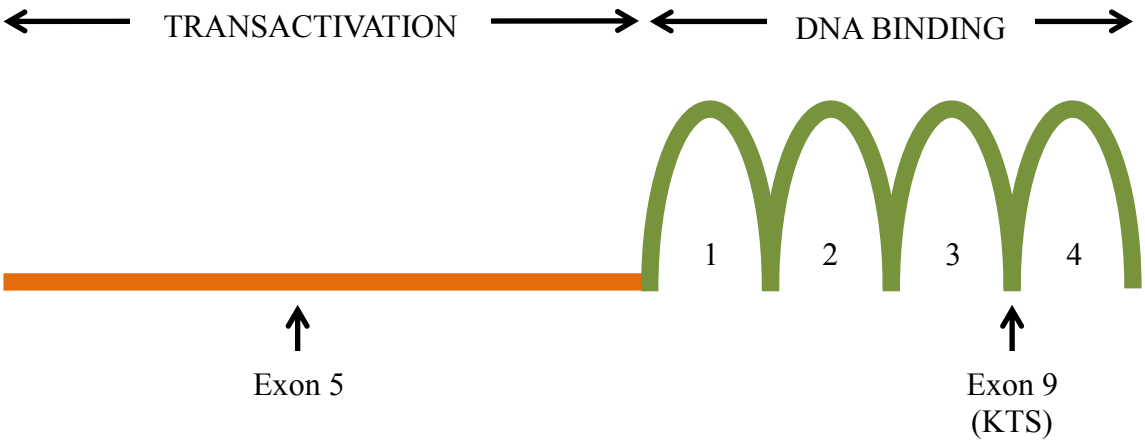


Background Figure 1: (left) histology of normal kidney with mesenchyme (M), s shaped (S) and comma-shaped (C) bodies, and glomeruli. (right) Triphasic histology of Wilms tumor exhibiting blastemal (B), stromal (F), and epithelial (E) components; lacks correct formation of glomeruli and kidney nephron.

known as comma-shaped and s-shaped bodies which ultimately become the mature nephron. Staining of early embryonic mouse kidney reveals *Wt1* expression localized to the uncondensed mesenchyme and urogenital ridge that includes the early mesonephros (Armstrong, 1992). During early phases of kidney development, *Wt1* expression levels are high with maximal expression in the comma-shaped and s-shaped bodies; *Wt1* expression decreases with continued epithelialization, and is eventually restricted to the podocytes of the mature nephron (Background Figure 2) (Pelletier et al., 1991; Pritchard-Jones, 1990).

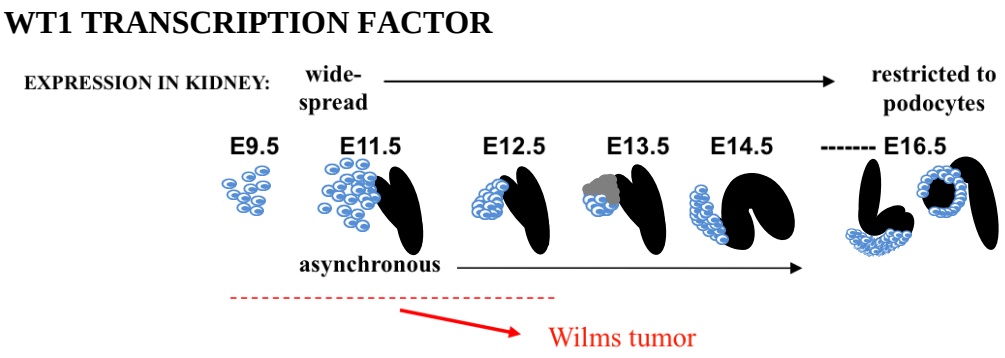
Conventional knockout of *Wt1* is embryonic lethal with failure to develop kidneys and gonads as well as phenotypes of the heart, liver, retina and spleen (Kreidberg, 1993). Conditional *Wt1* mouse models have confirmed the role of *Wt1* in the mesenchymal to epithelial transition in the kidney specifically (Miller-Hodges & Hohenstein, 2012); knockdown of *Wt1* results in incorrect formation of kidney nephron (Davies et al., 2004) and mice die pre or perinatally (Herzer, 1999; Kreidberg, 1993). *Wt1* conditional knockout mice induced for mutant WT1 expression at E11.5 in the context of *Igf2* biallelic expression have been shown to develop tumors closely resembling the histology of the human tumors and serve as the first model for Wilms Tumor disease (Hu et al., 2011).

Wilms tumors are characterized by a distinct triphasic histology consisting of blastemal, epithelial, and stromal components. Tumor histology closely resembles embryonic kidney and lack correct formation of glomeruli and kidney; leading to the hypothesis that tumors arise early during embryonic development, during the



Background Figure 2: The structure of WT1 ZNF transcription factor consists of a four zinc finger DNA binding domain with a three amino acid insert in exon 9, and a transactivation domain with an alternative splice site at exon 5.

mesenchymal to epithelial transition (Background Figure 3). Early mesenchymal progenitor cells are thought to give rise to tumors following genetic changes that drive aberrant cellular differentiation. In order to identify changes in cellular expression contributing to tumorigenesis, gene expression arrays and protein expression arrays have been carried out and identified a number of altered signaling pathways; tumors expressing genes *Six1*, *PAx2*, and *Eya1* suggest a nephrogenic mesenchyme progenitor origin for tumors (Hu et al., 2011). Recently, protein expression arrays of human Wilms tumors have identified several protein expression changes hypothesized to contribute to tumorigenesis (Figure 1). Reverse phase protein array (RPPA) data indicates that when grouped by mutation status, tumor samples harboring *WT1* mutations have a significant decrease in PRKCα (p-value, 0.00013) and p-PRKCα S657 (p-value, 0.00373) protein expression. These data suggest that the WT1 transcription factor regulates *PRKCα*, and that PRKCα plays a potential role in Wilms tumor tumorigenesis. We hypothesize that the WT1 transcription factor directly/indirectly regulates *PRKCα* and mutations occurring in *WT1* lead to decreased expression of PRKCα.



Background Figure 3: WT1 ZNF transcription factor is detectable in the metanephric mesenchyme of the developing kidney. Increased expression is seen in the condensing mesenchyme as the developing kidney progresses through formation of the early epithelial structures: the s-shaped and comma-shaped bodies. Expression is eventually restricted to the podocytes of the mature nephron.

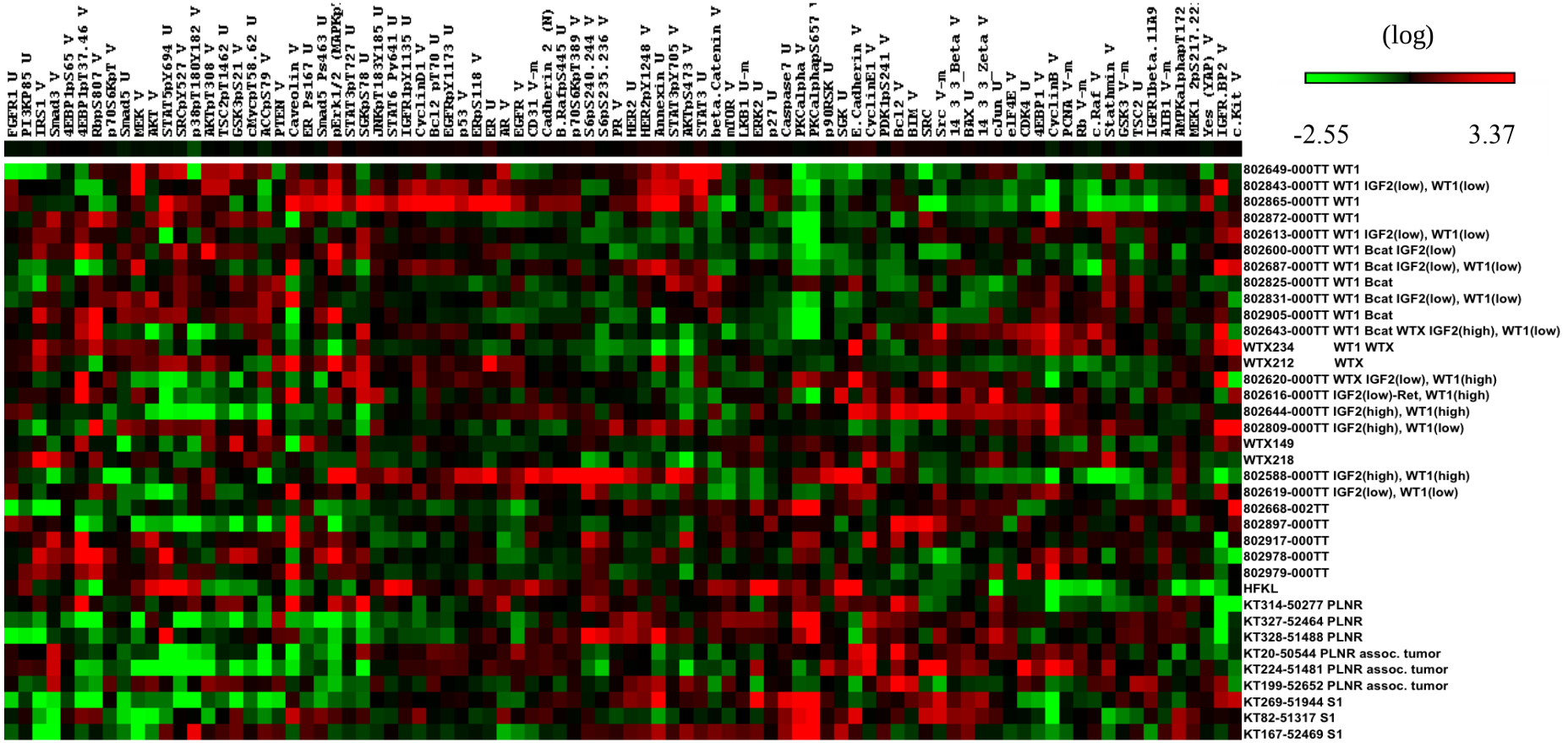
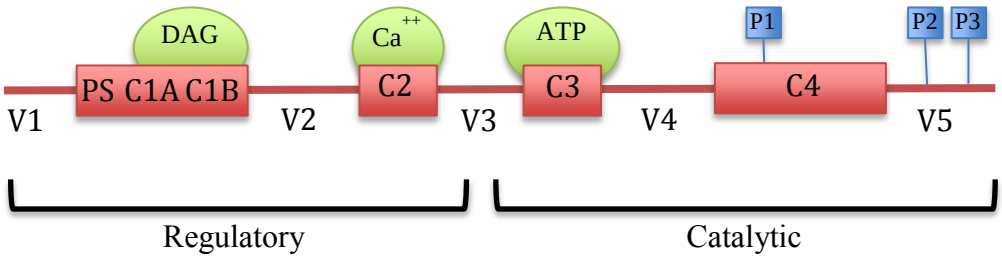


Figure 1: RPPA heatmap (supervised hierarchical clustering) of Wilms tumor protein expression shows that patients with a WT1 mutation have significantly decreased levels of PRKCα (p=0.00012) and p-PRKCα S657 (p=0.00372) expression.

Protein Kinase C alpha:

PRKCα belongs to a serine/threonine family of kinases ubiquitously expressed in vertebrates and responsible for multiple cellular processes (Nishizuka, 1988). PRKCα is a conventional (classical) type PKC and is implicated in a variety of biological responses such as apoptosis, proliferation, differentiation, and tumorigenesis, depending on tissue localization (Nakashima, 2002). PRKCα is primarily expressed in the heart, brain, liver and blood, although it can be detected in almost all organs. The PRKCα knockout mouse is viable with no external phenotypes – a surprising result based on its ubiquitous expression – but does show increased insulin signaling through the PI3K pathway (Letiges et al., 2002). Knockout mice have been used to identify a role for PRKCα in cardiovascular disease, pulmonary disease, platelet function, and immune cell function, as well as multiple types of cancer (Konopatskaya & Poole, 2010). In tumorigenesis specifically, PRKCα has been implicated in driving a more aggressive and metastatic phenotype for human breast cancer cells (Sun & Rotenberg, 1999; Ways et al., 1995) and overexpression of PRKCα has also been shown to contribute to the development of renal cell carcinoma (Chuize et al., 2010).

PRKCα conformation regulation is a well-described and tightly controlled process requiring diacylglycerol (DAG) and Ca^{2+} signals for activity (Background Figure 4) (Dekker & Parker, 1994; Jaken, 1996). An original phosphorylation event by PDK-1 in the activation loop of PRKCα at T497 leads to two secondary autophosphorylation events at T638 in the protein turn motif and S657 in the hydrophobic motif. Following the phosphorylation of PRKCα, conformational changes in the molecule result in a pseudosubstrate sequence occupying the active site of the



Background Figure 4: PRKCα protein structure consists of N-terminal regulatory, C-terminal catalytic, and V3 hinge regions. Active PRKCα is tightly regulated by three mechanisms: phosphorylation, cofactor binding, and intracellular localization. The N-terminus regulatory region requires DAG and Ca^{++} binding, and contains several phosphatidyl serine binding domains. The C-terminus catalytic domain is highly conserved across the three PKC subfamilies and binds ATP and substrate targets.

catalytic region; the PRKCα molecule is now considered 'primed,' but inactive (Griner & Kazanietz, 2007). Because of this N-terminal autoinhibitory mechanism, PRKCα is unable to bind and phosphorylate other substrates until DAG and Ca^{2+} signals are received (Parekh, Ziegler, & Parker, 2000). Once DAG and Ca^{2+} cofactor binding takes place, PRKCα is catalytically active and able to bind targets. Experiments have shown that of the three phosphorylation events that occur, phosphorylation at T497 is required to achieve full PRKCα activity, and therefore is most commonly used to assess for PRKCα catalytic status (Parekh et al., 2000).

Wilms Tumor Gene 1 and Acute Myeloid Leukemia:

A second part of this study is an investigation into the role of *WT1* in acute myeloid leukemia (AML) disease progression. Studies have suggested that *WT1* mutation status may serve as a novel, independent poor prognosis factor for leukemia disease progression and an appropriate target for therapy development (Virappane et al., 2008). Patients harboring a *WT1* mutation are more likely to have worse disease-free survival (DFS), worse overall survival (OS), and increased drug resistance (King-Underwood & Pritchard-Jones, 1998; Paschka et al., 2008). In AML patient mutation analysis studies, *WT1* mutations occur at a frequency of approximately 10%, cluster to exons seven and nine, and are associated with failure to achieve a complete remission (King-Underwood, Renshaw, & Pritchard-Jones, 1996). While the mechanisms by which WT1 mutant protein may contribute to leukemia disease progression are poorly understood, independent observations that leukemia patients can be categorized into high and low expressing PRKCa groups has suggested that the same relationship between *WT1* and *PRKCα*, hypothesized to contribute to Wilms tumor, may also play an important role in leukemia. In both diseases, we hypothesize that *Wt1* transcription factor is driving PRKCα-expression levels. If this is true, we expect that leukemia patients with *WT1* mutations also have decreased PRKCα expression.

We hypothesize that the WT1 transcription factor directly/indirectly regulates PRKC*α* and mutations occurring in WT1 lead to decreased expression of PRKC*α*. Understanding the relationship between WT1 and *PRKCα* will lead to increased knowledge of the mechanisms leading to Wilms tumorigenesis and better define the role of *WT1* mutation status as a possible prognosis factor in AML.

Chapter 2: WT1 co-localizes with PRKCα and Mutant WT1 affects PRKCα gene expression during kidney development

Introduction:

If *PRKCα* is a transcriptional target of *WT1* in the developing kidney, and an important protein for correct kidney development, it should be expressed in the same cell types as WT1. As previously discussed, WT1 expression is maximal in the embryonic kidney during the mesenchymal to epithelial transition as kidney progenitors give rise to early epithelial structures, comma-shaped and s-shaped bodies, before formation of the mature nephron. Wilms tumor histology shows an incomplete formation of the kidney nephron and a lack of epithelial structures, suggesting that tumors arise early as a result of disruption in correct signaling necessary for kidney development.

We hypothesize that WT1 is regulating *PRKCa* expression at this important developmental stage and mutation of WT1 results in changes in *PRKCα* expression that contribute to a lack of complete mesenchymal to epithelial transition. Using a combination of in-vivo and in-vitro models we should be able to establish that the two proteins are co-expressed within the same cell types during kidney development. Furthermore, utilizing *Wt1* knockout mice and other models for manipulating WT1 expression levels, we should be able to demonstrate that increasing or decreasing *WT1* expression results in reciprocal changes in *PRKCα* expression.

Materials/Methods:

Wt1 conditional knockout mice:

A mutant mouse strain carrying a *Wt1* allele in which exons 8 and 9 are flanked by *lox*P sites was generated by homologous recombination in previous studies (Gao et al., 2006) and combined with an Estrogen-Cre (Cre-ER) transgene. Tamoxifen treatment was shown to drive expression of Cre recombinase, resulting in an in-frame deletion of exons 8 and 9, and a functional knockout of *Wt1* activity. Exons 8 and 9 encode zinc fingers 2 and 3 of wild type WT1 protein and their deletion results in an allele ($Wt1^{\Delta}$) that acts as a null allele, as predicted by in-vitro studies (Hossain & Saunders, 2001; Nachtigal et al., 1998; Royer-Pokora et al., 2004). For this study, crosses were set up with females' genotype $Wt1^{fl/fl}$ and males' genotype $Wt1^{+/-}$; $Cre\text{-}ER^{TM}$. Pregnant females were injected with 3 mg (3mg/40g) Tamoxifen at E11.5 to ablate *Wt1* in the developing embryo and achieve $Wt1^{-/\Delta}$ mutant embryos with $Wt1^{+/\Delta}$ control embryos. Mice were genotyped by PCR to confirm *Wt1* ablation. Primer sets and PCR conditions used are listed in Table 1.

