

A Novel Approach to Tracing Oncogenesis Following von Hippel Lindau Inactivation in Mice

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Abstract

Defining the early events following cancer mutation in cells of normal tissues is an enduring challenge in understanding the origins of cancer. In clear cell renal cell carcinoma (ccRCC) evolution, the inactivation of the ubiquitously-expressed von Hippel Lindau (*VHL*) tumour suppressor gene is a truncal event. However, *VHL*-associated oncogenesis is highly tissue-specific and can require years or decades between *VHL* inactivation and presentation of cancer. This raises unanswered questions as to the behaviour of *VHL*-null cells prior to neoplastic transformation and the contexts in which such transformation is supported.

To study this behaviour, I have developed the use of a novel reporter mouse model that couples conditional *Vhl* inactivation to the expression of a tdTomato reporter within a single allele. This allows *Vhl*-null cells to be visualised and assayed in their native context and be retrieved accurately from tissues for *ex vivo* analyses and single-cell RNA sequencing. I have employed the model to study the early and time-accumulated survival, proliferative, and transcriptomic phenotypes of *Vhl*-null cells in tissues that are or are not associated with *VHL*-driven cancer. I have also tested whether co-inactivation of Polybromo-1 (*Pbrm1*), the second most inactivated gene in ccRCC, affects these phenotypes of *Vhl*-null cells in the kidney and accelerates oncogenesis.

These investigations provide the first description of the heterogeneous cellular and transcriptomic effects of *Vhl* inactivation *in vivo* with single-cell resolution. They 1) reveal that *Vhl*-null cells undergo cell-type specific elimination and time-restricted proliferation, 2) identify a novel class of renal proximal tubular cells as potential ccRCC cells-of-origin, and 3) suggest that *Pbrm1* inactivation promotes oncogenesis only in specific contexts. This work suggests that early events in normal cells can shape the cell-type specificity of oncogenesis and provide a focus for mechanistic understanding.

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Declaration

Except where acknowledgement is made in the text or figure legends, the work described in this thesis was performed by the author in the Nuffield Department of Medicine Research Building, the Ludwig Institute for Cancer Research, Oxford, or in the Wellcome Trust Centre for Human Genetics at the University of Oxford. The work reported in this thesis has not been submitted for any other degree in this or any other university or institute of learning.

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List of Abbreviations

Abbreviation	Term
4-OHT	4-hydroxytamoxifen
AP-1	Activator Protein 1
BAF	Brahma-Associated Factor complex
BAH	Bromo-adjacent Homology domain
BRD	Bromodomain
BrdU	5-bromo-2'-deoxyuridine
BSA	Bovine Serum Albumin
c-Casp-3	cleaved Caspase-3
CA9	Carbonic Anhydrase 9
ccRCC	Clear Cell Renal Cell Carcinoma
CDIC	Intercalated Cell of the Collecting Duct
CDPC	Principal Cell of the Collecting Duct
CI	Confidence Interval
cm	Centimetre
CNS	Central Nervous System
Control	<i>Vhl^{wt/pjr.fl}; Ubc-CreERT2</i> (Chapter 3), <i>Vhl^{wt/pjr.fl}; Pax8-CreERT2</i> (Chapter 4, 5)
Control <i>Pbrm1</i> KO	<i>Vhl^{wt/pjr.fl}; Pbrm1^{fl/fl}; Pax8-CreERT2</i>
D-PBS	Dulbecco's Phosphate Buffered Saline
DAB	Diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DCT	Distal Convolute Tubule
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic Reticulum
F	Female
FACS	Fluorescence Activated Cell Sorting
FBS	Foetal Bovine Serum
FFPE	Formalin-Fixed Paraffin-Embedded
g	Gram
g	Gravitational acceleration

GEM	Gel Beads-in-emulsion
GO	Gene Ontology
HIER	Heat Induced Epitope Retrieval
HIF	Hypoxia Inducible Factor ($\alpha+\beta$ subunits)
HIFA	Hypoxia Inducible Factor (α subunit)
HMG	High-mobility Group Domain
Homoflox	<i>Vhl^{pjr.fl/pjr.fl}; Pax8-CreERT2</i>
HRP	Horseradish Peroxidase
h	Hours
IF	Immunofluorescence
IHC	Immunohistochemistry
IP	Intraperitoneal Injection
kDa	Kilodalton
KO	<i>Vhl^{l^{jae}.KO/pjr.fl}; Ubc-CreERT2</i> (Chapter 3), <i>Vhl^{l^{jae}.KO/pjr.fl}; Pax8-CreERT2</i> (Chapter 4, 5)
LoH	Loop of Henle
M	Male
MEF	Mouse Embryonic Fibroblast
mg	Milligram
min	Minutes
ml	Millilitre
mM	Millimolar
mm	Millimetre
MRCA	Most Recent Common Ancestor
MW	Molecular Weight
NBF	Neutral Buffered Formalin
ncBAF	non-canonical Brahma-Associated Factor complex
<i>Ubc-CreERT2</i>	<i>Ndor1^{Tg(Ubc-cre/ERT2)1Ejb}</i>
Neg	tdTomato Negative
NK Cell	Natural Killer Cell
nm	Nanometre
OCT	Optimal Cutting Temperature Compound
P2A	Porcine Teschovirus 2A Self-cleaving Peptide
pA	Polyadenylation Site
PBAF	Polybromo-1 Brahma-Associated Factor complex