Isolation of mouse tissues:

Mutant ($Wt1^{-/\Delta}$;$Cre\text{-}ER^{TM}$) and littermate control mouse embryos were harvested at E13.5, E14.5, and E15.5 and kidneys isolated. For protein expression analyses kidneys were sonicated and stored in protein lysis buffer (20mM Tris pH 7.5, 150mM Nacl, 1mM EDTA, 1% Triton X-100 (Sigma-Aldrich), 1X Protease Inhibitor Cocktail (Sigma-Aldrich). Embryonic kidneys for qPCR analysis were stored in TRIzol (Invitrogen) and then homogenized. RNA was isopropanol precipitated, washed with 70% ethanol and

Table 1

Detected	Forward Primer	Reverse Primer	Product Size
Wt1 null allele	5'GTGACCCCGCAGCTAGCC3'	5'CCATTTGTCACGTCCTGC3'	250bps
Wt1 flox allele	5'TGCCTACCCAATGCTCATTG3'	5'GAAACTGTTTGTAACGAGAG3'	150 and 200bps
Cre-ER^{TM} transgene	5'TCCAATTTACTGACCGTACACCAA3'	5'CCTGATCCTGGCAATTTCGGCTA3'	600bps
Wt1$^{\Delta}$ allele	5'GCTAACATATGGGAGACATT3'	5'TGCCTACCCAATGCTCATTG3'	45bps
Cycle Conditions: 94 °C - 3 min, [94 °C - 45 sec, 58 °C - 45 sec, 72 °C - 50 sec] - 35 cycles, 72 °C - 7 min			

DNase 1 (Applied Biosystems) treatment to remove any DNA contamination. Embryonic kidneys for immunohistology/immunofluorescence analysis were collected and stored in 4% paraformaldehyde (PFA) overnight before paraffin embedding and sectioning for mounting on slides.

Tissue culture:

Kidney mesenchymal cells line F7346, Clone 10, E6, *Wt1*$^{+/fl}$; *Immorto* was generated from cells isolated from mouse embryo and characterized as metanephric mesenchyme cells expressing *Wt1* (Nosavanh MS Thesis 2011). Cells have been shown to be positive for mesenchymal cell marker, vimentin and negative for epithelial cell marker, cytokeratin. Cell cultures were maintained in 10% FBS (fetal bovine serum)(Hyclone) in 1X DMEM supplemented with interferon gamma purchased from Sigma-Aldrich and 100 ug/mL of antibiotics (penicillin, streptomycin, and amphotericin B) purchased from MediaTech at 33 °C. Cultures were genotyped using gene specific primers and characterized as maintaining a mesenchymal morphology of elongated, spindle-shaped, fibroblastic appearance (Table 1).

Human Embryonic Kidney cell line 293, HEK293, and M15, a mouse mesenchymal cell line, were cultured in 10% FBS (Hyclone) in 1X DMEM supplemented with 100 u/ML penicillin/streptomycin at 37 °C. T-Rex^{TM} -293(-KTS) and T-Rex^{TM} -293(+KTS) were further supplemented with Zeocin and Blasticidin and induced with 1ug/mL tetracycline for the indicated time. Cells lysates were collected for downstream applications using scraping spatulas in protein lysis buffer (described in

methods for isolating mouse tissues) for western blot analysis. RNA was isolated using Qiagen RNeasy kit according to the protocol.

Immunohistochemistry:

Wild-type E14.5 kidney sections were stained for protein expression using Vectastain ABC (avidin-biotin-peroxidase) kit (Vector Laboratories) according to the protocol. Antibody to WT1 (mouse monoclonal antibody clone 6F-H2) was purchased from Dako. Antibodies to PRKCα (rabbit monoclonal antibody Y124) and p-PRKCα T497 (rabbit monoclonal antibody) were purchased from Abcam. Deparaffinized slides were treated with 95-100 °C citrate buffer for antigen retrieval and allowed to cool to room temperature before blocking with 3% hydrogen peroxide in PBS for 10 min. Slides were washed three times in PBS and incubated for 1 hr in 10% BSA (bovine serum albumin) in PBS at room temperature followed by incubation with primary antibody in 10% BSA in PBS overnight. Following primary antibody incubation, the slides were washed three times in PBS and species-specific secondary antibodies were added in PBS for 1 hr at room temperature (1:400). Vectastain ABC reagents were used according to the protocol for color development and slides were counterstained with hematoxylin and clear mounted using Permount (Fisher) after xylene dehydrating.

Immunofluorescence:

For immunofluorescence studies of cultured cells, cells were trypsinized, added to 24-well plates containing glass discs, and allowed to adhere overnight before methanol fixation at -20 °C for 15 min. For immunofluorescence studies of tissues

sections, slides were deparaffinized and treated with citrate buffer as described in immunohistochemical analysis above. After cooling to room temperature, slides were incubated with 1% glycine in 0.1% TWEEN-20 (Sigma-Aldrich) in PBS for 1 hr. Both discs of cultured cells and tissue slides were then washed twice in PBS followed by a 1 hr incubation with 15% NGS (normal goat serum) and slides/discs were incubated with primary antibodies diluted in 2% NGS and left at 4 °C overnight. Slides/discs were then washed three times in PBS and incubated with secondary antibodies Alexa Fluor-594 and/or Alexa Fluor-488 (Invitrogen, according to species-specificity) for 1 hr in the dark. Subsequently, the slides/discs were washed three times in PBS and stained using ProLong Gold antifade reagent with DAPI (Invitrogen) and allowed to air-dry overnight at room temperature in the dark.

Western Blot Analysis:

Protein blot analyses were carried out as previously described (Gao et al., 2006; Hu et al., 2011) with modification. Briefly, protein lysates were denatured for 3 min at 97 °C with 10 uL β-mercaptoethanol reducing agent before loading into BIORAD Mini-PROTEAN Tetra cell assembly using Mini-PROTEAN TGX Precast Gels with Laemmli running buffer at 90 volts for 2 hrs. Following gel electrophoresis, proteins were transferred to PVDF membranes using BIORAD Mini Trans-Blot cell assembly run at 70V for 2 hrs at 4 °C. Membranes with bound proteins were blocked for 1 hr in 5% milk in TBST (1X Tris buffered saline/0.1% Tween-20) followed by incubation with primary antibody in 5% milk in TBST overnight at 4 °C (primary antibodies described in methods for immunohistochemical analysis). Primary antibody incubation was followed

by 3 washes in TBST and a 1 hr incubation with species-specific secondary antibody diluted 1:5000 in 5% milk in TBST at room temperature. Following a 3 washes in TBST, membranes were developed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) according to the protocol.

RT-PCR/qPCR Analysis:

RNA for gene expression by SYBR Green assays (Applied Biosystems). First, RNA was quantified, normalized across samples and mixed with MultiScribe Reverse Transcriptase and PCR Master Mix according to the protocol for cDNA synthesis (Invitrogen). cDNA samples were mixed with SYBR Green reagent and forward and reverse primers for detecting expression levels of specific genes and amplified using ABI 7900 HT sequence detection system (Applied Biosystems). Gene specific Ct values were compared across samples relative to *Gapdh* endogenous control levels and calculated as fold differences to a control reference value whose expression was designated as 1. qPCR primer sequences are listed in Table 2.

siRNA Transfection:

Knockdown of *Wt1* in mouse mesenchymal cell line M15 was achieved by transient transfection of scrambled *Wt1* siRNA (ON-TARGET plus SMARTpool duplex, Dharmacon) using media supplemented with 10uL of HyperFect Transfection Reagent (Qiagen). Cells were plated into 6 well plates at $8x10^4$ cells per well and allowed to grow overnight before adding 50nm siRNA. Following 24hrs of *Wt1* siRNA treatment, RNA and protein lysates were collected as described in methods for tissue culture.

Table 2

	Forward Primer	**Reverse Primer**
Wt1 (mouse)	5’AGTTCCCCAACCATTCCTTC3’	5’TTCAAGCTGGGAGGTCATTT3’
Prkcα (mouse)	5’TTGGGAAGGTGATGCTTGCT3’	5’CAGGATCTTGATGGCGTACAGTT3’
Gapdh (mouse)	5’CTCTCTGCTCCTCCCTGTTC3’	5’CGTTCACACCGACCTTCAC3’
WT1 (human)	5’CAGTTCCCCAACCACTCATT3’	5’AAGCTGGGATGTCATTTGGT3’
PRKCα (human)	5’CCAGTGGATGGTACAAGTTGCTTA3’	5’CCTTCCGGAATGGGTACGT3’
GAPDH (human)	5’GCTCCTCCTGTTCGACAGTCA3’	5’ACCTTCCCCATGGTGTCTGA3’

Results:

Immunohistochemical staining of wild type E14.5 mouse kidney for WT1 and PRKCα confirms that both proteins are expressed in the uncondensed mesenchyme during kidney development. Robust WT1 expression is seen in the uncondensed mesenchyme of the fetal kidney as well as in the comma and s-shaped body early epithelial structures and is eventually restricted to the podocytes of the mature nephron (Figure 2, top). Cells and epithelial structures positive for WT1 expression show strong nuclear staining. Likewise, PRKCα staining shows a positive signal in the uncondensed mesenchyme of E14.5 mouse kidney. Positive cells show largely cytoplasmic staining for PRKCα protein. Additionally, PRKCα is expressed in proximal and distal tubules and mesangeal cells of the fully formed glomerulus; again, the signal is largely cytoplasmic (Figure 2, middle). Staining for the phosphorylated isoform of PRKCα, p-PRKCα T497 (Abcam, rabbit monoclonal antibody), shows a positive signal in the uncondensed mesenchyme, proximal and distal tubules, and mesangeal cells, an expression pattern identical to pan PRKCα (Figure 2, lower).

Double immunofluorescent staining of E14.5 wild type mouse kidney for WT1 and PRKCα confirms co-localization of the proteins in the developing glomerulus. Early epithelial structures show strong nuclear staining for WT1 and cytoplasmic staining for PRKCα. Both proteins are expressed in the same cells of the comma-shaped body, but largely within different cell compartments (Figure 3).

Similarly, double immunofluorescent staining of mesenchymal cell cultures confirms co-localization of WT1 and PRKCα. Kidney mesenchymal cell line F7346 shows strong nuclear expression of WT1 and strong nuclear/cytoplasmic expression of

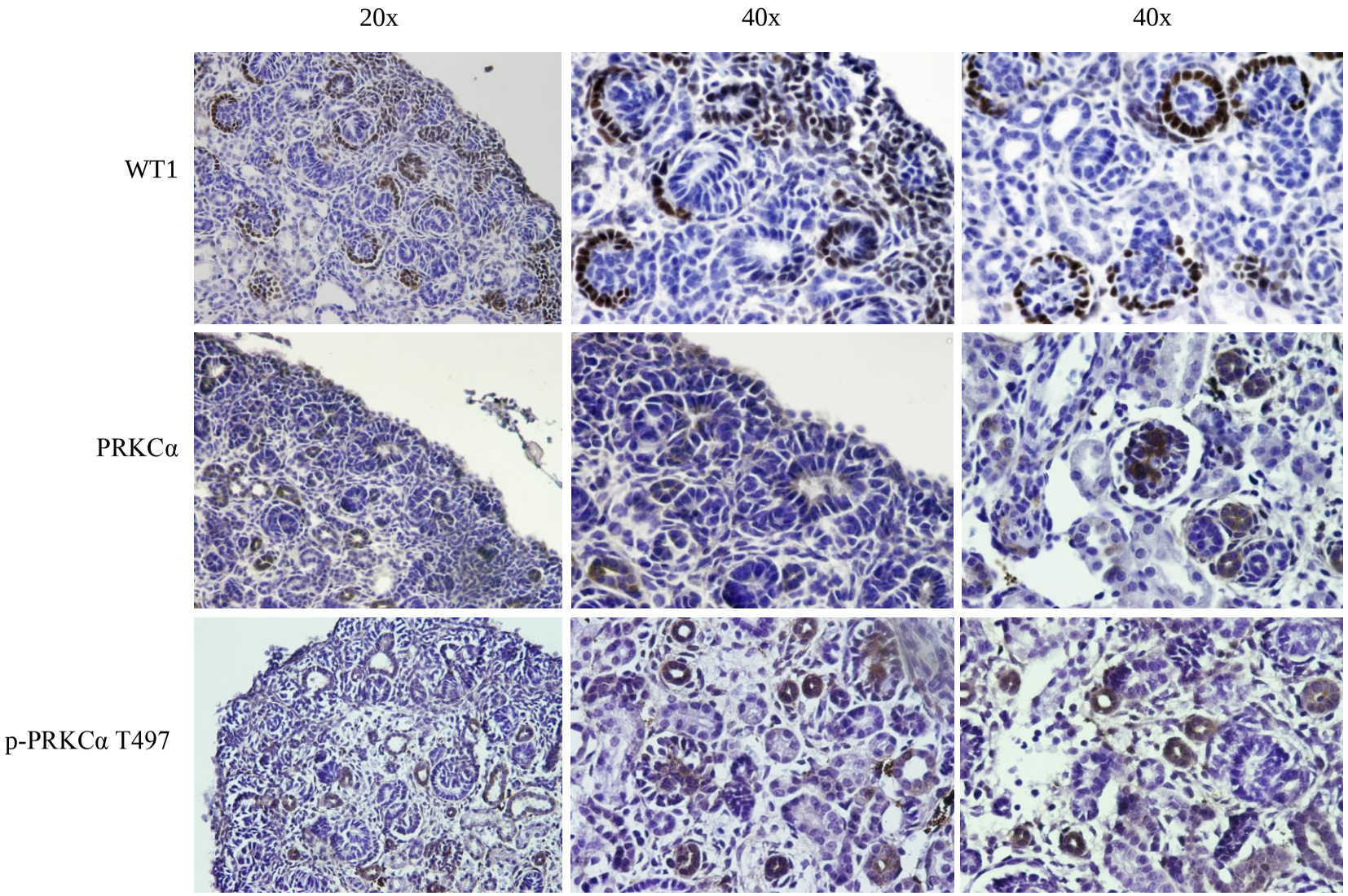


Figure 2: (top panel) WT1 IHC of wild type E14.5 mouse kidney shows staining of condensing mesenchyme, early epithelial structures, and podocytes. (middle panel) PRKCα IHC of wild type E14.5 mouse kidney shows light staining of condensing mesenchyme, proximal and distal tubules, and mesangial cells of the glomerulus. (lower panel) p-PRKCα T497 IHC of wild type E14.5 mouse kidney shows identical staining as total PRKCα IHC; the condensing mesenchyme, proximal and distal tubules, and mesangial cells are all positive.

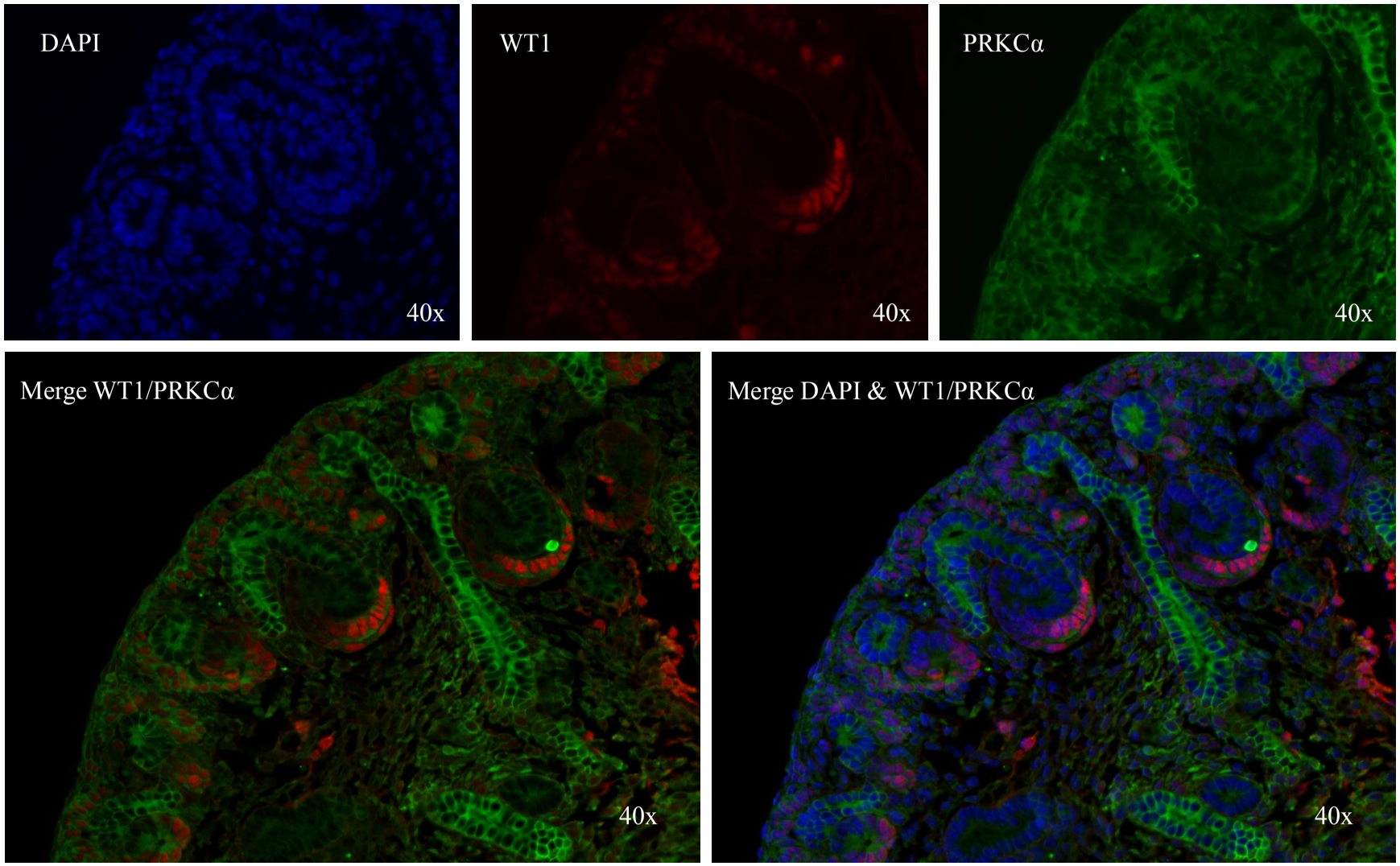


Figure 3a: Double IF staining shows co-localization of WT1 (red) and PRKCα (green) in the condensing mesenchyme of embryonic kidney; PRKCα expression can also be seen in other structures of the developing kidney.