<i>Pbrm1^{fl}</i>	<i>Pbrm1^{tm1c(EUCOMM)Wtsi}</i>
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PEC	Parietal Epithelial Cell
PHD	Prolyl Hydroxylase
pM	Picomolar
Pos	tdTomato Positive
PT	Proximal Tubule
RNA	Ribonucleic Acid
RNA-seq	RNA Sequencing
rpm	Revolutions Per Minute
RPTEC	Renal Proximal Tubular Epithelial Cells
RT	Room Temperature
RT-qPCR	Reverse Transcriptase Quantitative Polymerase Chain Reaction
RTE	Renal Tubular Epithelium
scRNA-seq	Single-cell RNA Sequencing
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	SDS Polyacrylamide Gel Electrophoresis
SNP	Single Nucleotide Polymorphisms
TBS-T	Tris buffered Saline with Tween-20
<i>Pax8-CreERT2</i>	<i>Tg(Pax8-cre/ERT2)CAmat</i>
TMX	Tamoxifen
TSG	Tumour Suppressor Gene
TUNEL	Terminal Deoxynucleotidyl Transferase dUTP Nick End Labelling
UMAP	Uniform Manifold Approximation and Projection
UMI	Unique Molecular Identifier
VCB	VHL-Elongin C-Elongin B complex
Veh	Vehicle control
VHL	von Hippel Lindau (protein)
<i>VHL</i>	von Hippel Lindau (human gene)
<i>Vhl</i>	von Hippel Lindau (mouse gene)
<i>Vhl</i> KO	<i>Vhl^{fl/fl}; Ksp-Cre</i> (Chapter 1); <i>Vhl^{flae.KO/pjr.fl}; Pax8-CreERT2</i> (Chapter 6)

<i>Vhl Pbrm1</i> KO	<i>Vhl^{fl/fl}; Pbrm1^{fl/fl}; Ksp-Cre</i> (Chapter 1); <i>Vhl^{flae.KO/pjr.fl}; Pbrm1^{fl/fl}; Pax8-CreERT2</i> (Chapter 6)
<i>Vhl^{flae.KO}</i>	<i>Vhl^{tm1Jae}</i> (recombined form)
<i>Vhl^{pjr.fl}</i>	<i>Vhl^{tm1.1b(tdTomato)Pjr}</i> (unrecombined form)
<i>Vhl^{pjr.inrec}</i>	<i>Vhl^{tm1.1b(tdTomato)Pjr}</i> (incompletely recombined form)
<i>Vhl^{pjr.KO}</i>	<i>Vhl^{tm1.1b(tdTomato)Pjr}</i> (recombined form)
<i>Vhl^{wt}</i>	<i>Vhl</i> (wild-type allele)
VSMC	Vascular Smooth Muscle Cell
w/v	Weight-by-Volume
α -SMA	Smooth Muscle Actin
μ g	Microgram
μ l	Microlitre
μ m	Micrometre
μ M	Micromolar
μ m	Micrometre

Chapter 1: Introduction

Decades of cancer research have advanced our understanding of how a set of mutations that occur in a single cell cause it to form a tumour. Yet it remains a puzzle as to how this “renegade” cell¹ eschews its physiological functions, proliferates without restraint, and alters its interactions with neighbouring cells in response to the genetic perturbations it incurs². One reason that the origin of cancer remains unknown is that few studies have assayed the earliest consequences of oncogenic mutation in cells in normal tissues *in vivo*, largely owing to technological limitations. I have embarked on such a study focusing on the effects of inactivation of the von Hippel Lindau (*VHL*) tumour suppressor gene in the context of genesis of clear cell renal cell carcinoma (ccRCC). In this chapter, the background literature and specific aims of the thesis are discussed.

1.1 Tissue-specificity of cancer

Solid cancers arise when cells gain metabolic, signalling, proliferative, and immunomodulatory properties. These properties allow those cells to override tissue homeostatic mechanisms, form tumours, and ultimately break tissue barriers to invade other tissues. The emergence and sustenance of these properties are driven by the dysregulated actions (or inactivation) of oncogenes and tumour-suppressor genes (TSGs) in these cells^{2,3}. Unsurprisingly, many of these driver genes are members of regulatory frameworks that control fundamental aspects of cellular function - cell cycle regulation, DNA damage repair, metabolism, and cellular signalling. Yet paradoxically, their dysregulation often drives cancer in a tissue-specific manner⁴⁻⁶. For instance, the DNA repair genes *BRCA1* and *BRCA2* work in concert to signal DNA damage and facilitate homologous recombination to repair double stranded DNA breaks in all cells of the body. However, *BRCA1*- and *BRCA2*-inactivating mutations overwhelmingly cause breast, ovarian and prostate cancers over other malignancies⁷. Such tissue-specificity in tumorigenesis has been observed for many other

cancer-driving genes such as *APC* (for colorectal cancer)⁸, *PPP6C* (for melanoma)⁹, or *CDH1* (for diffuse gastric cancer)¹⁰. These observations suggest that tissue identity impacts or shapes the tumour-driving capacity of these genes^{4,5}.

Several hypotheses have been generated to explain the tissue-specific oncogenesis entrained by particular cancer drivers. These include 1) the tissue-specific upregulation of TSGs by the actions of oncogenes which then restrains cell proliferation and transformation¹¹; 2) tissue-specific hormonal signalling that protects against TSG loss¹²; 3) tissue-specific upregulation of additional oncogenes as a result of specific chromatin accessibility in particular cell lineages¹³; and 4) tissue-specific kinetics of signalling networks that determine which downstream pathways are activated by the same oncogenic stimulus⁵. While these have provided some leads, the mechanisms underlying tissue identity and tumorigenic context for most cancer-driving genes remain largely unidentified.

Another enduring question in cancer biology, especially for solid cancers such as renal cancer, is of the latency associated with oncogenesis. For instance, ‘normal’ or ‘healthy’ tissues are known to contain cells which carry cancer driver mutations; these occur through exposure to mutagens or simply from errors in DNA replication¹⁴. Such cells can persist in tissues for years or decades before developing into a tumour. While multiple advances in the molecular and genetic analysis of cancer have transformed understanding of the disease over recent decades², most work has focused on either the later stages of cancer or on short-term models of aggressive disease. Much less is known about the earliest events and the cellular responses to those events, particularly when progression to cancer is slow. The factors that determine the path from mutation to overt cancer in such cells in normal tissues, the tissue-specificity therein and requirement of any external factors, have not been widely studied.