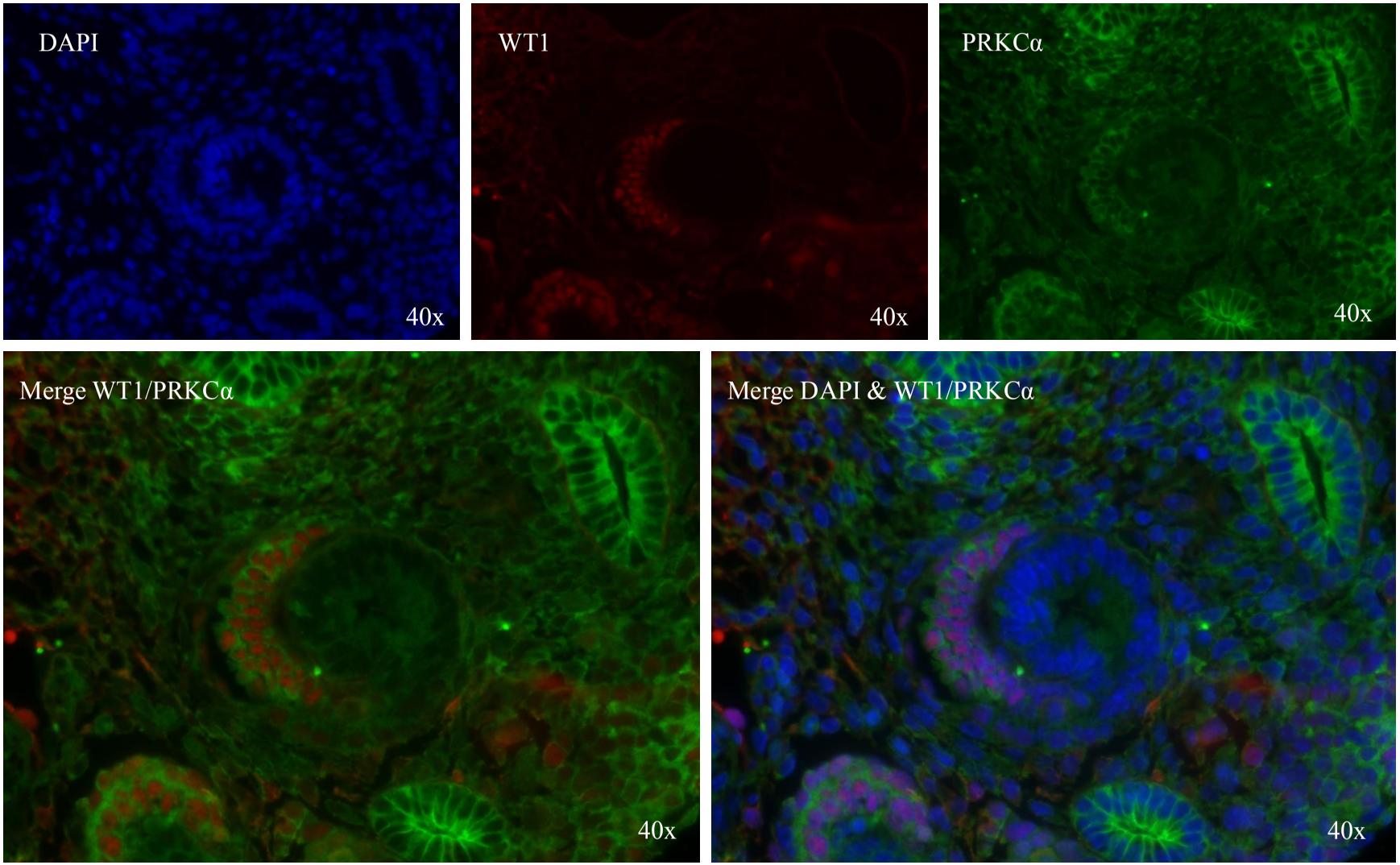


Figure 3b: Double IF staining shows co-localization of WT1 (red) and PRKCα (green) in the condensing mesenchyme of embryonic kidney; specifically, within a comma-shaped body early epithelial structure.

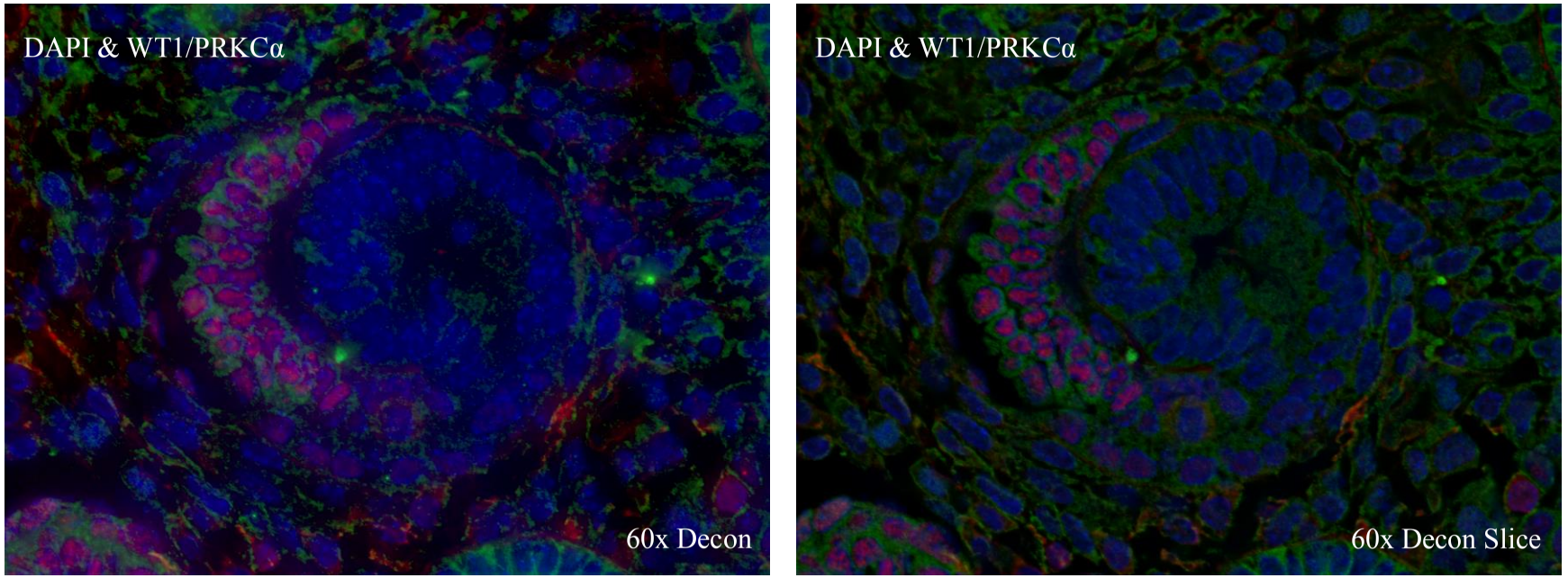


Figure 3c: Deconvolution microscopy double IF staining shows co-localization of WT1 (red) and PRKCα (green) in the condensing mesenchyme of embryonic kidney; specifically, within a comma-shaped body early epithelial structure.

PRKCα. p-PRKCα T497 also shows robust expression (Figure 4). These data confirm that PRKCα co-localizes with WT1 during embryogenesis and kidney development.

In previous experiments I have shown M15 cells to co-express nuclear WT1 and cytoplasmic PRKCα. Following 24hrs of *Wt1* siRNA treatment, a 21% decrease in *Wt1* RNA levels and a resulting 78% decrease in *Prkcα* RNA levels was measured by qPCR. At the protein level, by western blot analysis, a decrease in WT1 protein was also detected, as well as a decrease in PRKCα protein expression (Figure 5). In the reverse experiment T-Rex^{TM} -293(-KTS) cells, in which WT1 was over-expressed using 1ug/mL tetracycline induced WT1(-KTS) expression for 24hrs, an 11-fold increase in WT1 RNA expression was assessed by qPCR. Following the significant increase in WT1 RNA expression, a 40% increase in PRKCα RNA expression was observed. Western blot analysis of protein expression confirms this relationship at the protein level; using cell lines T-Rex^{TM} -293(-KTS) and T-Rex^{TM} -293(+KTS), both –KTS and +KTS isoforms of WT1 are increased after being induced with tetracycline and result in an increase in PRKCα protein level. Interestingly, results shows that at the protein level the +KTS isoform of WT1 results in a much greater increase in PRKCα expression, as compared to the –KTS isoform (Figure 6).

In-vivo deletion of *Wt1* on *Prkcα* gene expression was measured using *WT1* functional knockout mice. A breeding strategy producing *Wt1*$^{-/fl}$*; Cre-ER*^{TM} mutant mice was used to ubiquitously ablate WT1 function at E11.5 days of development in mice embryos. In-utero tamoxifen injection (3mg/40g) at the E11.5 timepoint, disrupts kidney development and results in a 64% decrease in wild type *Wt1* RNA by E13.5. This decrease in *Wt1* RNA results in a 70% decrease in *Prkcα* RNA. Likewise, ablation of

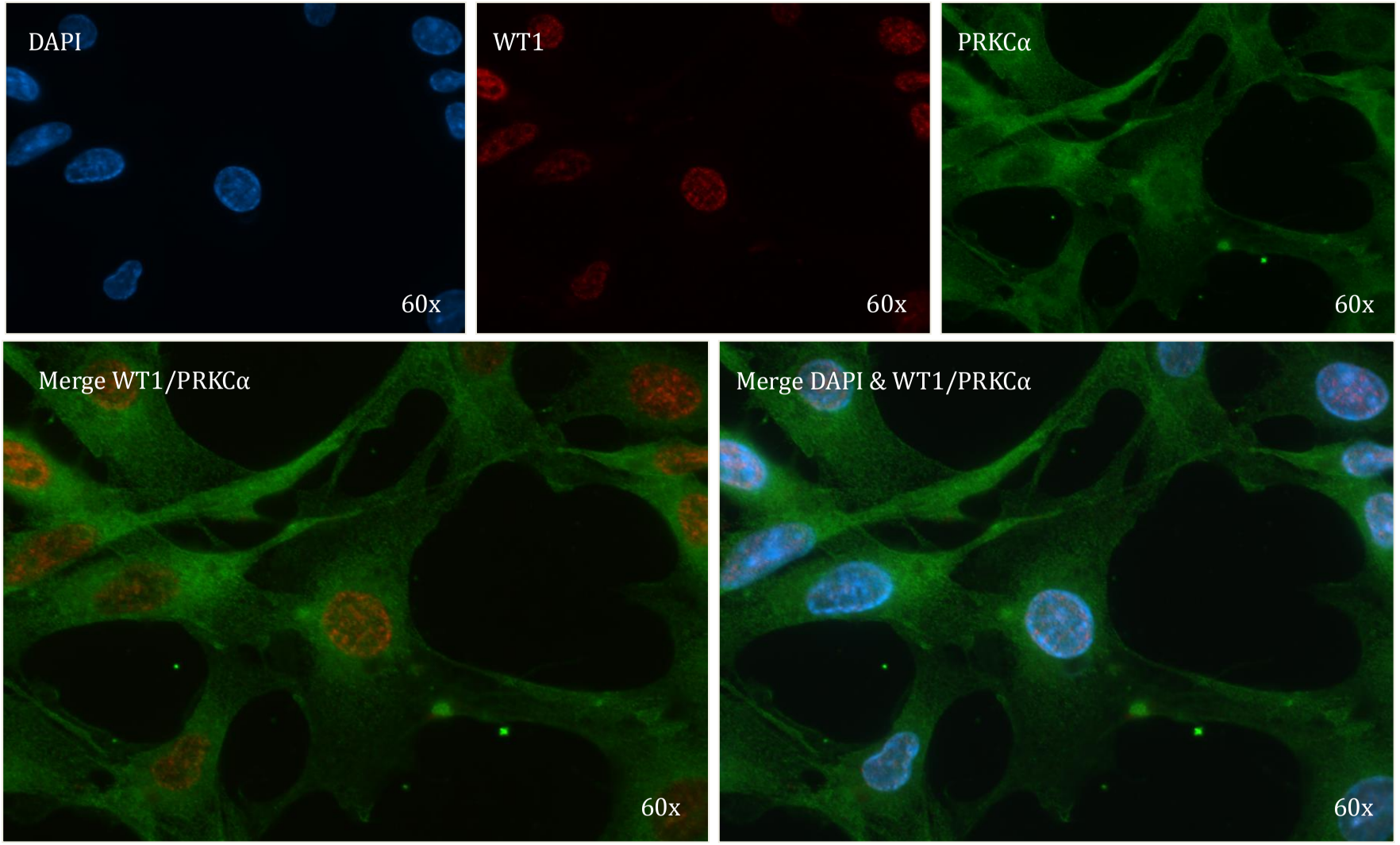


Figure 4a: Double IF staining shows co-localization of WT1 (red) and PRKCα (green) in kidney mesenchymal cell line M15.

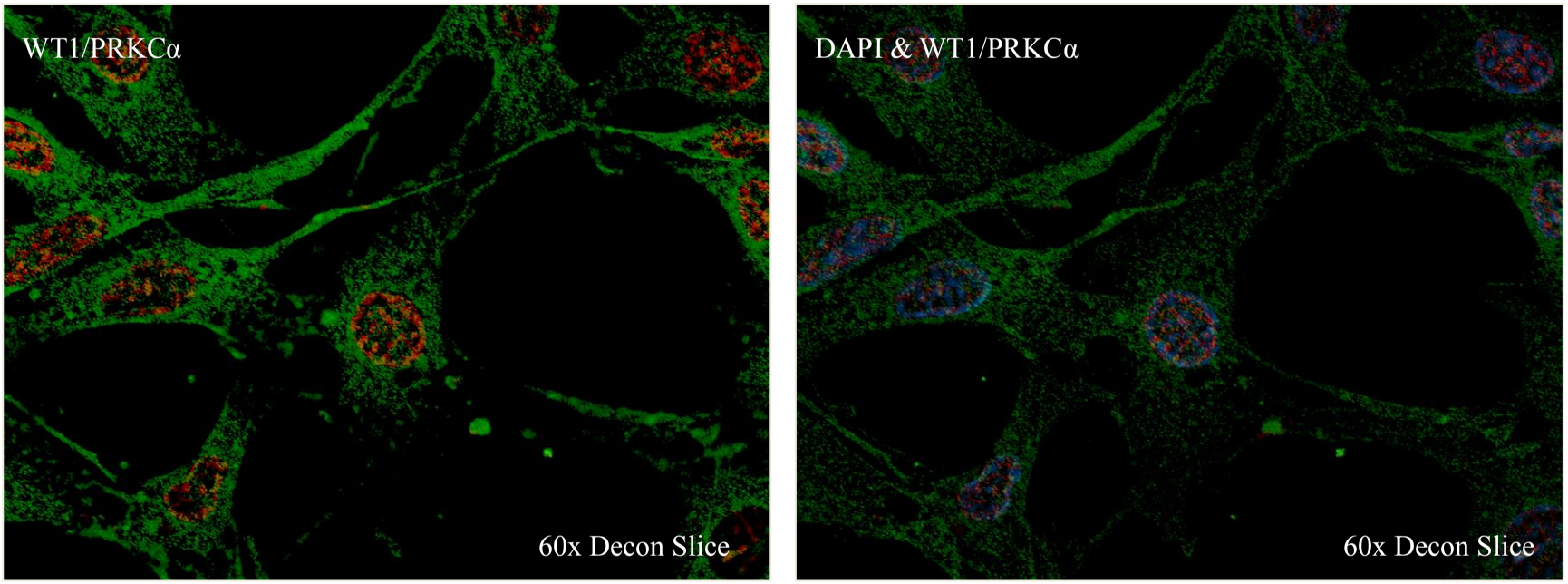


Figure 4b: Deconvolution microscopy double IF staining shows co-localization of WT1 (red) and PRKCα (green) in the kidney mesenchymal cell line M15.

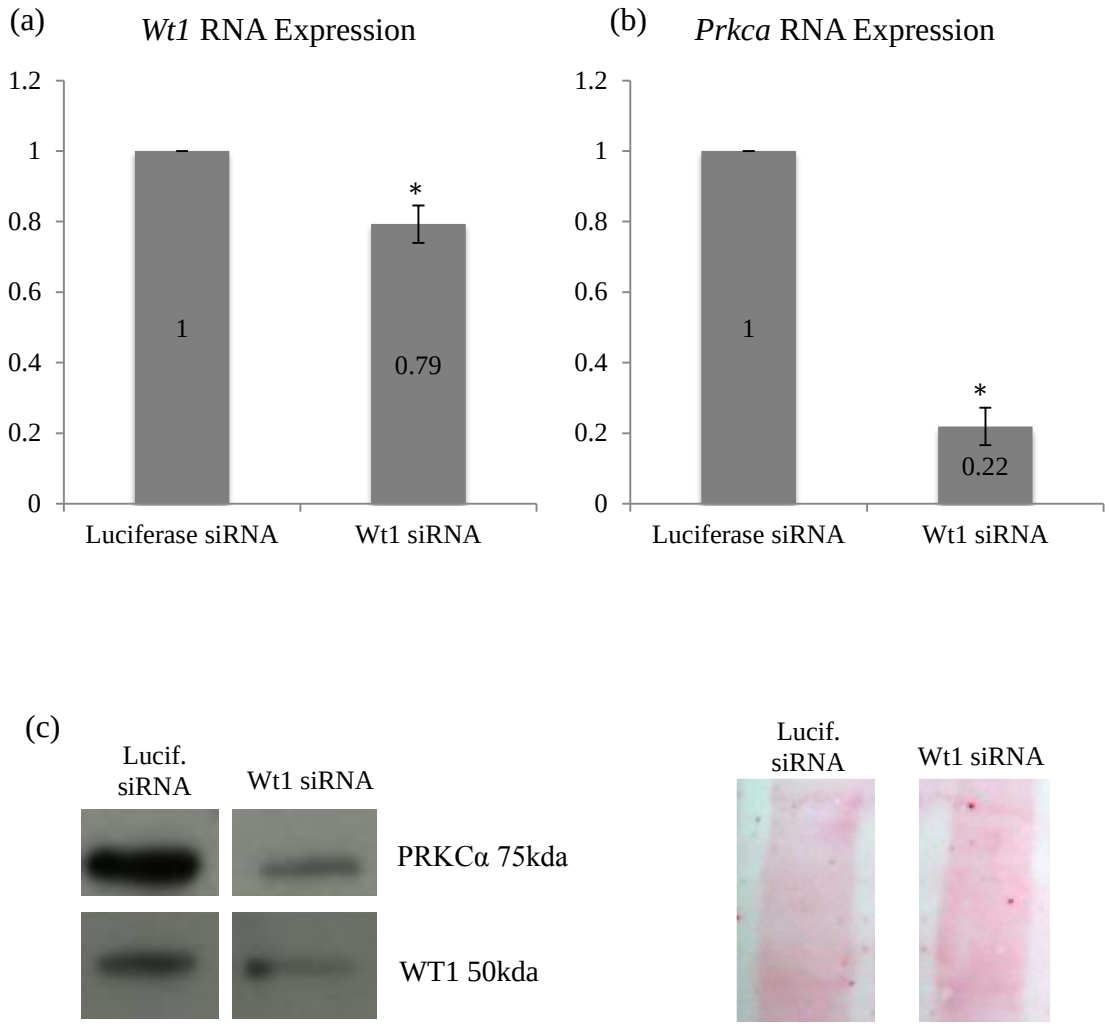


Figure 5: (a) qPCR analysis, siRNA knockdown of *Wt1* in M15 cell culture resulted in 20% decrease in *Wt1* RNA expression (p=0.021) and (b) an 80% decrease in *Prkcα* RNA expression (p=0.042) in comparison to luciferase siRNA control experiments. (c) Knockdown of *Wt1* expression is confirmed at the protein level, and a resulting decrease in *Prkcα* protein expression is seen.

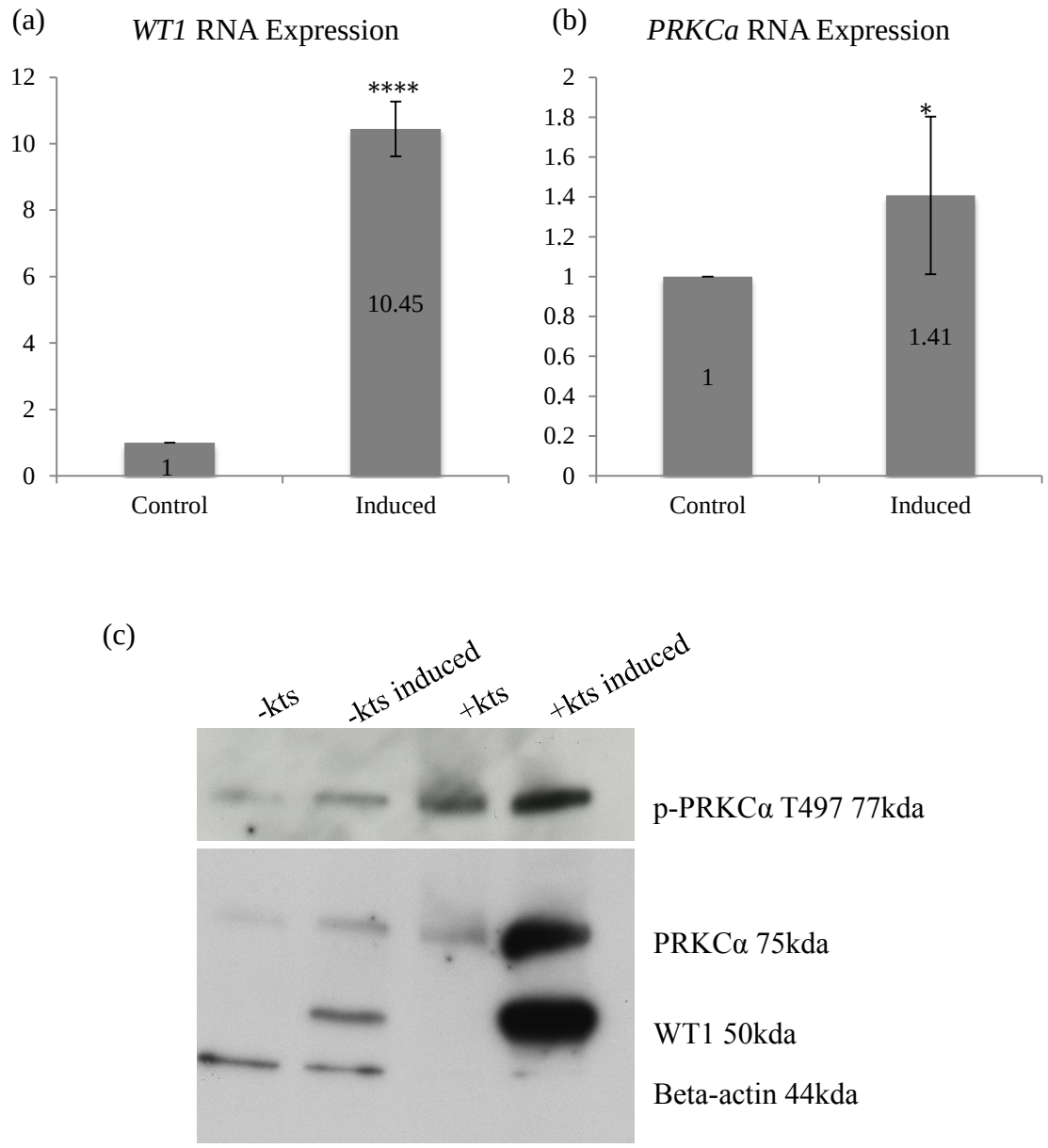
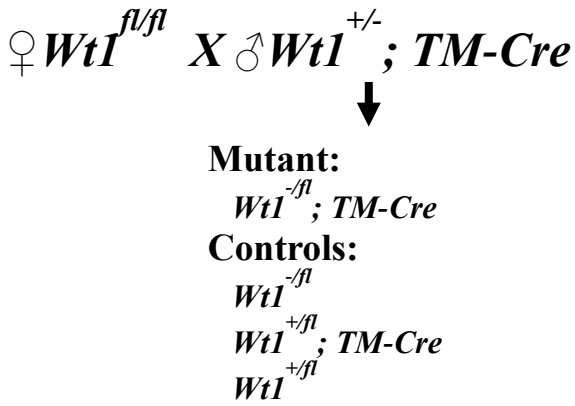


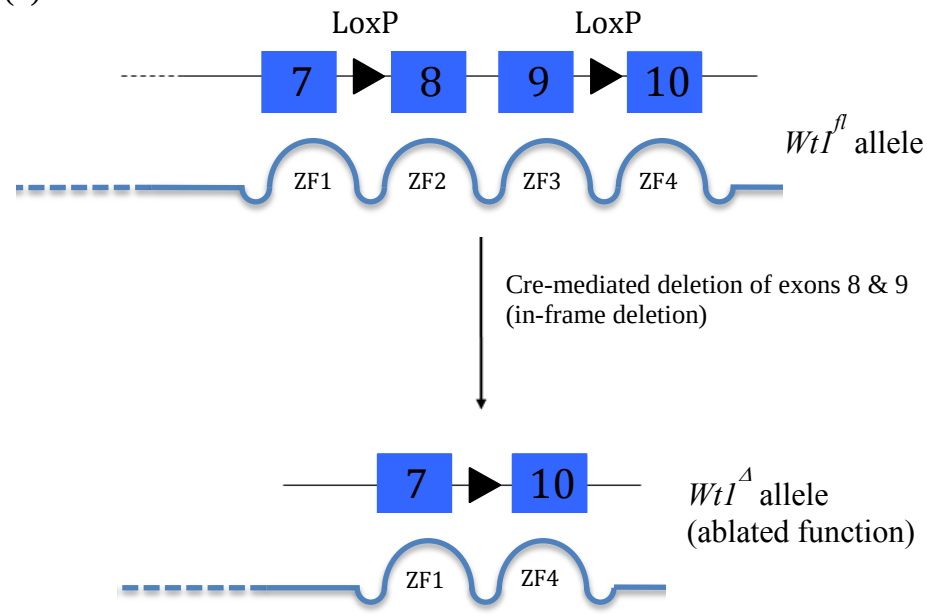
Figure 6: (a) qPCR analysis, tetracycline inducible WT1 expression in HEK293 cells increases *WT1* RNA expression by more than 10-fold (p=0.000019) and (b) a resulting 40% increase in PRKCα RNA expression (p=0.032) is observed. (c) Protein expression data show an increase in WT1 after cell lines are induced with tetracycline. Both -kts and +kts isoforms of WT1 protein are induced and result in downstream increase in PRKCα protein expression, particularly the +kts isoform.

WT1 measured at E15.5 shows a 78% decrease in wild type *Wt1* RNA and a 40% decrease in *Prkc*α RNA. Western blot analysis also confirms this trend, with a significant decrease in PRKCα protein expression in E14.5 *Wt1* knockout embryos observed (Figure 7). Furthermore, the effects of mutant WT1 on PRKCα were evaluated using human Wilms tumor patient RNA. qPCR analysis of PRKCα RNA expression confirms changes in expression originally seen at the protein level in RPPA experiments. It was found that patients with a mutation in *WT1* have a 74% decrease in PRKCα RNA expression (Figure 8).

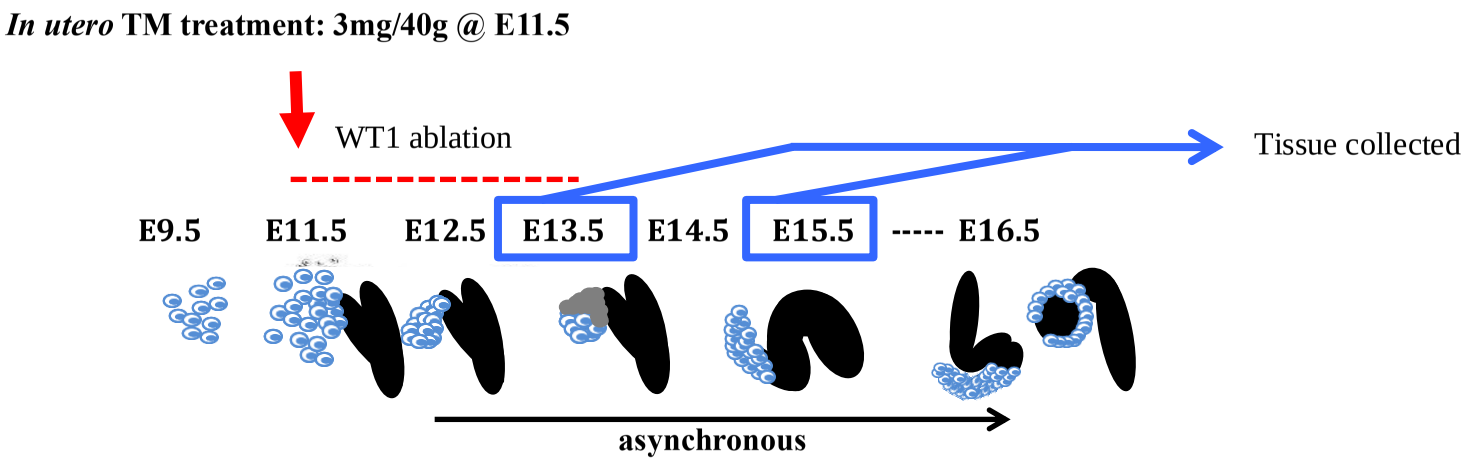
(a)


♀Wt1fl/fl X ♂Wt1+/- ; TM-Cre
Mutant:
Wt1-/fl ; TM-Cre
Controls:
Wt1-/fl
Wt1+/fl ; TM-Cre
Wt1+/fl

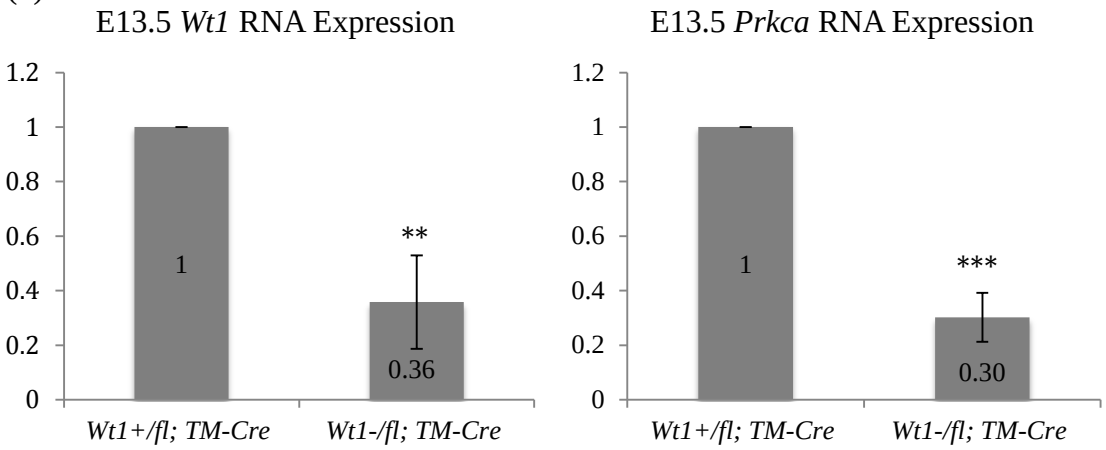
(b)


LoxP
LoxP
7
8
9
10
Wt1fl allele
ZF1
ZF2
ZF3
ZF4
Cre-mediated deletion of exons 8 & 9
(in-frame deletion)
7
10
Wt1Δ allele
(ablated function)
ZF1
ZF4

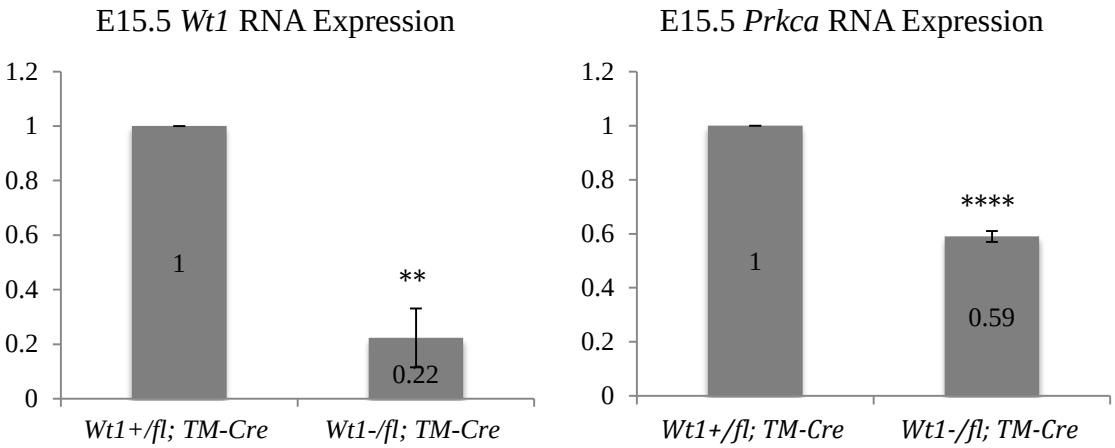
(c)


In utero TM treatment: 3mg/40g @ E11.5
WT1 ablation
Tissue collected
E9.5
E11.5
E12.5
E13.5
E14.5
E15.5
----- E16.5
asynchronous

(d)



(e)



(f)

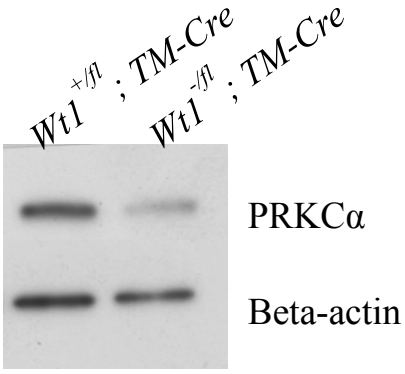


Figure 7: (a) Breeding strategy used to achieve *Wt1* mutant mouse, (b) and Cre-ER, a tamoxifen-inducible Cre-expressing transgene used to ablate *Wt1* function. (c) Mice were injected at E11.5 with 3mg TM, and tissues were collected at E13.5 and E15.5. (d) qPCR analysis, at E13.5 a 60% ablation in *Wt1* RNA expression (p=0.0017) is observed, with a resulting 70% decrease in *Prkcα* RNA expression (p=0.0001). (e) At E15.5 an 80% decrease in *Wt1* RNA expression (p=0.0064) is observed, with a resulting 40% decrease in Prkca RNA expression (p=5.87E-6). (f) Western analysis shows a decrease in *Prkcα* expression at the protein level following *Wt1* ablation.

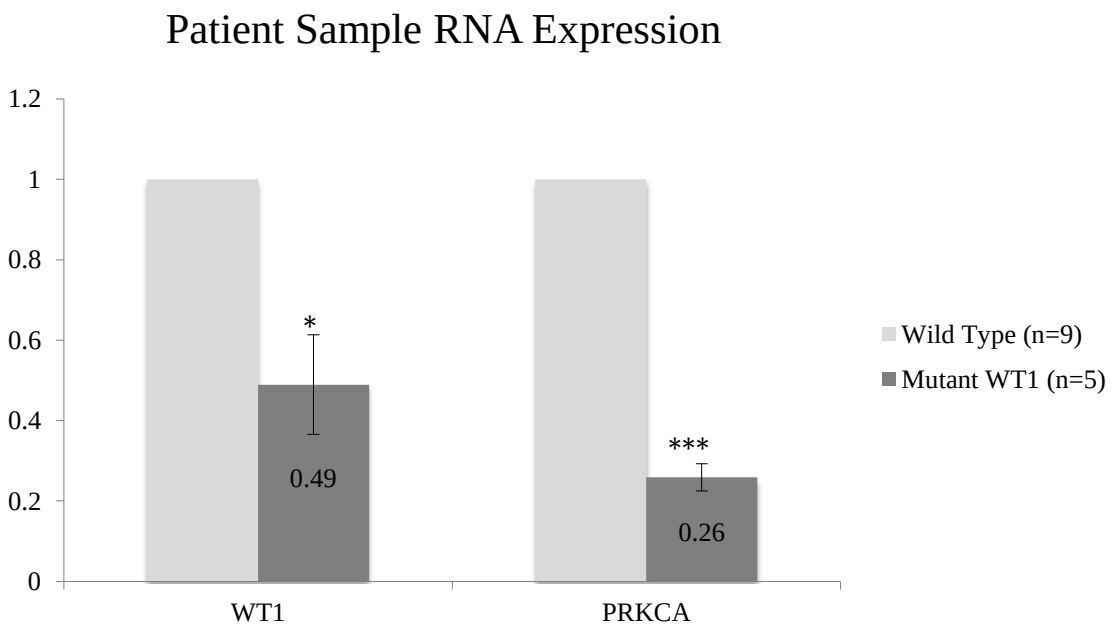


Figure 8: qPCR analysis of Wilms tumor patient samples shows a 50% decrease in *WT1* expression (p=0.028) at the RNA level and a 70% decrease in *PRKCα* RNA expression (p=0.0010). These data support results of the original RPPA heatmap data that showed comparable changes at the protein level.