Investigation of these early events in cancer and their associated tissue-specificity requires technologies that allow assessment of cell states – cell biological and genetic – in tissue context with accurate cellular and temporal resolution. In this thesis, I embark on such

a study of tissue-specific oncogenesis associated with the von Hippel Lindau (*VHL*) tumour suppressor gene.

1.2 The von Hippel Lindau (*VHL*) tumour suppressor

1.2.1 von Hippel Lindau tumour suppressor gene and its product

The von Hippel Lindau (*VHL*) tumour suppressor gene is an 11.9 kbp protein-coding gene located on human chromosome 3 at the 3p25.3 locus and contains three major exons (E1, E2, and E3) and two introns (I1, I2) (<https://www.ncbi.nlm.nih.gov/gene/7428/>)¹⁵. *VHL* codes for a 213 amino acid 24.1 kDa protein (VHL) that migrates at 30 kDa by SDS-PAGE (pVHL₃₀)¹⁶; E1, E2 and E3 code for 1-113, 114-154, 155-213 residues respectively¹⁷. An alternative 160 amino acid 18.5 kDa VHL isoform that migrates at 19 kDa by SDS-PAGE (pVHL₁₉)¹⁸ is also observed and is translated from an alternative translation initiation site on the *VHL* transcript at Methionine-54. A minor alternatively-spliced *VHL* transcript that includes a cryptic exon (E1') within I1 has also been described¹⁹; the protein product of this transcript has been detected only rarely in tissues.

VHL is conserved across all multi-cellular animals^{20,21}. In the mouse, *Vhl* is a 7.6 kbp gene located on chromosome 6 (<https://www.ncbi.nlm.nih.gov/gene/22346/>) and codes for either 181- or 162-amino acid protein isoforms (pVHL₂₅ and pVHL₂₁) that are orthologous to pVHL₃₀ and pVHL₁₉ respectively²². The murine and human *VHL* genes differ mainly in the first 54 N-terminal codons; while the human pVHL₃₀ contains eight copies of an acidic pentameric repeat in this region, murine pVHL₂₅ contains only one. The shorter isoforms are highly similar and contain a stretch of 140 amino acids that share 98% sequence identity between the human and the mouse²². *Vhl* contains three exons and two introns and alternative splicing at the cryptic E1' exon has not been reported (<https://www.ncbi.nlm.nih.gov/gene/22346/>).

1.2.2 VHL regulates oxygen-dependent stabilisation of hypoxia inducible transcription factors (HIF)

The VHL protein interacts with Elongin C that in turn interacts with Elongin B to form a stable ternary complex called VCB²³. The VCB complex further interacts with the scaffold protein CUL2 that also interacts with the RING-domain containing protein RBX1^{23,24}. The VCB-CUL2-RBX1 protein complex acts as an E3 ubiquitin ligase of which VCB is the targeting subunit. Interaction with VCB with a target protein places the target in proximity to RBX1, which then catalyses the sequential transfer of a ubiquitin moiety from an E2 ubiquitin ligase to a lysine residue on the target protein²³⁻²⁵. The polyubiquitinated VCB target is then recognised by the proteasome for degradation.

The best characterised targets of VCB are the α -subunits of hypoxia inducible transcription factors (HIFA; HIF1A and HIF2A)^{26,27}. A third HIF3A isoform has been described but is not well characterised²⁸. VCB interacts with HIFA only when HIFA is hydroxylated at a proline residue in an oxygen-dependent manner by prolyl hydroxylase (PHD) enzymes²⁹⁻³¹. The extent of HIFA hydroxylation and by extension its proteasomal degradation is directly proportional to O₂ concentration and varies significantly over O₂ concentrations observed physiologically³². In low oxygen environments (hypoxia), HIFA hydroxylation is reduced, and thus VCB-HIFA interactions are abrogated. This prevents proteasomal degradation of HIFA which then allows it to form a dimeric complex with HIF β -subunits (HIF1B) and entrain a transcriptional programme that drives cellular adaptation to hypoxia. The VCB/HIF/PHD system thus acts as a conserved cellular oxygen sensor³³.

The hydroxy-proline residue on HIFA is recognised by the VCB complex *via* interactions with a single binding pocket lined by 9 amino acid residues on VHL³⁴. All these residues are part of a β -sheet domain formed by amino acids 63-150 and 195-204^{23,34}. Interactions between VHL and Elongin C are dependent on 15 surface-exposed amino acid residues, 10 of which are present in an α -helical domain formed by amino acids 158-190²³.

All these residues (and both these domains) are present in both pVHL₃₀ and pVHL₁₉ isoforms of VHL and are conserved across species^{18,22,35,36}. Mutations of these VHL residues, truncation of VHL exons 2 or 3, or loss of *VHL* expression all lead to the stabilisation of HIF1A or HIF2A even at normal oxygen concentrations (normoxia)³⁷, and makes cells enter a ‘pseudohypoxic’ state. VHL is thus a critical protein in VCB function and is directly responsible for oxygen-dependent HIF1A regulation in cells.

1.2.3 Non-HIF1A interaction partners of VHL

Individual studies have identified non-HIF1A targets of the VCB-CUL2-RBX1 complex, many of which were reported to be hydroxylated by PHD enzymes. However, neither the prolyl-hydroxylation of these targets nor their VCB-mediated ubiquitination and degradation has been consistently reproduced in the literature³⁸. HIF1A and HIF2A thus remain the only consistently identified targets of VCB-CUL2-RBX1-mediated oxygen-dependent degradation. However, VHL has been shown to act as an adaptor or stabilising protein for other targets³⁹.

The VCB complex interacts with Collagen IV; this interaction is dependent on the hydroxy-proline binding site on VHL but does not result in polyubiquitination and degradation of collagen^{40,41}. VCB interacts with fibrillar but not triple-helical folded collagen⁴⁰ at the endoplasmic reticulum (ER)⁴¹ and has in fact been proposed to play a role in the stabilisation and export of collagen fibrils to the extra-cellular matrix⁴⁰⁻⁴². Notably, both isoforms (pVHL₃₀ and pVHL₁₉) of VHL participate in this interaction.