Discussion:

My ability to demonstrate that WT1 and PRKCα are both expressed in the uncondensed mesenchyme of the developing kidney strengthens the possibility that WT1 transcription factor regulates *Prkcα* expression. WT1 staining is largely nuclear in the uncondensed mesenchyme of E14.5 mouse kidney while PRKCα expression is cytoplasmic, confirming that regulation of *Prkcα* at the transcription level by WT1 takes place within the nucleus before PRKCα is shuttled to cytoplasmic regions where it is catalytically active (Nakashima, 2002). Phosphorylation of PRKCα at the T497 residue is considered essential for kinase activity (Cazaubon, 1994) and therefore p-PRKCα T497 is expected to localize to regions beyond the cell nucleus. By immunostaining for the T497 residue and localizing its expression to the cytoplasm, I have demonstrated that the phosphorylated form is present in the kidney, consistent with the hypothesis that WT1 is regulating a catalytically active form of PRKCα. WT1-PRKCα immunofluorescent staining of embryonic kidney structures provides further evidence for protein co-localization. Positive staining for WT1 and PRKCα can be clearly seen in the epithelializing cells of a comma-shaped body in E14.5 mouse kidney. As discussed earlier, WT1 expression becomes restricted to the epithelial structures of the developing kidney as organogenesis proceeds (Pelletier et al., 1991; Pritchard-Jones, 1990). At the E14.5 stage in mouse kidney, WT1 expression is mainly found in early epithelial structures of the developing kidney. While immunohistochemical staining clearly showed PRKCα and WT1 co-expression in the uncondensed mesenchyme, double immunofluorescence staining of comma-shaped bodies further confirms that the two proteins are expressed within the same cells at later stages in kidney development. This

staining pattern suggests that transcriptional control of *Prkc*α by WT1 continues throughout the mesenchymal-to-epithelial transition of kidney organogenesis. A final method for confirming co-localization of WT1 and PRKCα in kidney mesenchymal cells was achieved by in vitro staining of a kidney cell line F7346, derived from harvested kidney mesenchymal cells. Mesenchymal kidney culture shows a clear co-expression of WT1 and PRKCα and confirms both immunohistochemical and immunofluorescent data.

I was able to demonstrate by a number of methods that WT1 and PRKCα co-localize at a timepoint in kidney development at which cells can give rise to Wilms tumor. Mutations in *WT1* occurring before complete mesenchymal-to-epithelial transition disrupt cellular differentiation and drive tumorigenesis. By showing that PRKCα is expressed alongside WT1 in the uncondensed mesenchyme, before formation of early epithelial structures and at a time during mesenchymal differentiation at which Wilms tumors are though to arise, we have shown that it is possible that WT1 is regulating *Prkcα.* While further evidence is needed to implicate *Prkcα* as a contributor to Wilms tumorigenesis, these data, along with RPPA expression patterns in Wilms tumor patients harboring *WT1* mutations, strengthens the possibility of WT1 regulating *PRKCα* expression. *Prkcα* is therefore a potential novel target of WT1 during kidney development, and decreased expression of *Prkcα* may contribute to aberrant kidney development and/or Wilms tumor.

Both knockdown and up-regulation experiments show that PRKCα is regulated by WT1 in-vitro. M15 cells were previously shown to express both WT1 and PRKCα, although in different subcellular components (Figure 4). In-vitro knockdown experiments using *Wt1* targeted siRNA show that a decrease in *Wt1* RNA expression

results in a significant decrease in *Prkcα* RNA expression. This result suggests that WT1 is in fact regulating *Prkcα* gene expression. Furthermore, changes in gene expression at the RNA level are mirrored at the protein level with a decrease in PRKCα protein expression a result of decreased WT1 protein expression. M15 cell culture is a mouse mesenchymal cell culture; this data, demonstrates that the regulatory relationship between WT1 and PRKCα, originally seen in human tumors, is maintained in cells that are important to kidney formation and implicated in tumorigenesis.

In the reverse experiment, *WT1* increased expression is driven by tetracycline-inducible system in human embryonic kidney cells line, HEK293. As predicted by our hypothesis, a significant increase in WT1 RNA and protein levels results in a significant increase in PRKCα RNA and protein levels. Together, knockdown and up-regulation experiments in kidney cells specific to embryogenesis support our hypothesis that WT1 is regulating *PRKCα* expression. In-vitro experiments provide a system not confounded by complex histology or genotype and retain the positive regulatory relationship between the genes in question, providing compelling evidence in support of our hypothesis.

By using a mouse model for *Wt1* functional knockout, we were able assess WT1 regulation of *Prkcα* and validate in-vitro findings in-vivo. In order to achieve the highest level of biological relevance with regard to Wilms tumorigenesis, gene expression levels were assessed at E13.5-E15.5 days, a period important for correct cellular formation of the embryonic kidney. RNA levels assessed at E13.5 and E15.5 showed the expected knockout of wild-type WT1 and a resulting decrease in *Prkcα* RNA levels. It is important to note that a greater level of decreased *Prkcα* RNA expression was observed at E13.5 as opposed to E15.5 timepoint. This data can be explained histologically; as the

kidney undergoes signaling between uncondensed metanephric mesenchyme and the invading uteric bud, cell populations differentiate and give rise to diverse structures not implicated in Wilms tumorigenesis. Previously, we identified cells populations not implicated in tumorigenesis as expressing *Prkcα.* As the embryonic kidney differentiates, these populations increase and therefore there is greater PRKCα in the total kidney. In order to confirm in-vivo changes at the protein level, embryonic kidney expression was assessed at the E14.5 timepoint. As expected, there was a decrease in PRKCα protein levels following knockout of wild-type WT1 protein.

For further analysis of WT1 and PRKCα expression levels we assessed human Wilms tumor patient samples, originally used for RPPA analysis, for RNA expression levels. We were able to confirm changes in gene expression in patients harboring *WT1* mutations, originally seen at the protein level, at the RNA level. Patients with mutations in *WT1* were found to have significantly decreased PRKCα RNA levels.

While human RPPA data has already demonstrated that a decrease in wild type WT1 correlates with decreased PRKCα protein expression in Wilms tumor, we have been able to show in a variety of experimental systems that decreased WT1 expression leads to decreased PRKCα expression. In both human and mouse systems we were able demonstrate that PRKCα expression is positively correlated with WT1 expression. Similarly, by utilizing in-vitro models, we were able to eliminate variables that exist in in-vivo models such as multiple cell types, to confirm that intra-cellular control of PRKCα expression is maintained by WT1. It is still imperative to determine if regulation of PRKCα by WT1 is a direct or indirect process. It is possible that WT1 transcription factor binds the PRKCα promoter region directly to control transcriptional activation,

but it is also possible that PRKCα falls further downstream of WT1 transcriptional activity. Overall, there is compelling evidence that WT1 does in fact regulate *PRKCα* expression directly or within close proximity as we observed strong correlation between WT1 and PRKCα expression levels.

Chapter 3: Identification of WT1 binding sites in PRKCα regulatory regions

Introduction:

The data presented in the previous chapter show that PRKCα expression at both the RNA and protein level is dramatically reduced or increased following, respectively, loss of WT1 function or up-regulation of WT1 expression. However, further evidence is needed to support the hypothesis that WT1 directly transcriptionally regulates *PRKCα*. The Wilms tumor gene encodes a DNA-binding protein containing four c-terminal Cys_2His_2 zinc fingers shown biochemically to bind target DNA (Background Figure 2) (Stoll et al., 2007). While originally thought to behave like a typical Early Growth Response (EGR) family transcription factor, the function of WT1 has proven to be more complex (Ladomery & Dellaire, 2002). The primary WT1 transcript is alternatively spliced into four isoforms depending on the presence or absence of exon 5 and a three amino acid insert (KTS) between zinc fingers 3 and 4 of the protein (Call et al., 1990; Gessler et al., 1990). Previous studies have found no major developmental implications concerning the exclusion of exon 5 from the final transcript, and therefore is not a focus of this study (Natoli et al., 2002); however, there are significant implications to protein activity based on +KTS vs. –KTS splice variants and their expression ratios (Hammes et al., 2001; Klamt et al., 1998). +/- KTS isoforms have been show to localize to different areas of the cell and preform unique roles. The –KTS isoform is localized to the cell nucleus and has been shown to be transcriptionally active, while the +KTS isoform is localized to the splicesome complex and largely plays a post-transcriptional role (Haber et al., 1991; Larsson et al., 1995).

Previous work has shown that WT1 protein preferentially binds GC-rich regions similar to EGR-1 consensus binding site 5'GCG-(T/G)GG-GCG3,' although with loose consensus and isoform specificity (Bickmore et al., 1992; Rauscher, Morris, Tournay, Cook, & Curran, 1990). Alternatively, WT1 transcription factor has also been shown to bind sequences 5'TCCTCCTCCTCCTCTCC3' and 5'GCGTGGGAGT3,' as well as others, depending on cell type and expression levels (Nakagama, Heinrich, Pelletier, & Housman, 1994; Wang, Qiu, Enger, & Deueul, 1993). Due to the loose consensus and poorly characterized binding sites of WT1, it is necessary to experimentally demonstrate WT1 binding of target sequences to prove transcriptional regulation. In this case it is necessary to predict possible WT1 binding sites within the PRKCα promoter and demonstrate transcriptional regulation by WT1 using reporter assays.

The human PRKCα gene shows a high level of conservation, >98% homology between species, with shared homology to the murine transcription start site and promoter specifically (Clark, Haridasse, & Glazer, 2002; Desai, Hirai, Karnes, Niles, & Ohno, 1999; Finkenzeller, Marme, & Hug, 1990). Glazer et al. mapped and characterized the human PRKCα promoter in great detail and identified a number of important features. Basal promoter activity is restricted to a region from -227 to +77 relative to the transcription start site, which was narrowed further by deletion constructs to the sequence from -227 to -106. Within this region an activator protein-2 (AP-2) binding site was identified and shown to increase transcription when overexpressed, and multiple other AP-2 and Sp1 binding sites were identified throughout the promoter region. While post-transcriptional activation of PRKCα gene is a well-understood process, few studies have been carried out to better understand transcriptional regulation

of PRKCα due to its ubiquitous expression. In order to determine if WT1 transcription factor binds and regulates *PRKCα,* the cloned human sequence was obtained from Glazer et al.

Materials/Methods:

Promoter Constructs and Luciferase Reporter Assay:

Three PRKCα promoter constructs, -1571/+77, -227/+77, and -106/+77, were received from Robert Glazer, Ph.D. (Professor, Oncology and Pharmacology, Georgetown University) (Figure 9) and sequenced to confirm 100% sequence identity to human PRKCα. HEK293 cells were transfected with one of three pGL3-PRKCα promoter vectors. In addition, luciferase activity control experiments using empty PGL3 vector were carried out to assess for background activity. In 24-well plates, $7.5x10^4$ HEK293 cells were plated per well before experimentation. Into each well was added 300ng of PRKCα promoter construct as well as 5ng of *Renilla* (sea pansy luciferase) construct for normalization. Following transfection using SuperFect Transfection Reagent (Qiagen), the cells were incubated for 36hrs before trypsinization and assessing for both firefly and *Renilla* activity using Dual-Luciferase Reporter Assay System (Promega) according to the protocol.

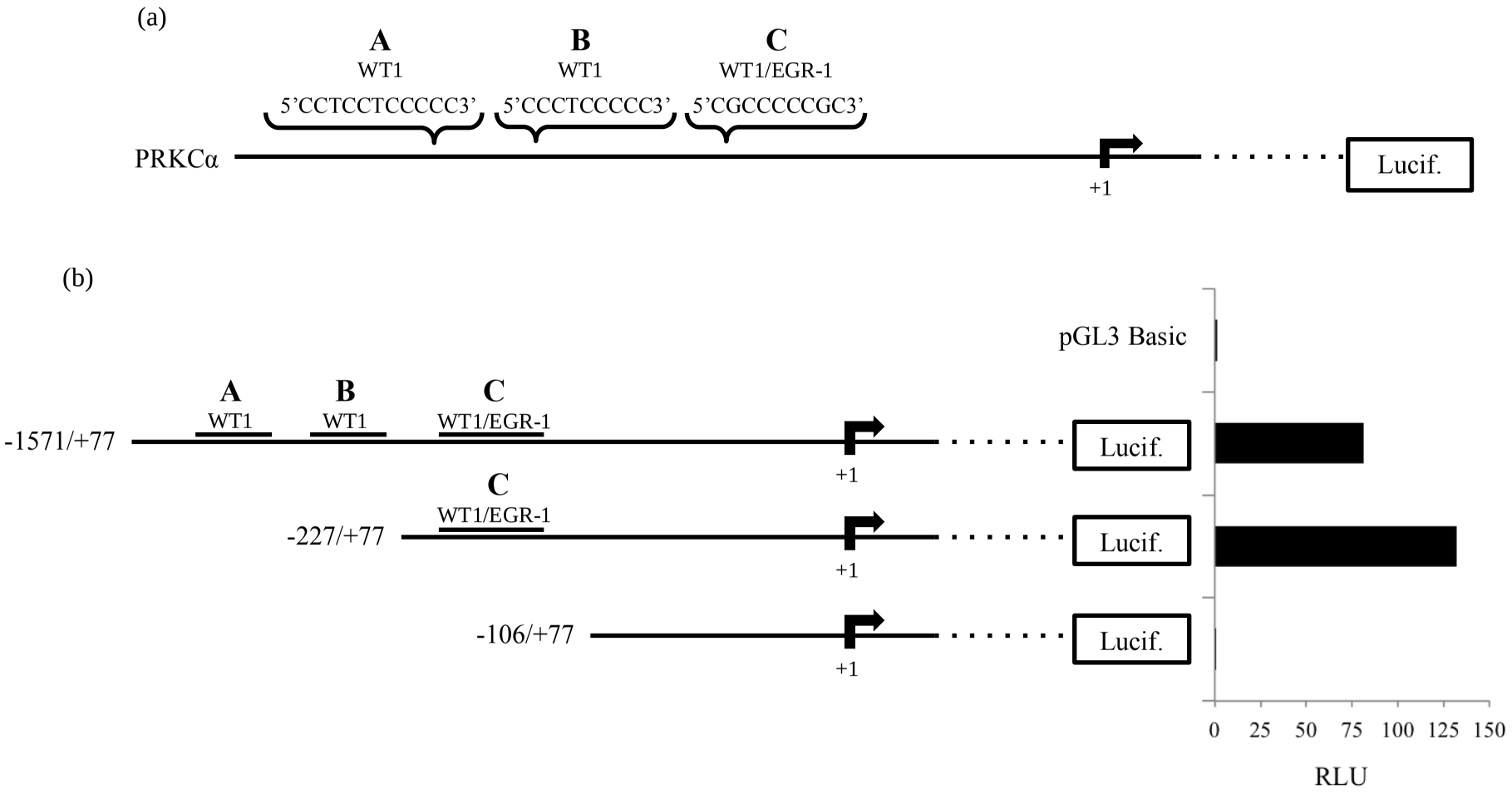


Figure 9: (a) WT1 binding sites identified in the *PRKCα* promoter region. Site A and B identified as BIOBASE predicted WT1 binding sites, and Site C as a WT1/EGR-1 consensus site. (b) Diagram of promoter constructs used in luciferase reporter experiment; -227/+77 has the highest affinity for WT1 binding and -1571/+77 construct is also positive for WT1 binding. Both pGL3 Basic and -106/+77 negative control constructs show no WT1 binding activity.

Results:

BIOBASE TRANSFAC© bioinformatics software was first used to assess PRKCα regulatory regions for possible WT1 binding sites. Two predicted WT1 binding sites were identified, located upstream of the PRKCα transcriptional start site and within the predicted PRKCα promoter: 5'CCTCCTCCCCC3' and 5'CCCTCCCCC3'. Both sites are GC rich sequences, a feature commonly reported for WT1 target sequences. A third site, closer to the transcription start site and not originally predicted by BIOBASE, was identified by hand as a possible WT1 binding site. The third site, an EGR-1 site binding site 5'CGCCCCCGC3,' showed by consensus that it could possibly serve as a WT1 binding sequence (Figure 9). Three PRKCα promoter constructs, -1571/+77, -227/+77, and -106/+77, were assessed for WT1 binding activity (Figure 9). The -1571/+77 PRKCα promoter construct contains all three predicted WT1 binding sites, the -227/+77 PRKCα promoter construct contains only the EGR-1 consensus site, and the -106/+77 does not contain a predicted WT1 binding site. Luciferase reporter experiments, in which HEK293 cells were transfected with one of three pGL3-PRKCα promoter vectors, tested for up-regulation of *PRKCα* transcript by WT1. Cells transfected with the -1571/+77 construct showed significantly increased luciferase activity in comparison to the -106/+77 negative control construct, demonstrating that endogenous WT1 can drive PRKCα expression and possibly binds the promoter directly. Moreover, cells transfected with the -227/+77 construct showed significantly increased luciferase activity in comparison to the -106/+77 negative control, as well as the -1571/+77 construct. Interestingly, while the -1571/+77 construct contains all three predicted WT1 binding

sites, it is the -227/+77 construct containing the EGR-1/putative WT1 binding site that produced the strongest luciferase signal (Figure 9).

Discussion:

Bioinformatic assessment predicts direct binding of WT1 to the *PRKCα* promoter and Luciferase reporter experiments demonstrate that *PRKCα* expression is regulated by WT1 transcription factor. While a number of possible WT1 binding sites were predicted, the highest level of transcriptional activity was localized to a region from -227 to -106 of the PRKCα promoter. Interestingly, this is the same region, previously described by Glazer et al., that is required for basal activity (although this is attributed to AP-2 binding site). In order to confirm the predicted 5‘CGCCCCCGC3’ WT1 binding site as the actual site of WT1 binding, it will be necessary to carry out mutation analysis of this site. By altering the target sequence by even a few nucleotides we should be able to obliterate WT1 binding ability (Shahidul Makki, Cristy Ruteshouser, & Huff, 2013).

Direct regulation of *PRKCα* by WT1, in conjunction with PRKCα expression levels in Wilms tumors harboring *WT1* mutations, implicates PRKCα as a possible important player in tumorigenesis. Developmentally, *WT1* mutations have been shown to contribute to tumorigenesis through poorly defined signaling mechanisms - these results suggest that WT1 signaling through PRKCα is a process important to normal kidney organogenesis and that when normal signaling cascades downstream of WT1 and PRKCα are disrupted, an environment favorable for tumor development is created.

Chapter 4: Identification of WT1 and PRKCα downstream targets in-vitro

Introduction:

In Wilms tumorigenesis, mutations in WT1 lead to decreased PRKCα expression in the developing kidney. We have shown that WT1 directly regulates PRKCα and that gene expression is significantly decreased following WT1 knockdown. In order to better understand the relationship between WT1 and PRKCα expression and the downstream consequences of their dysregulation, it is important to determine targets of the two proteins. According to our model, mutations leading to aberrant WT1 expression drive gene changes through decreased PRKCα expression that contribute to Wilms tumorigenesis; identifying and mapping downstream targets is important to understanding the cellular micro-environments giving rise to Wilms tumor development.