VHL also interacts with the extracellular glycoprotein fibronectin^{40,43,44}. Interestingly, this interaction occurs with pVHL₃₀ but not with pVHL₁₉^{18,35,40}, and is also independent of the ability of VHL to form the VCB complex⁴³. Like its interactions with Collagen IV, VHL interacts with fibronectin at the ER and is proposed to facilitate fibronectin export to the extra-cellular matrix. Consistent with this function, silencing of *VHL* is associated with defective fibronectin and collagen deposition in the extra-cellular matrix of

cultured cells, and homozygous *Vhl* deletion impacts fibronectin organisation in the developing mouse embryo⁴³.

VHL has also been shown to interact and stabilise microtubules by preventing their depolymerisation. This interaction occurs with both isoforms of VHL, is dependent on the presence of residues 95-123, and is independent of the ability of VHL to form the VCB complex⁴⁵. VHL-microtubule interactions have been shown to play a role in regulating the mitotic spindle⁴⁶, and homozygous deletion of *VHL* in cell culture or of *Vhl* in murine renal cells *in vivo* affects the orientation of the mitotic spindle and results in chromosome segregation errors^{46,47}. Other studies have linked the VCB complex with the ubiquitination of atypical protein kinase C (aPKC)⁴⁸ and Aurora kinase A (AURKA)⁴⁹. These proteins play a role in regulating cell polarity and regulating mitotic progression both of which involve precise organisation of microtubules. While the role of aPKC and AURKA ubiquitination is not fully known, these observations suggest a convergence of VHL function on microtubule regulation and organisation.

1.3 VHL-associated cancer

1.3.1 VHL is a classical tumour suppressor gene for ccRCC

VHL is mutated in ~80% of cases of clear cell renal cell carcinoma (ccRCC) and its expression is silenced by promoter hypermethylation in an additional ~10% of ccRCC cases⁵⁰⁻⁵⁵. Though some mutational hotspots are observed, *VHL* mutations in ccRCC occur throughout the gene. They are predominantly comprised of frameshift, deletion, or nonsense mutations that either alter the identity and structural integrity of the translated protein product or abolish *VHL* expression, and of missense mutations that alter key residues that mediate VHL interactions with HIF or Elongin C^{50,54}. Most of these mutations abrogate the ability of VHL to regulate HIF1A or HIF2A in normoxic conditions³⁷. *VHL* is located on human chromosome 3 at the 3p25 locus⁵⁶⁻⁵⁸ and allelic deletion of this chromosomal region (3p loss)

is also detected in ~90% of ccRCC^{50-52,59,60}. Almost all cases of ccRCC thus carry biallelic loss-of-function or inactivation of *VHL*.

Between 2-5% of ccRCC cases occur in patients with ‘VHL disease’ that is caused by inheritance or *de novo* occurrence of *VHL* mutations in the germline⁶¹⁻⁶³. These germline *VHL* mutations predispose patients to haemangioblastoma in the retina, cerebellum, and spine, ccRCC in the kidney, pheochromocytomas in the adrenal medulla, and cysts or tumours in the pancreas^{64,65}. The clinical presentation of VHL disease depends on the nature of the *VHL* mutation carried by the patient in the germline, and by extension, in every cell of the soma⁶⁴. Susceptibility to ccRCC is specifically associated with germline frameshift, nonsense, missense mutations, or deletions that are effectively loss-of-function *VHL* mutations^{37,66-68}. Interestingly, mutations found in VHL disease-associated ccRCC are not necessarily identical to those found in sporadic ccRCC⁵⁰, suggesting that convergence on *VHL* loss-of-function contributes to ccRCC more than a precise change in *VHL* sequence. These ccRCC also exhibit 3p loss that always occurs on the chromosome carrying the non-mutated *VHL* allele^{60,69-72}. Thus, both sporadic and VHL disease-associated ccRCC almost universally carry biallelic inactivation of *VHL*, where one copy of *VHL* is mutated or silenced and the other copy is generally lost as part of a chromosome 3p deletion event.

VHL disease-associated ccRCC occurs at a younger age than sporadic ccRCC. Additionally, VHL disease-associated and sporadic ccRCC exhibit a first-order linear and second-order logarithmic relationship respectively between age and incidence of ccRCC⁷³. This statistical relationship is consistent with Knudson’s ‘two-hit model’ described for *RBI* in retinoblastoma⁷⁴ and provides further support for biallelic *VHL* inactivation in driving the progression of ccRCC. Re-introduction of wild-type *VHL* cDNA into *VHL*-deficient ccRCC cells inhibits their ability to form tumour xenografts in immunodeficient mice¹⁶.

Taken together, evidence of biallelic *VHL* inactivation, convergent molecular genetics of sporadic and VHL disease-associated ccRCC, consistency with Knudson’s two-

hit model, and sufficiency of wild-type *VHL* to restrict xenograft formation confirm the role of *VHL* as a classical tumour suppressor gene in ccRCC.

1.3.2 *VHL*-associated cancers are tissue-specific

Neither *VHL* mutations nor 3p loss were detected in a survey⁵⁰ of sporadic cancers arising in the lung, breast, prostate, cervix, ovary, colon, bladder or thyroid. Furthermore, interrogation of The Cancer Genome Atlas⁷⁵ shows *VHL* to be mutated predominantly in clear cell renal cell carcinoma (ccRCC) compared to other major types of cancers (<https://portal.gdc.cancer.gov/genes/ENSG00000134086>; accessed on 09 August 2023).

Tissue-specificity is also seen with *VHL* mutations and tumours associated with VHL disease⁶⁶⁻⁶⁸. Retinal and central nervous system (CNS) haemangioblastomas, the most common clinical feature of VHL disease, are associated with the inheritance of loss-of-function *VHL* mutations^{67,76}. Additionally, 60% of both sporadic and VHL disease-associated sporadic CNS haemangioblastoma exhibit 3p loss that results in the deletion of the *VHL* locus⁷⁷. Pancreatic tumours occur in 10-20% of VHL patients, are associated with the inheritance of loss-of-function *VHL* mutations^{78,79}, and are more likely to be observed in patients presenting with CNS haemangioblastoma and renal tumours than those with adrenal pheochromocytoma⁸⁰. Both sporadic and VHL disease-associated pancreatic tumours exhibit 3p loss involving the *VHL* locus^{72,81}. These observations suggest that biallelic *VHL* inactivation might contribute to the development of haemangioblastoma and pancreatic tumours as it does for ccRCC. In contrast, *VHL* inactivation does not appear to be necessary for the development of pheochromocytoma. For instance, between 70-80% of patients carrying loss-of-function germline *VHL* mutations develop ccRCC by the age of 50, but only 10-20% of these patients develop adrenal pheochromocytoma^{44,67,76}. Conversely, 50-60% of patients carrying missense mutations that do not affect regulation of HIF1A or formation of the VCB complex, develop pheochromocytoma by the age of 50, but only 10-20% of these patients develop ccRCC^{44,67,76}. Furthermore, 3p loss involving the *VHL* locus^{50,82} and *VHL*

mutations are observed in only a minority of sporadic pheochromocytoma⁸³, indicating tumorigenic mechanisms distinct from biallelic *VHL* inactivation in driving these tumours.

VHL is expressed in a wide range of tissues and cell types in human and mouse, adults and during development (<https://www.proteinatlas.org/ENSG00000134086-VHL/tissue+cell+type>; accessed on 25 September 2023), including in tissues that do not exhibit *VHL*-associated cancer^{84,85}. This suggests that despite ubiquitous expression and the conserved molecular function of *VHL*, inactivation of the gene has highly tissue-specific effects that are oncogenic only in the cells-of-origin for ccRCC, haemangioblastoma, and pancreatic islet tumours. ccRCC arises in the renal cortex, specifically in cells of proximal tubule of the renal tubular epithelium (RTE)⁸⁶⁻⁹⁰. Haemangioblastomas in VHL disease are thought to arise in embryologically arrested mesoderm cells (haemangioblasts) with haematopoietic and vasculogenic potential⁹¹. The cells-of-origin of pancreatic tumours have not conclusively been identified, though ductal or centro-acinar cells of the pancreas are implicated in oncogenesis⁷⁸. Whether and how *VHL* inactivation supports oncogenesis specifically in these cell types remains to be explored.

1.3.3 Genetics of ccRCC

VHL inactivation (by mutation or silencing) and 3p loss are observed in >90% of ccRCC. Interestingly, the 5-10% of ccRCC that do not contain *VHL* mutations and/or exhibit 3p loss often carry mutations in the *TCEB1* gene (that codes for Elongin C) and allelic deletion of the other *TCEB1* copy *via* loss of Chr 8q at the 8q21 locus^{51,92,93}. These prevent the formation of functional VCB ubiquitin ligase complexes in affected cells analogous to the effects of *VHL* inactivation and 3p loss in ccRCC cells⁹³. Furthermore, *VHL* and *TCEB1* inactivation occur in a mutually exclusive manner^{51,93}, possibly because inhibition of VCB function by inactivation of either *VHL* or *TCEB1* makes further inactivation of *TCEB1* or *VHL* respectively redundant.

VHL inactivation in ccRCC is almost always accompanied by other mutations and chromosomal abnormalities that occur heterogeneously in these cancers^{51,55,69,94}. The 2nd to 4th most commonly mutated genes in ccRCC are Polybromo-1 (*PBRM1*)⁹⁵, *BRCA1*-associated protein 1 (*BAP1*)⁹⁶, and SET-domain containing protein 2 (*SETD2*)^{97,98}. Interestingly, all three of these genes are located on human chromosome 3p at the 3p21 locus⁹⁹ and are thus often deleted as part of the 3p loss event in ccRCC. *PBRM1* and *BAP1* mutations correlate with loss of PBRM1 and BAP1 protein expression in ccRCC respectively¹⁰⁰. Mutations in *SETD2*, the only known histone methyltransferase responsible for trimethylating Lysine-36 on Histone H3 (H3K36me3)¹⁰¹, correlate with loss of H3K36 trimethylation in ccRCC¹⁰⁰. These observations indicate that the *PBRM1*, *BAP1*, and *SETD2* mutations observed in ccRCC are loss-of-function, and thus implicate these genes as additional tumour suppressors in ccRCC.

Genes other than *VHL*, *PBRM1*, *BAP1*, and *SETD2* are mutated individually in <10% ccRCC. ccRCC also exhibit copy number alterations across several chromosomal regions such as loss of 1p, 4q, 6q, 9p, and 14q and gain of 1q, 2q, 5q, 7q, 8q, 12p, 20q^{70,102,103} but these too are observed individually in only in a minority of tumours. However, these minor mutational and copy number alteration events converge on pathways involved in p53 signalling, mTOR activation, and SWI/SNF chromatin remodelling complexes. Genes involved in p53 signalling (e.g., *TP53*, *MDM*, *CDKN2A* and *ATM*), in PI3K/mTOR activation (e.g., *MTOR*, *PTEN*, *PIK3CA*, *TSC1* and *TSC2*) and in formation of SWI/SNF chromatin remodelling complexes (e.g., *PBRM1*, *ARID1A*, *SMARCB1* and *ARID2*) are collectively mutated or exhibit copy-number alterations in 40%, 26-28%, and 22% (58% including *PBRM1*) of ccRCC respectively^{51,55,69}.

The genetics of human ccRCC have been corroborated in the mouse. While mice harbouring *Vhl* inactivation in the RTE do not develop renal tumours^{47,104-108}, those with concomitant *Vhl* and *Pbrm1* inactivation or with concomitant *Vhl* and *Bap1* inactivation in

the RTE develop renal tumours resembling ccRCC over the course of 9-20 months after gene inactivation^{104,109-111}. Furthermore, activation of mTOR signalling by *Tsc1* inactivation exacerbates tumour grade in mice modelling concomitant *Vhl* and *Pbrm1* inactivation¹⁰⁴. Simultaneous dysregulation of pRb signalling, p53 signalling, and *VHL* inactivation in the RTE also models ccRCC in the mouse^{112,113}.