Materials/Methods:

Creation of Stable Cell lines:

M15, mesenchymal cells were transduced using lentirviral delivered shRNA. Two separate MISSION TRC shRNA Custom Lentiviral Particles, pLKO-puro-IPTG-3xLacO, targeting *Wt1* and *Prkc*α were ordered (Sigma-Aldrich); as well as lentivirus carrying non-target sequence negative control (NTC) shRNA. In 6 well plates, $1.5x10^4$ M15 cells were seeded per well along with lentivirus at 0.5 MOI targeting *Wt1*, *Prkcα*, or NTC. Cells were incubated in 1X DMEM with 10% FBS (Hyclone) media containing lentivirus and 1000ng/mL puromycin selection antibiotic for 24hrs before fresh media and antibiotic without lentivirus was added. Transduced cells were grown for an additional six days under puromycin selection to ensure uptake of viral construct before trypsinization and selection of single cells for subcloning into M15-*Wt1*-shRNA, M15-*Prkcα*-shRNA, and M15-NTC-shRNA cell lines. Each cell line was then evaluated and selected for low copy number by qPCR with primers to the Lac operon of the viral construct. Low copy number was desired for creating a biologically relevant system in which there is decreased probability of inserting sequence into genes important for regular cell behavior. Also, too high of a copy number could possibly tax normal cellular activities to a point of arbitrarily affecting experimental outcome. M15-*Wt1*-shRNA and M15-*Prkcα*-shRNA cell lines were assessed by immunofluorescence for retention of wild type M15 mesenchymal cell markers; cell lines remained positive for WT1 (Dako, mouse monoclonal antibody clone 6F-H2), vimentin (Sigma-Aldrich, mouse monoclonal antibody clone LN-6), and PRKCα (Abcam, rabbit monoclonal antibody Y124) proteins,

and remained negative for cytokeratin (Sigma-Aldrich, pan mouse monoclonal antibody) protein (Figure 10a/b).

shRNA Knockdown:

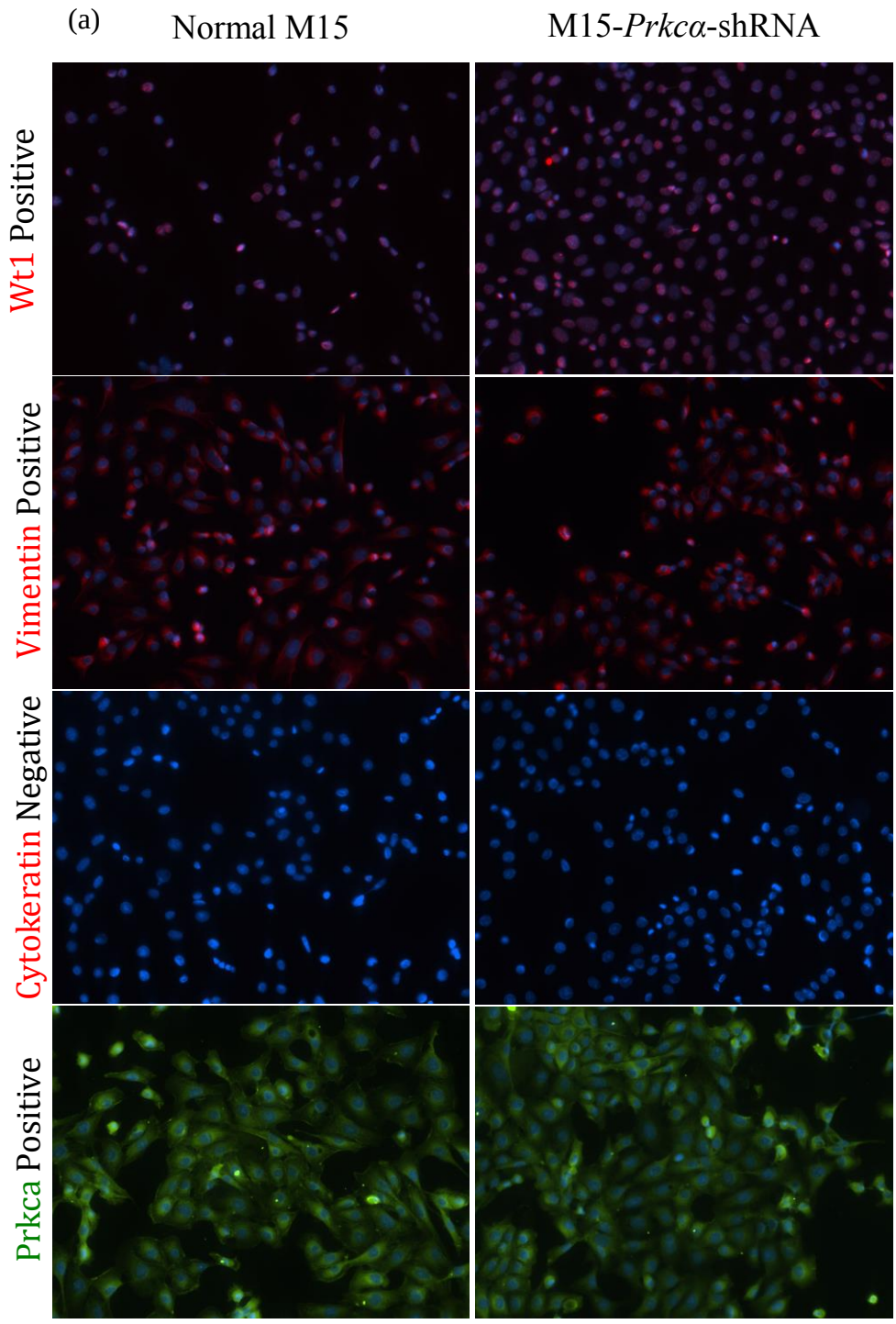
qPCR analysis confirmed knockdown of appropriate gene targets after 48hrs of 50uM IPTG induction.

PCR Array:

RNA lysates from both cell lines, following 48hrs of IPTG induction, were each used in the Mouse Cancer PathwayFinder RT^2 Profiler PCR Array (Qiagen) to profile the expression of 84 genes representative of biological pathways involved in transformation and tumorigenesis.

RPPA Analysis:

Changes in the protein expression of both cell lines were also assessed by Reverse Phase Protein Assay (RPPA). Protein cell lysates were prepared according to the protocol provided by MD Anderson Cancer Center, Dept. of Systems Biology, Reverse Phase Protein Array Core Facility and submitted for analysis of differential gene expression with 157 antibodies. Experiments were submitted in replicates of five in order to obtain statistically significant results. Conversely, HEK 293 cells containing a tetracycline inducible WT1 construct, T-Rex^{TM} -293 WT1(-kts), were assessed for changes in protein expression following induced expression with 1ug/mL tetracycline


(a)
Normal M15
M15-*Prkcα*-shRNA
Wt1 Positive
Vimentin Positive
Cytokeratin Negative
Prkca Positive

(b)

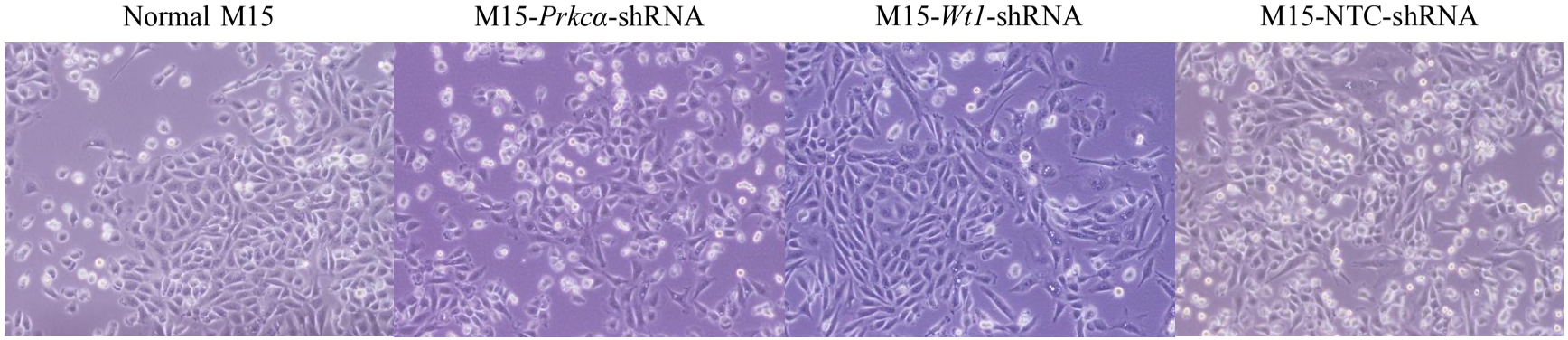


Figure 10: (a) Stable cell lines created using lentiviral delivered shRNA continue to express mesenchymal cell markers following transduction. (b) Stable cell lines retain a cell morphology identical to original M15 cell population following transduction and puromycin treatment.

for 24hrs. Protein cell lysates for induced and un-induced (control) experiments were submitted in triplicate and evaluated with the same 157 antibodies.

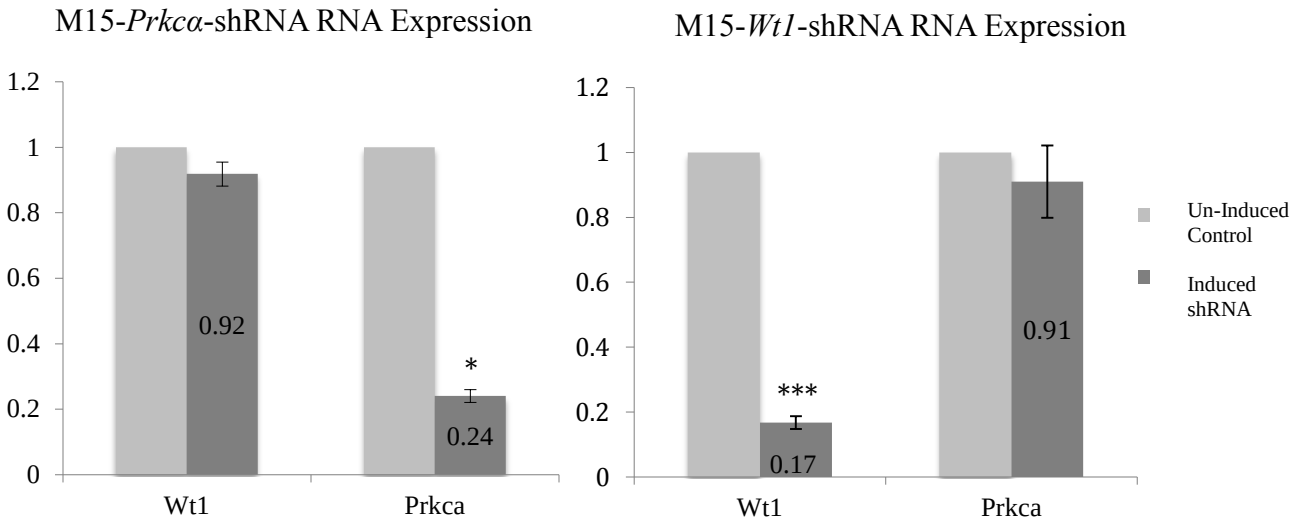
Results:

qPCR analysis of induced stable cell lines confirmed knockdown of appropriate gene targets after 48hrs of 50uM IPTG treatment. M15-*Prkcα*-shRNA cell line showed a 76% knockdown of *Prkcα*, and M15-*Wt1*-shRNA cell line showed an 83% knockdown of *Wt1* (Figure 11a). M15-*Wt1*-shRNA cell lines were also assessed for RNA expression of *Prkcα* since we hypothesize it to be a target of WT1. Previous work suggests that a resulting decrease in *Prkcα* expression should be seen. However, no changes in *Prkcα* RNA levels were identified. Western blot analysis of PRKCα protein levels following *Wt1* shRNA expression did show a decrease in PRKCα protein level (Figure 11b). No change in cell morphology was seen in stable cell lines following 48hrs of IPTG treatment (Figure 11c).

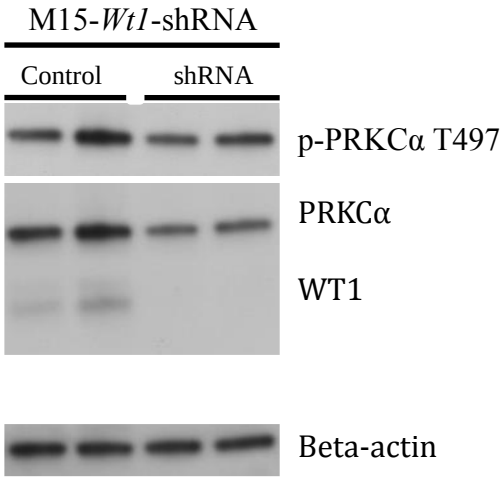
RNA lysates from both cell lines, following 48hrs of IPTG induction, were each used in the Mouse Cancer PathwayFinder RT^2 Profiler PCR Array (Qiagen) to profile the expression of 84 genes representative of biological pathways involved in transformation and tumorigenesis. 11 differentially expressed target genes were identified following knockdown of *Wt1* (Figure 12), and 17 differentially expressed target genes were identified following knockdown of *Prkcα* (Figure 13). In both arrays, the Vegf family of proteins was shown to have changed expression levels. No changes in expression levels for genes encoding signal transduction molecules or transcription factors were detected.

Changes in the protein expression levels of both stable cell lines were also assessed by Reverse Phase Protein Array (RPPA) (Figure 14). Array results show a number of changes following gene targeted knockdown. Significant gene expression changes are listed in Tables 4.

(a)


M15-*Prkcα*-shRNA RNA Expression
1.2
1
0.8
0.6
0.4
0.2
0
0.92
*
0.24
Wt1
Prkca
M15-*Wt1*-shRNA RNA Expression
1.2
1
0.8
0.6
0.4
0.2
0
***
0.17
0.91
Wt1
Prkca
Un-Induced Control
Induced shRNA

(b)


M15-*Wt1*-shRNA
Control
shRNA
p-PRKCα T497
PRKCα
WT1
Beta-actin

(c)

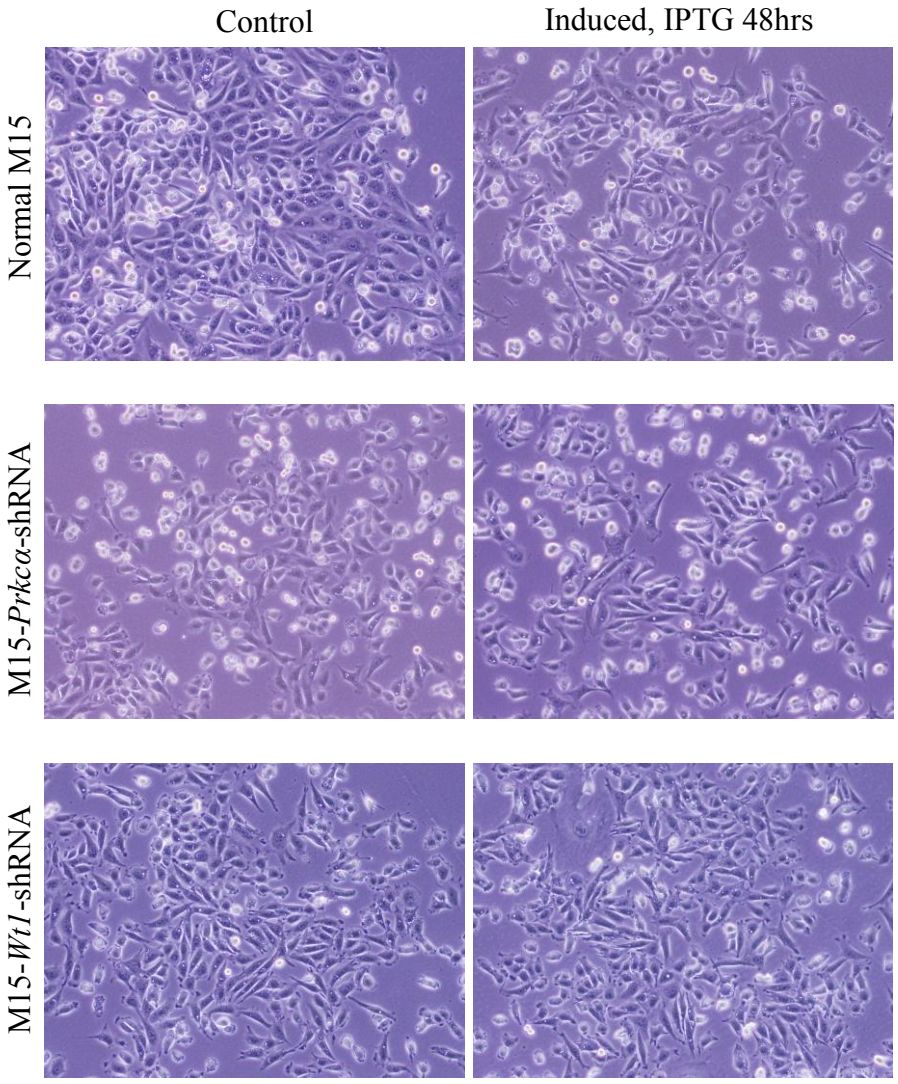


Figure 11: (a) 48hrs induction of shRNA by addition of IPTG into culture media produces knockdown of target genes in both cell lines. In M15-*Prkcα*-shRNA cell line, 76% knockdown of *Prkcα* (p=0.011) is achieved. In M15-*Wt1*-shRNA cell line, 83% knockdown of *Wt1* (p=0.00019) is achieved. (b) Western blot analysis of protein levels following induction of shRNA and knockdown of *Wt1* target gene, confirms decreased protein expression of both WT1 and PRKCα (total and unphosphorylated). (c) No change in cell morphology is seen in stable cell lines following 48hrs of IPTG treatment.

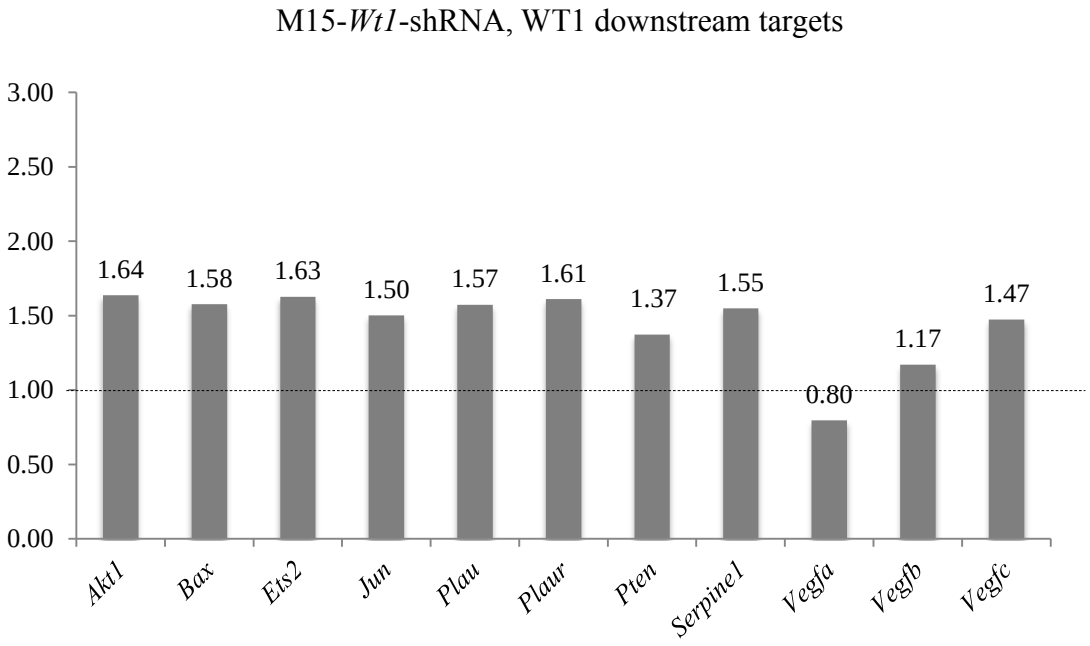


Figure 12: PCR Array of M15-*Wt1*-shRNA (un-induced vs. induced plate) identified 11 differentially expressed target genes.