1.3.4 Evolution of ccRCC

Multi-regional sampling across a range of disease stages, and matched comparisons between primary tumours and metastases of ccRCC have revealed that the mutational and copy number alteration events in ccRCC occur with deterministic timing and evolutionary trajectories^{70,102,103}. 3p loss is the first genomic event in the evolution of most cases of sporadic ccRCC. Interestingly, this event occurs as part of a chromothripsis process involving Chr 3p and Chr 5q in 40% of the cases⁷⁰. 3p loss followed by subsequent *VHL* mutation define the ‘most recent common ancestor’ (MRCA) of most ccRCC clones⁷⁰; this is true for VHL disease-associated ccRCC too, though in these cases *VHL* mutation is inherited and 3p loss follows later⁷⁰. The evolution of ccRCC following on from the MRCA depends on the nature of the 2nd mutation, the most common of which is of *PBRM1*. Evolution of *VHL/PBRM1*-dual mutated clones with 3p loss then diverges either towards *SETD2* mutation and subsequent chromosomal alterations or towards *PI3K/MTOR* mutations and subsequent chromosomal alterations¹⁰².

Comparisons of mutational profiles, projected evolutionary pathways and known rates of incidence in sporadic and VHL disease-associated ccRCC⁶⁷ have allowed the timing of 3p loss and MRCA generation to be determined⁷⁰. The 3p-5q chromothripsis event is projected to occur in childhood or adolescence in cases of both sporadic and VHL disease-associated ccRCC. Subsequent *VHL* mutation and generation of the MRCA is projected to require up to 30 years after 3p loss⁷⁰ in sporadic ccRCC. Strikingly, a median latency of 28 years (range 4-49 years) is projected between the generation of the MRCA and the clinical

presentation of ccRCC¹⁰². This latency period is projected to shrink to a median of 5 years (range 1-34 years) after the acquisition of *PBRM1* mutation in the MRCA¹⁰².

The presence of “latent” non-neoplastic normal cells harbouring biallelic *VHL* inactivation (*VHL*-null cells) has been demonstrated in the kidney by histological examination of renal biopsies of VHL patients^{114,115}. These cells exhibit HIF activation and express carbonic anhydrase IX (CA9)^{114,115}, which is a known HIF-target gene upregulated in ccRCC¹¹⁶. Interestingly, these cells are never found in the collecting duct or Loop of Henle, are always present as single cells in the proximal tubules and are often present as multi-layered epithelia in distal tubules¹¹⁴, suggesting that their survival and phenotype is dependent on their tissue location and cell type.

Collectively, these studies have indicated that:

1. Cancer associated with biallelic *VHL* inactivation is remarkably tissue-specific even though *VHL* is expressed and is functional in most tissues and cell types.
2. Biallelic *VHL* inactivation (with 3p loss) is a near-universal, truncal event in the evolution of ccRCC that can occur in cells decades before overt presentation of cancer.
3. Cancer progression in *VHL*-null cells is accelerated by the acquisition of a second driver event (e.g., *PBRM1* inactivation), though a latency of years is still observed.

The mechanisms underlying either the tissue-specificity or the latency associated with *VHL*-driven cancers remain to be explored.

1.4 Experimental approaches to understanding the cellular effects of *VHL* inactivation

Studying how *VHL* inactivation affects cell survival, proliferation, and senescence in cells of different tissues over time might provide insights into the tissue-specificity and latency of *VHL*-associated oncogenesis. A substantial amount of work characterising *VHL*-associated phenotypes has been performed in cell culture, in mouse models harbouring

developmental inactivation of *Vhl* (constitutive), and in mouse models in which *Vhl* is somatically inactivated *in vivo* in defined segments of the RTE. However, as reviewed below, while these have generated knowledge on the cellular actions of *VHL*, they have not resolved how *VHL* inactivation affects normal renal cells and direct oncogenic or tumour-suppressive fates.

1.4.1 Cell Culture

Most cell culture work has involved reintroduction of *VHL* in cell lines derived from *VHL*-deficient renal cancers such as RCC4 and 786-O. These cell lines carry significant genomic alterations, are already transformed^{117,118}, and thus represent neither the genomic status nor the biology of renal cells that have incurred only *VHL* inactivation.

Other studies have skirted this issue with cancer cell lines by using primary cell cultures (human and mouse renal proximal tubular cells (RPTECs) as well as mouse embryonic fibroblasts (MEFs)) that are non-cancerous, have defined genetic features but can still be manipulated in culture. *Vhl* inactivation was found to reduce growth rates and increase expression of senescence markers in MEFs and RPTECs within three weeks of culture under atmospheric (21% O₂) oxygen tension^{112,119-121}. However, most tissues experience lower oxygen concentrations *in vivo* (3% - 7% in the kidney)¹²². When experiments were performed at these physiological oxygen tensions (5% O₂), *Vhl*-null MEFs and RPTECs were found to exhibit similar growth patterns to *Vhl*-competent MEFs and RPTECs, suggesting that *Vhl* inactivation does not affect cell survival and growth under physiological conditions^{120,121}. Notably, one study contrasted this finding by reproducing *Vhl*-associated growth defects in MEFs at 5% O₂¹⁰⁹.

Cell growth in culture further depends on seeding density, substratum, and confluency. When the growth behaviour of human RPTECs was compared in sub-confluent *versus* confluent culture conditions, it was found that inactivation of *VHL* at confluence conferred a proliferative advantage to cells by relieving contact inhibition¹²³. Considering

that cells in tissue are inherently subjected to contact inhibition and are under physiological oxygen tensions, these observations suggest that *Vhl* inactivation might confer a proliferative advantage to proximal tubular cells in the kidney that would otherwise be subjected to mitotic restraints¹²⁴ placed by their presence in differentiated tissue. This hypothesis has not been adequately tested.

Though insightful, the relevance of findings in primary cells to biology *in situ* is limited by the fact that renal cells in culture are stripped of their regulated renal microenvironments, apico-basal polarity, and physiological functions as part of a tubular structure. Additionally, these cells cannot be indefinitely maintained in culture for over a month and thus cannot inform on the growth and proliferative phenotypes of *VHL*-null cells over the longer time periods relevant for oncogenesis.

1.4.2 Developmental *Vhl* inactivation *in vivo* in murine RTE

This highlights the need to study *Vhl* inactivation *in vivo*, which has been performed using mouse models of *Vhl* inactivation. Complete *Vhl* inactivation is embryonically lethal in mice¹²⁵, so these models have employed conditional *Vhl* alleles that are inactivated by Cre recombinases with tissue-specific expression. Mice in which *Vhl* is inactivated *in utero* either predominantly in distal tubules and collecting ducts (with *Ksp1.3-Cre*^{106,126} or *Hoxb7-Cre*¹²⁷), in proximal tubules (with *Pepck-Cre*¹⁰⁸, or *Sglt2-Cre*¹⁰⁴), or across the RTE (with *Pax8-Cre*¹⁰⁴) developed functional kidneys and presented no tubular defects into adulthood, suggesting that *Vhl* inactivation is not deleterious to the development and differentiation of RTE cells.

These models did not develop renal cancer^{47,104-108}, and only one, in which *Vhl* was inactivated in proximal tubules *in utero* using *Pepck-Cre*¹⁰⁸, developed renal cysts without requiring deletions in other genes, indicating that constitutive *Vhl* inactivation alone has little oncogenic effect in the RTE. Inactivation of *Vhl* in the distal nephron using *Hoxb7-Cre* was associated with gross tubular abnormalities, inflammation, and fibrosis¹²⁷⁻¹²⁹. However, this

Cre recombinase allele has been shown to have extra-renal activity in granulocytes and thus, it is difficult to ascertain the extent to which these phenotypes were driven specifically by *Vhl*-null tubular cells.

The proliferative behaviour of cells in *Vhl*-null nephron segments has been studied in some of these models. *Vhl*-null proximal tubules (modelled with *Pepck-Cre*) did not exhibit increased senescence unless subjected to oxidative stress by administration of the potent and toxic oxidising agent paraquat¹³⁰ to mice¹²⁰; the age at which they were analysed was not reported. Such investigations have not been reported with *Vhl* inactivation in the distal nephron and collecting duct. Three studies have assayed proliferation of epithelial cells in the renal cortex following *Vhl* inactivation using *Ksp1.3-Cre*. Intriguingly, two of the studies (one of which induced proliferation in the renal tubules by subjecting the kidney to ischaemic injury) observed no *Vhl*-specific phenotype^{47,106}, but the third found that cortical epithelial cells in kidneys with *Vhl* inactivation were twice as likely to be in cell cycle compared to those in kidneys from wild-type mice¹⁰⁷. Proliferation has not been assessed in kidneys with *Vhl* inactivation induced *in utero* in the proximal tubules.

Overall, these studies have failed to highlight any obvious contribution of developmental *Vhl* inactivation to oncogenesis or tumour suppression. However, there is a key caveat to interpreting this work in the context of cancer. These studies model biallelic *Vhl* inactivation during development and in entire segments of the nephron. Neither this extent nor this temporality of *VHL* inactivation is seen in human disease. Analysis of expected somatic *VHL* mutation rates against age-dependent incidence has suggested that biallelic *VHL* inactivation occurs in only 200-400 cells in the kidneys of sporadic ccRCC patients⁷⁰. Even in renal biopsies of patients with VHL disease-associated ccRCC, CA9-positivity is detected in only a small proportion of total RTE cells^{114,115}. In either case, biallelic *VHL* inactivation is predicted to occur in cells in early childhood and not during embryological development⁷⁰. Survival and proliferation of epithelial cells harbouring oncogenic mutations

has been shown to depend on whether they are immediately surrounded by other mutated cells or by wild-type cells in tissues *in vivo* or in cell culture¹³¹⁻¹³⁴. Hence, it is hence possible that the behaviour of *Vhl*-null cells surrounded by *Vhl*-competent cells in the RTE might differ from that of *Vhl*-null cells in renal tubules composed entirely of *Vhl*-null cells. The study of the cellular effects of *Vhl* inactivation using constitutively active Cre recombinase alleles is thus of limited relevance to understanding tissue-specificity and latency of oncogenesis.

1.4.3 Somatic *Vhl* inactivation *in vivo* in murine RTE

Inducible Cre recombinase alleles¹³⁵ have been used to inactivate *Vhl* somatically in a subset of cells within targeted segments of the RTE. These *Vhl* mouse models were developed after much of the cell culture and constitutive mouse work had already shown no phenotypes of interest with *Vhl* inactivation alone. Therefore, most studies with inducible Cre systems have primarily focussed on reproducing renal cancer by combining *Vhl* inactivation with deletion of other genes such as *Trp53* and *Rb1*^{112,113}, *Pbrm1*¹⁰⁹, and *Myc* and *Cdkn2a*¹³⁶. As such, only four of these studies have reported data on the cellular effects of somatic *Vhl* inactivation on its own. These have used three Cre recombinase alleles each targeting different segments of the nephron and have performed experiments at different timepoints following gene inactivation – tamoxifen-inducible *Pax8-CreERT2*¹³⁷ (pan-RTE; assayed at 12-20 months¹⁰⁹), tamoxifen-inducible *Ksp1.3-CreERT2*¹³⁸ (distal nephron, rarely proximal tubules; assayed at 2 months¹³⁹ and 7 months¹³⁶), and testosterone-inducible *Kap2-iCre*¹⁴⁰ (proximal tubules; constitutive in males, inducible in females; assayed at 6 and 12 months¹⁴¹). In agreement with previous work, somatic loss of *Vhl* alone did not result in the formation of renal tumours or cysts. No fibrotic lesions were reported in any study^{109,136,141}, marking a contrast with the *Hoxb7*-Cre study¹²⁷.