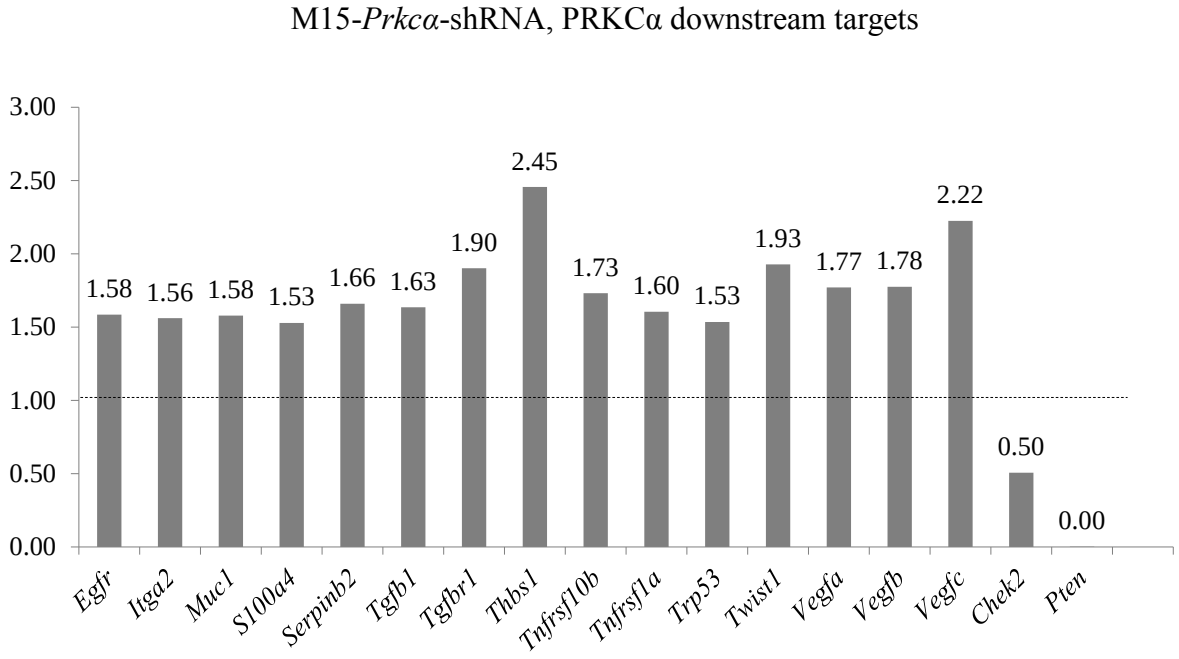


Figure 13: PCR Array of M15-*Prkcα*-shRNA (un-induced vs. induced plate) identified 17 differentially expressed target genes.

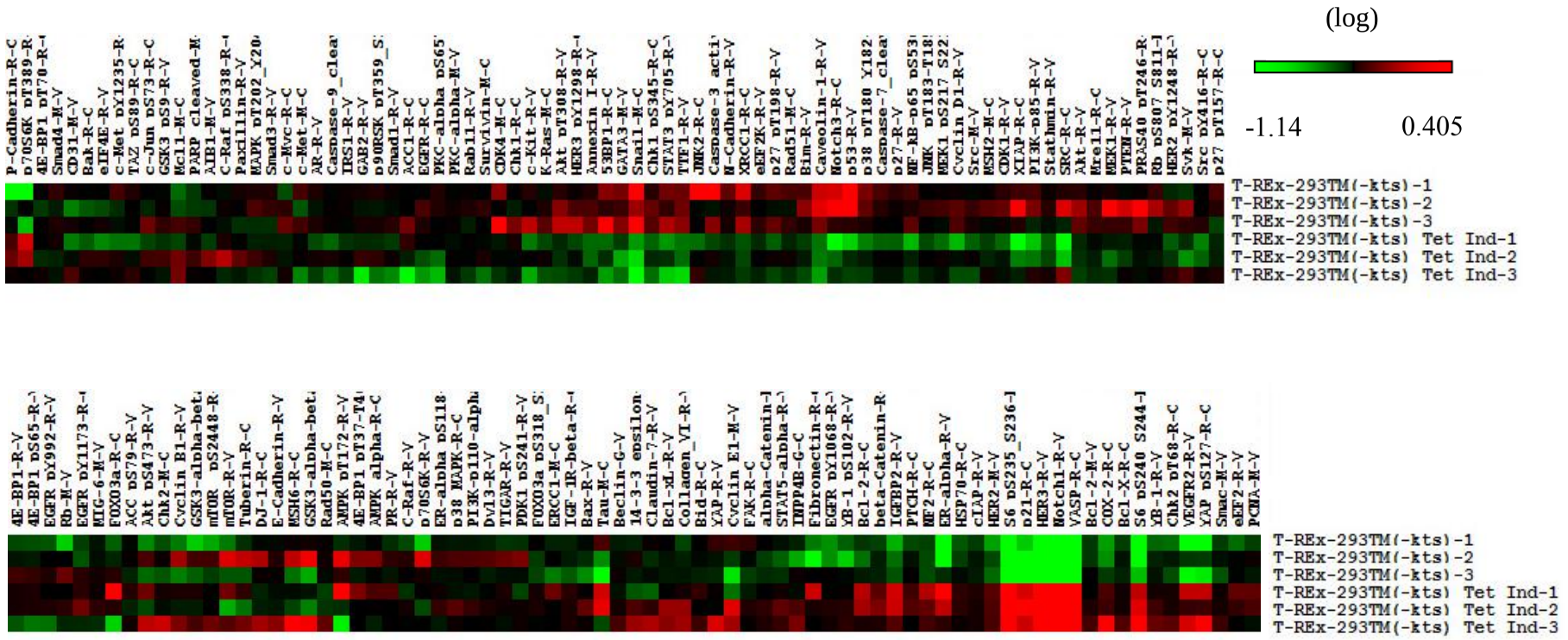


Figure 14a: RPPA heatmap (supervised hierarchical clustering) of HEK 293 protein expression following induction of WT1 expression.

Table 3

Significantly Increased	p-value
Bcl-2	0.04
Bcl-X	0.04
beta-Catenin	0.03
Chk2 pT68	0.03
cIAP	0.03
ER-alpha	0.03
HER2	0.02
HER3	0.00
HSP70	0.04
IGF-1R-beta	0.05
IGFBP2	0.01
NF2	0.03
Notch1	0.00
p21	0.00
S6 pS235 S236	0.01
S6 pS240 S244	0.01
Smac	0.03
STAT5-alpha	0.03
VASP	0.00
VEGFR2	0.04
YAP pS127	0.02
YB-1	0.03

Significantly Decreased	p-value
53BP1	0.03
Akt	0.04
Annexin I	0.01
Bim	0.04
Caveolin-1	0.00
Chk1 pS345	0.01
GATA3	0.01
Mre11	0.05
Notch3	0.03
p38 pT180 Y182	0.02
p53	0.04
Snail	0.00
STAT3 pY705	0.02
TTF1	0.01
XIAP	0.06
XRCC1	0.04

Conversely, HEK 293 cells containing a tetracycline inducible WT1 construct, T-Rex™ -293 WT1(-kts), were assessed for changes in protein expression RPPA analysis (Figure 14). RPPA heatmap data shows significant changes in protein expression in a number of proteins, listed in Table 3.

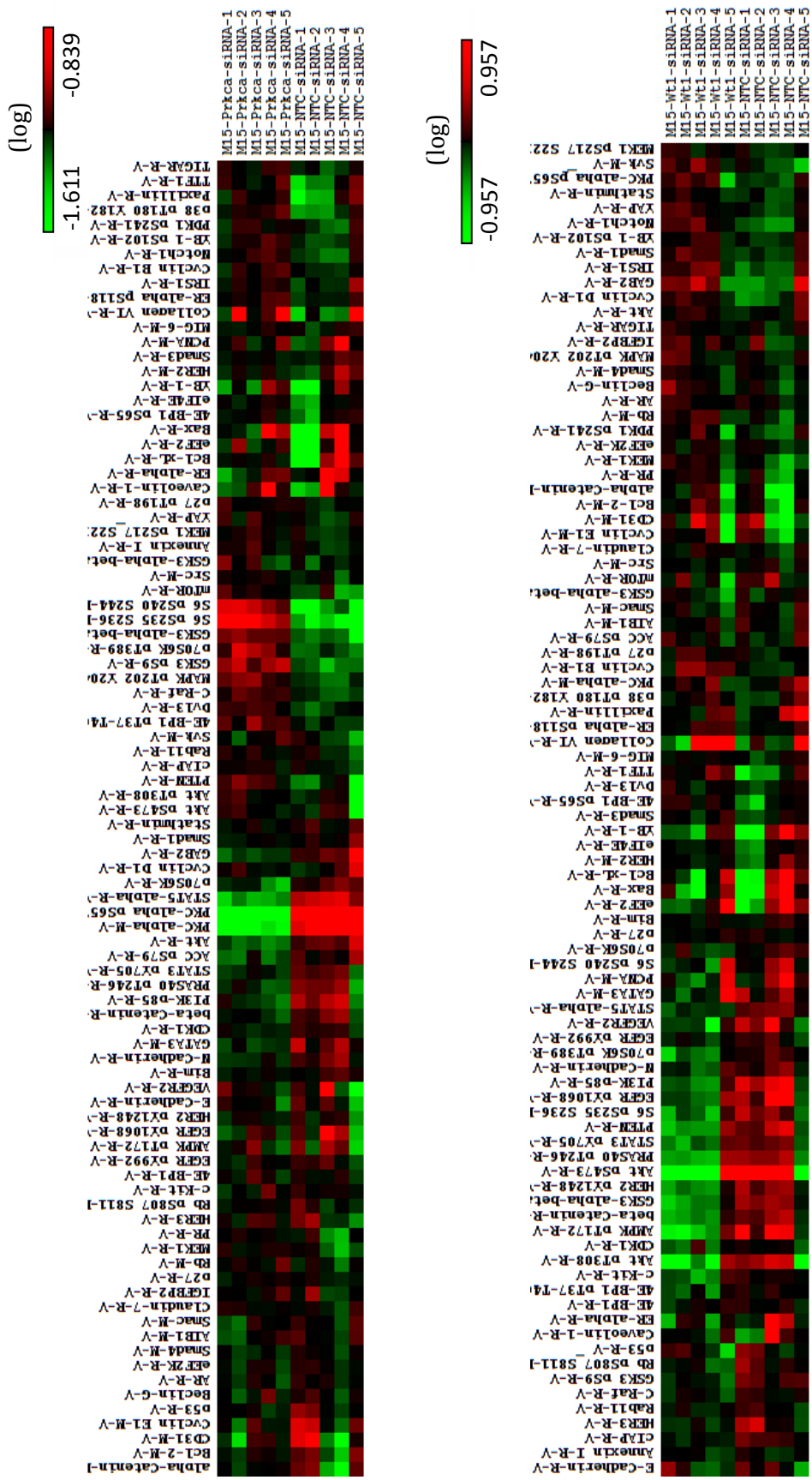


Figure 14b: RPPA heatmap (supervised hierarchical clustering) of M15-*Prkcα*-shRNA and M15-*Wt1*-shRNA following induced expression of shRNA.

Table 4

Putative *Prkcα* Targets	Increased (Inc.) vs. Decreased (Dec.) Expression
4E-BP1 pT37 T46	Inc., p = 0.005
ACC pS79	Dec., p = 0.010
Akt	Dec., p = 0.000
C-Raf	Inc., p = 0.000
Dvl3	Inc., p = 0.005
GAB2	Dec., p = 0.045
GATA3	Dec., p = 0.023
GSK3-alpha-beta pS21 S9	Inc., p = 0.001
GSK3 pS9	Inc., p = 3.94E-05
MAPK pT202 Y204	Inc., p = 0.000
N-Cadherin	Dec., p = 0.023
Notch1	Inc., p = 0.032
p38 pT180 Y182	Inc., p = 0.030
p70S6K	Dec., p = 0.009
p70S6K pT389	Inc., p = 0.002
PKC-alpha	Dec., p = 0.000
PKC-alpha pS657	Dec., p = 1.68E-07
PTEN	Inc., p = 0.030
S6 pS235 S236	Inc., p = 8.64E-05
S6 pS240 S244	Inc., p = 0.000
STAT5 alpha	Dec., p = 9.19E-07

Table 4 cont.

Putative *Wt1* Targets	Increased (Inc.) vs. Decreased (Dec.) Expression
AMPK pT172	Dec., p = 0.012
beta-Catenin	Dec., p = 0.006
cIAP	Dec., p = 0.010
Cyclin B1	Inc., p = 0.024
GSK3-alpha-beta pS21 S9	Dec., p = 0.001
GSK3 pS9	Dec., p = 0.004
HER2 pY1248	Dec., p = 0.003
Notch1	Inc., p = 0.006
p70S6K pT389	Dec., p = 0.011
PI3K p85	Dec., p = 0.012
PTEN	Dec., p = 0.041
Rb pS807 S811	Dec., p = 0.039
S6 pS235 S236	Dec., p = 0.041
STAT3 pY705	Dec., p = 0.050
STAT5-alpha	Dec., p = 0.000
Syk	Inc., p = 0.029
YAP	Inc., p = 0.046

Discussion:

One of the major focuses of this study is determining the functional consequences of decreased PRKCα in the developing kidney and its contribution to Wilms tumorigenesis. Because previous experiments discussed in Chapter 2 demonstrated that WT1 does, in fact, regulate *PRKCα* expression, it is important to determine how this signaling pathway affects downstream expression of target genes. As discussed, the ubiquitous nature of *PRKCα* expression complicates the identification of its tissue and cellular specific roles. Therefore, in order to better understand how WT1 and PRKCα together affect kidney development, separate RPPA and PCR Array experiments can be evaluated with the purpose of comparing results. Comparing gene changes following *WT1* knockdown to gene changes following *PRKCα* knockdown should identify genes in common between the two groups. If WT1 and PRKCa are the beginning of a signaling pathway important to kidney function and implicated in tumorigenesis, affected gene signaling pathways can then be constructed to determine biological consequences of decreased *WT1*/*PRKCα* expression.

In assessing the targets identified by *Prkcα* knockdown PCR Array alone, a few important observations arise. First, many of the targets identified play important roles in increased angiogenesis and proliferation, such as the differentially expressed *Vegf* genes (Figure 13). VEGF is a major regulator of angiogenesis that can aberrantly bind and activate downstream tyrosine kinase receptors to drive tumor formation in multiple cancer models (Lee, Dublin, Bobrow, & Poulsom, 1998; Salven et al., 1998; Takahashi, Kitadai, Bucana, Cleary, & Ellis, 1995). Interestingly, experiments at the RNA level show that VEGF expression is increased in our system following knockdown of *PRKCα*.

Additionally, other differentially expressed targets were identified, such as: *Thbs1*, *Tgfb*, and *Itga2*. The role of *Itga2* in cellular remodeling and angiogenesis is well defined (Grzesiak & Bouvet, 2006; Ruosiahti, 1991) and some studies also implicate *Thbs1* and *Tgfb* as cofactors involved in tumor cell attachment and invasion (Albo et al., 1994). Overall, the majority of the PRKCα targets identified by PCR Array and listed in Figure 13 are known to act within the signaling pathways affecting cellular growth, with many of the involved in increased angiogenesis and proliferation, increased vascular permeability, and inhibited apoptosis (Doerks, Copley, Schultz, Ponting, & Bork, 2002).

Interestingly, and as predicted by our hypothesis, the *Wt1* knockdown PCR Array identified some of the same targets as those identified in the *Prkcα* knockdown PCR Array. Both arrays show the *Vegf* family to be differentially expressed at the RNA level; it is likely that signaling through these genes, mutant WT1 eventually leads to increased angiogenesis in the developing kidney - possibly contributing to tumorigenesis (Dohi et al., 2010; Ozluk, Kilicaslan, Gulluoglu, Ayan, & Uysal, 2006; Skoldenberg et al., 2001). A full list of gene changes identified by *Wt1* knockdown PCR Array can be found in Figure 12 for comparison.

Further insight into the downstream effects of WT1 and PRKCα is revealed through analysis of protein changes following the knockdown and up-regulation of the two genes in RPPA experiments. High-throughput RPPA experiments identified a number of protein targets differentially expressed following knockdown of either *Wt1* or *Prkcα* in the mouse mesenchymal cell line M15 (Table 4) or induced expression of *WT1* in human embryonic cell line HEK293 (Table 3). Much like PCR Array data, a number of the genes identified are involved in known cancer pathways. Results of protein

analysis are best visualized in heatmap representations (Figure 14) and show a number of expression changes. Notably, knockdown of *Prkcα* identified disregulation of genes involved in insuling signaling, MAPK/Erk, and mTOR pathways – all of which are hypothesized to contribute to increased cell growth, increased protein synthesis, and increased proliferation.

Chapter 5: Investigating the relationship between WT1 mutations and PRKCα expression in Acute Myeloid Leukemia

Introduction:

Apart from Wilms tumor, *WT1* also plays an important role in Acute Myeloid Leukemia (AML) disease progression. *WT1* mutations occur at a frequency of approximately 10% and cluster to exons seven and nine in cytogenetically normal patients (King-Underwood et al., 1996). *WT1* mutation status has been implicated, controversially, as an independent poor-prognosis factor in leukemia, leading to decreased probability of overall survival, complete remission, and disease free survival (King-Underwood & Pritchard-Jones, 1998; Paschka et al., 2008). RPPA analysis of AML patient samples showed similar decreases in PRKCα/p-PRKCα protein expression as seen in the human Wilms tumor heatmap (Dr. Steven Kornblau, Department of Leukemia, The University of Texas MD Anderson Cancer Center, personal communication). Therefore, the possible role of WT1 and PRKCα in leukemia disease progression is an additional focus of this study.

Materials/Methods:

Mutation Analysis:

Mutation analysis was carried out on 54 diploid AML leukemia patient samples, chosen based upon high or low PRKCα and p-PRKCα S657 expression levels, to identify patients harboring *WT1* mutations. Patient DNA samples were obtained from Dr. Steven Kornblau (Department of Leukemia, The University of Texas MD Anderson Cancer Center) and given unique identification codes to create an unbiased test environment during mutation analysis. Amplification of *WT1* exons one through ten were carried out using primer sets and PCR conditions listed in Table 4. PCR products were analyzed by Sanger-based sequencing at the MD Anderson Cancer Center Department of Genetics, Sequencing and Microarray Facility, followed by qPCR validation of results and assessment for loss of heterozygosity.

Creation of Cell lines:

HL60 (human promyelocytic cells) cell lines were transduced using lentirviral delivered shRNA. Two separate MISSION TRC shRNA Custom Lentiviral Particles, pLKO-puro-IPTG-3xLacO, targeting *WT1* and *PRKCα* were used (Sigma-Aldrich), as well as lentivirus carrying non-target sequence negative control (NTC) shRNA. In 48 well plates, $2.0x10^4$ cells were seeded per well along with lentivirus at 20 MOI targeting *WT1*, *PRKCα*, or NTC. Cells were incubated in media containing Polybrene (Sigma) for 4hrs at 37 °C before 30min centrifugation followed by fresh virus application and overnight incubation. Transduced cells were grown for an additional six days under

puromycin selection to ensure uptake of viral construct selection of colonies for HL60-*WT1*-shRNA, HL60-*PRKCα*-shRNA, and HL60-NTC-shRNA cell lines.

shRNA Knockdown:

qPCR analysis confirmed knockdown of appropriate gene targets after 48hrs of 50uM ITPG induction.

Table 5

Detected	Forward Primer	Reverse Primer	Product Size
5’ Exon 1	5’AGCCAGAGCAGCAGGGAGTC3’	5’AACGACCCGTAAGCCGAAGC3’	277bps
3’ Exon 1	5’CGCCGGTGCTGGACTTTG3’	5’CGGTCAAAAGGGGTAGGAGA3’	432bps
Exon 2 & 3	5’CGTCTTCCTGCCGAAAGTC3’	5’TAGAGTGGAGTCGAGGCGTCT3’	979bps
Exon 4	5’ATCCCTTCTGTCGTGTATGAA3’	5’AAGGAGGAAAGCGTTCTA3’	425bps
Exon 5	5’TTGAGGGGCTTTTCACTGG3’	5’GTCCTAACTCCTGCATTG3’	225bps
Exon 6	5’GCATTTCCAAATGGCGACTG3’	5’TCAGACCCAGGGGACGAGCA3’	317bps
Exon 7	5’CCCTCAAGACCTACGTGAATG3’	5’AACCTGGGTCCTTAGCAGT3’	303bps
Exon 8	5’CCTAACAAGCTCCAGCGAAGT3’	5’GAATCATGAAATCAACCCTAGCC3’	282bps
Exon 9	5’GGACTGGGGAAATCTAAG3’	5’AATCCCTCTCATCACAAT3’	320bps
Exon 10	5’GGGTGCCTTGTGATGACTTC3’	5’GGCCTGTGAGTCAACTAA3’	429bps
Cycle Conditions:			
5’ Exon 1: 95 °C - 3 min, [94 °C - 30 sec, 61 °C - 30 sec, 72 °C - 30 sec] - 40 cycles, 72 °C - 3 min			
3’ Exon 1: 95 °C - 3 min, [95 °C - 30 sec, 62 °C - 30 sec, 72 °C - 30 sec] - 40 cycles, 72 °C - 3 min			
Exon 2 & 3: 95 °C - 3 min, [95 °C - 30 sec, 62 °C - 30 sec, 72 °C – 1 min 30 sec] - 40 cycles, 72 °C - 5 min			
Exon 4 -10: 95 °C - 3 min, [94 °C - 30 sec, 55 °C - 30 sec, 72 °C - 30 sec] - 40 cycles, 72 °C - 3 min			

Results:

WT1 mutation analysis of 54 diploid leukemia patient samples identified two patients with mutations hypothesized to lead to a mutant WT1 protein. Patient 1 has a homozygous frameshift deletion, c. 939delG, in exon 7 identified by sanger-based sequencing and analyzed using Mutation surveyor software. The frameshift mutation results in 67 novel amino acids before reaching a stop codon. Patient 2 was identified to with a heterozygous point mutation at the exon 8 splice site, c.1046-1G>A (Figure 15). The overall frequency of *WT1* mutations in our cohort was 2/54 or 3.7%. However, considering cytogenetically normal patients alone, the frequency of mutations is 2/23 or 8.6%.

qPCR analysis of lysates from HL60-*WT1*-shRNA, HL60-*PRKCα*-shRNA, and HL60-NTC-shRNA cell lines induced for shRNA expression for 48hrs confirmed knockdown of target proteins (Figure 16). 70% knockdown of *PRKCa* was achieved in cell line HL60-*PRKCα*-shRNA and 67% knockdown of *WT1* was achieved in HL60-*WT1*-shRNA cell line. Additionally, qPCR analysis showed that following the 67% knockdown of *WT1* in HL60-*WT1*-shRNA, a 60% decrease in *PRKCα* was observed.

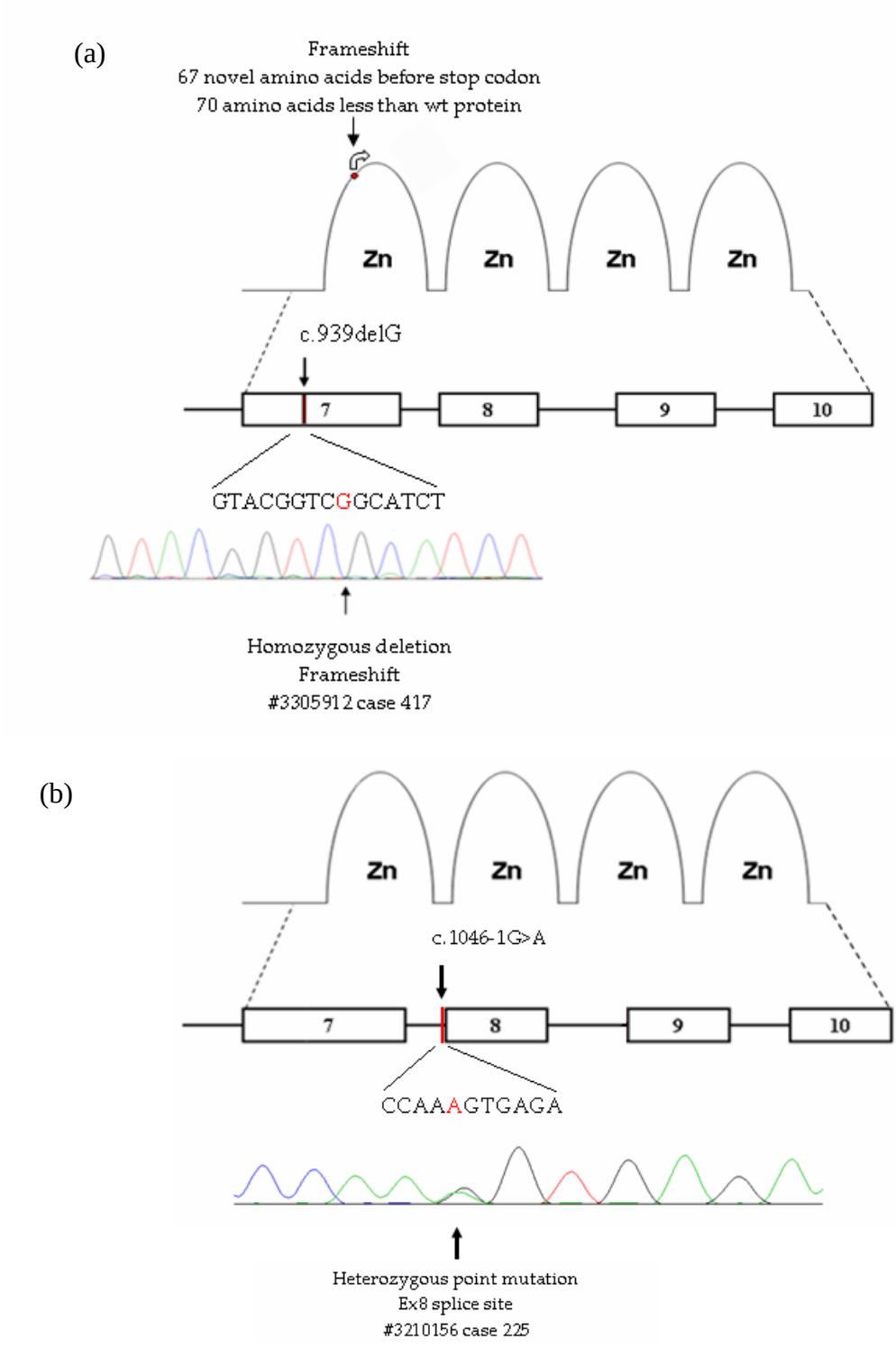


Figure 15: (a) Patient 1 has a homozygous deletion in exon 7. The frameshift mutation results in 67 novel amino acids before a stop codon is reached; 70 amino acids shorter than the wild type WT1 protein. (b) Patient 2 has heterozygous point mutation at the exon 8 splice site; and is predicted to results in a mutant WT1 protein.

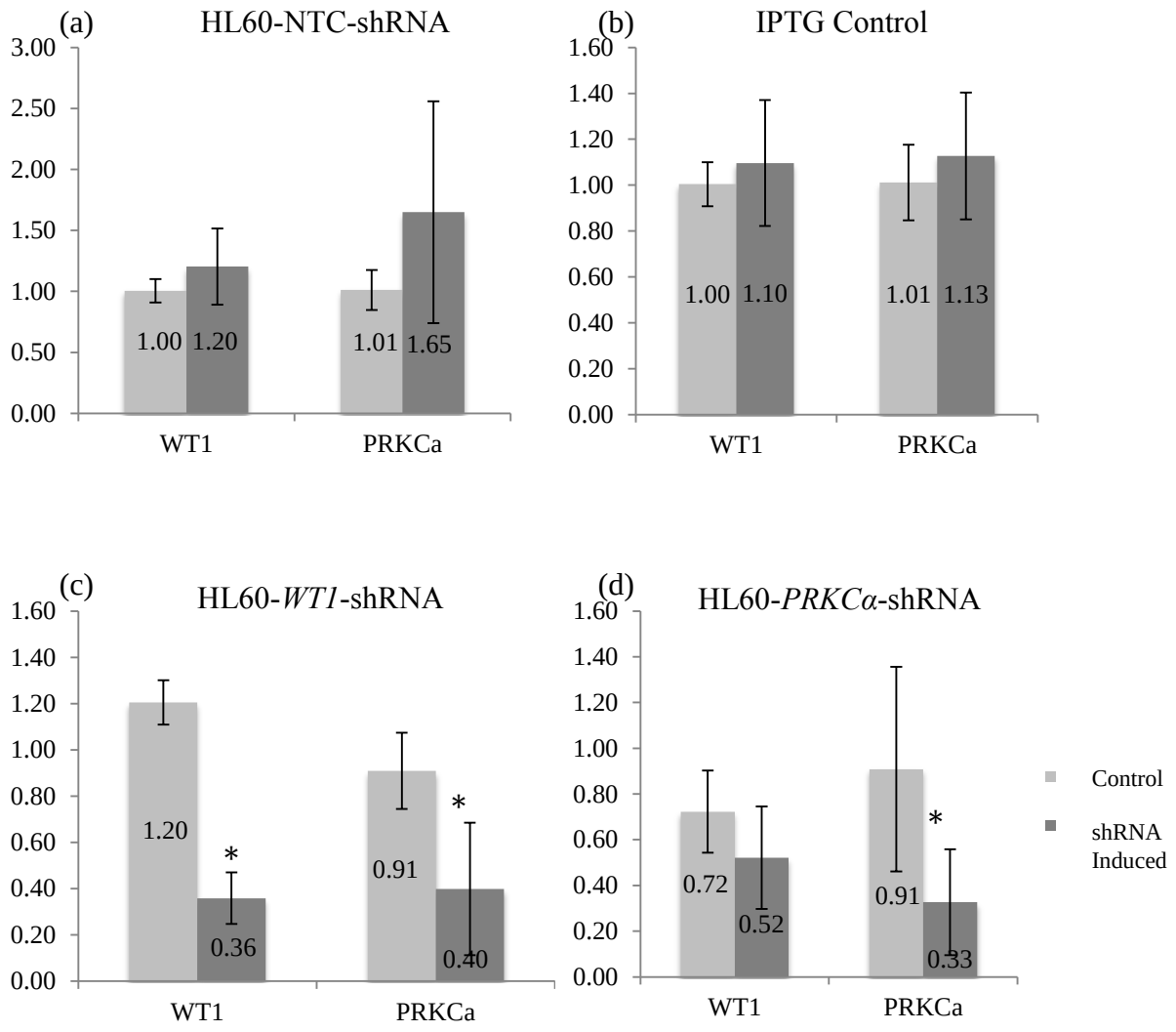


Figure 16: (a) No significant changes in *WT1* or *PRKCα* RNA expression were seen following induction of non-target control shRNA in HL60 cell culture. (b) No changes in RNA expression were seen after treating control HL60 cells with IPTG. (c) Following 48hrs of induced shRNA expression in HL60-*WT1*-shRNA cell culture, a significant decrease in *WT1* (p=0.0396) and *PRKCα* (p=0.0421) RNA expression was observed. (d) Following 48hrs of induced shRNA expression in HL60-*PRKCα*-shRNA cell culture, a significant decrease in *PRKCα* RNA expression (p=0.023) was observed.

Discussion:

According to our hypothesis, a mutation analysis of 54 leukemia patient samples should reveal that patients harboring a *WT1* mutation also have decreased *PRKCα* expression. We predict the frameshift mutation identified in Patient 1 would produce a nonfunctional, mutant WT1 protein a full 70 amino acids shorter than the wild type protein, resulting in a truncated protein lacking correct formation of the four zinc fingers that allow WT1's DNA binding ability. While we can be sure that the first patient, with a frameshift mutation, does in fact express a non-functional WT1 protein, the same is not definitive for the second patient whose mutation occurs at the exon 8 splice site. We only predict that a mutation at this location results in a truncated WT1 protein.

Correlating the *WT1* mutation status of the two patients with protein expression levels, we would expect both to have low PRKCα expression. Interestingly, Patient 1 does in fact express very low levels of PRKCα, but Patient 2 does not have low PRKCα expression. One possible explanation for the high level of PRKCα expression in Patient 2 is that the mutation does not affect WT1 function; instead, the patient has a normally functioning WT1 protein product and so we would not expect decreased PRKCα expression, but without further experimentation we cannot conclude this definitively.

Lentiviral constructs applied to the HL60 cell line achieved significant knockdown of targets. HL60-*WT1*-shRNA cells showed a significant decrease in *WT1* RNA expression and a resulting significant decrease in *PRKCα* expression. Once again, we would expect to see this decrease in *PRKCα* expression following *WT1* knockdown. HL60-*PRKCα*-shRNA cells also achieved knockdown of the target gene, with a significant decrease in *PRKCα* expression (and no significant change in *WT1*

expression). Altogether, these results confirm the hypothesis that WT1 is regulation *PRKCα* expression; and in leukemia patients, the observed decrease in *PRKCα* expression can possibly be attributed to mutant *WT1* expression.

IMPLICATIONS

The research I presented in this Thesis identifies *PRKCα* as a novel target of WT1 transcription factor. By first identifying the temporal and spatial expression of PRKCα, I was able to confirm its expression in embryonic kidney at a timepoint important to kidney development. Importantly, I showed that it is co-expressed in mesenchymal cells with WT1 transcription factor during the mesenchymal to epithelial transition – cell from which Wilms tumors are hypothesized to arise. Various in-vitro systems confirmed the observation originally made in human patient protein expression data that patients harboring *WT1* mutations have significantly decreased PRKCα expression. Both knockdown and up-regulation experiments showed that manipulating *WT1* expression resulted in similar changes in *PRKCα* expression. Biological significance was achieved by also being able to show decreased *PRKCα* expression in the embryonic kidney of *WT1* conditional knockout mice.

Another focus of this study was determining the functional consequences of decreased PRKCα and its potential contribution to Wilms tumorigenesis. The downstream targets of WT1 transcription factor are largely unidentified and efforts are being made to better characterize the consequences of mutant protein expression on signaling pathways. This works has identified *PRKCα* as a target of WT1 transcriptional activity and elucidated the consequences of disrupted signaling following decreased PRKCα expression. High-throughput experiments identified a wide variety of putative targets downstream of the aberrant expression of PRKCα, many of which play a role in cell-growth and proliferation. Known cancer pathways contributing to angiogenesis, protein synthesis, and cell proliferation are all identified here as aberrantly expressed and

point to the implication of mutant WT1 – decreased PRKCα signaling pathway in Wilms tumorigenesis. I predict that continued investigation into the role of PRKCα in the developing kidney and its potential contribution to Wilms tumorigenesis will offer interesting insight into disease etiology. Additionally, high-throughput WT1 knockdown and up-regulation experiments can be evaluated independently in order to identify new targets of WT1 transcription factor, independent of PRKCα signaling, that may play a role in tumor formation.

Furthermore, this Thesis has addressed the relationship between WT1 and *PRKCα* in Acute Myeloid Leukemia Patients. The role of the WT1 transcription factor in leukemia is particularly interesting in that it may serve as a novel predictor of disease outcome. Research has indicated that by assessing for WT1 expression, a determination of the probability of OS and DFS of patients can be made. Here I have confirmed that in the developing kidney, WT1 is regulating *PRKCα*, directly or indirectly. The importance of this investigation is obvious in its implications to patient survival and treatment. I was able to identify at least one patient whose WT1 mutation is associated with decreased PRKCα - who later suffered disease relapse. It is my opinion that future investigations will identify WT1 mutation status and *PRKCα* expression levels as important drivers in acute myeloid leukemia disease.

Through my research I have provided new insight into the role of *WT1* and its regulation of *PRKCα* either directly or indirectly. Identification of a novel target of WT1 transcription factor provides not only a new avenue for future studies on kidney development and Wilms tumorigenesis but other diseases as well. Ongoing research into the role of WT1 has implicated the transcription factor as an important regulator in a

wide variety of tissues and diseases, and this observation, combined with the ubiquitous expression of *PRKCα* offers great potential for the importance of WT1 – PRKCα signaling in human disease.

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