Proliferation following *Vhl* inactivation has been assessed by only one study. It found no difference in the number of cycling cells in the renal cortex of kidneys bearing *Vhl*-null

cells in proximal tubules compared to those with no *Vhl* inactivation at six months after recombination was induced¹³⁶. No assessment was made at earlier timepoints after *Vhl* inactivation. Furthermore, proliferative effects specifically within *Vhl*-null cells (which might have been only a minority of all cortical cells) were not distinguished from those in their *Vhl*-competent neighbours. As such, a proliferative effect of *Vhl* inactivation in RTE cells cannot be excluded and needs further study.

The timed induction of *Vhl* recombination in these models allows assessment of the long-term survival of *Vhl*-null cells in otherwise *Vhl*-competent kidneys. Using CA9¹¹⁶ immunohistochemistry (IHC) as a readout of *Vhl* inactivation, two studies detected the presence of *Vhl*-null cells in the renal cortex at 12 months specifically in the proximal tubules¹³⁶ and at 20 months across the RTE¹⁰⁹, suggesting that at least some *Vhl*-null cells are retained in the cortex over long periods of time. No data was shown for other regions in the kidney nor for the renal cortex at earlier timepoints. Interestingly, analysis of genomic DNA extracted from kidneys of mice in which *Vhl* was inactivated in RTE cells demonstrated a downward trend (though not statistically significant) in the amount of recombined *Vhl* allele present in the kidney between 12 and 20 months following recombination¹⁰⁹. This suggests perhaps that the total number of cells harbouring *Vhl* inactivation in the kidney might have decreased between the two timepoints. The numbers of CA9-positive cells in the renal cortex from the same mice were reportedly unchanged between these two timepoints¹⁰⁹. Although not formally tested in the study, this suggests that time-dependent loss of *Vhl*-null cells from non-cortical regions of the kidney may have contributed to the observed downward trend. Whether this data arises from a region-specific survival phenotype entrained in *Vhl*-null cells remains to be answered.

While few and varied in scope, studies on *Vhl*-null cells induced in the RTE in this way have generated a key hypothesis that *Vhl* inactivation might affect survival and proliferation of cells in the RTE in a region-specific manner. If true, this might underlie the

tissue-specificity observed for *VHL*-associated cancers. This hypothesis merits further testing by systematically assaying *Vhl*-null cells in different renal regions and extra-renal tissues at multiple timepoints.

1.5 Effects of *Vhl* inactivation on gene expression

1.5.1 HIF-dependent gene expression is relevant to *VHL*-associated oncogenesis

The known role of *VHL* in regulating HIF activity and the near-universality of VCB inhibition in ccRCC highlight HIF-mediated gene regulation as a key consequence of *VHL* inactivation relevant to oncogenesis.

Genome-wide association studies have identified single nucleotide polymorphisms (SNPs) that confer increased susceptibility to ccRCC. Notably, many of these SNPs directly promote HIF transcription factor binding to enhancer elements of genes such as *CCND1* and *BHLHE41* that are upregulated in ccRCC^{142,143}. Interestingly, such a relationship is not observed for susceptibility polymorphisms for cancers associated with environmental hypoxia but without *VHL* inactivation¹⁴⁴. RTE cells stabilise both HIF1A and HIF2A in response to *in vivo* *Vhl* inactivation but are thought to stabilise only HIF1A when exposed to hypoxia¹⁴⁵. Specific pharmacological inhibition of HIF2A has adverse effects on ccRCC growth¹⁴⁶ and is now an approved treatment for VHL disease-associated ccRCC^{147,148}. These observations further suggest that HIF-dependent gene expression mediated specifically by *VHL* inactivation promotes tissue-specific oncogenesis in the RTE.

HIF-dependent gene expression has been extensively characterised in cell culture and *in vivo* by a combination of RT-qPCR, microarray analysis, and bulk RNA sequencing (RNA-seq)¹⁴⁹⁻¹⁵⁵. Though a module of genes largely involved in glycolytic metabolism (e.g., *PGK1*, *LDHA*) was consistently identified as being regulated by HIF in all assayed cell types, most HIF-dependent gene regulation was found to be cell-type specific¹⁴⁹. Additionally, the mode of HIF stabilisation – hypoxia *versus* PHD inhibition *versus* VHL inhibition – also

appeared to affect gene expression within the same cell line¹⁵⁴. These observations highlight the need for precise identification of gene expression changes induced specifically by *VHL* inactivation in cancer-associated *versus* non-associated cell types.

1.5.2 Gene expression in *VHL*-null cells

Genome-wide changes in gene expression following *Vhl* inactivation in the RTE *in vivo* have not been studied widely. Most studies have focussed on transcriptomic analysis in the context of cancer¹¹¹⁻¹¹³, rhabdomyolysis-induced injury¹⁵⁶, and cystic or fibrotic disease¹²⁷. Only one of these studies was performed with an inducible Cre recombinase system¹⁵⁶ (*Pax8-rtTA* tetO-Cre¹⁵⁷; pan-RTE) and reported data from kidneys of healthy mice harbouring *Vhl* inactivation but not subjected to any other intervention. Four days after inducing *Vhl* recombination, kidneys carrying *Vhl*-null cells upregulated genes involved in glucose metabolism, MAPK signalling, and the Delta/Notch signalling pathway and downregulated genes involved in glutathione and folate metabolism¹⁵⁶; several of the upregulated genes were known to be HIF targets. Gene expression was not assayed at any other timepoint in this study. Furthermore, gene expression was not compared between different segments of the nephron (e.g., in the cancer-associated proximal tubule *versus* cancer non-associated collecting duct), and changes in *Vhl*-null cells were not distinguished from those occurring indirectly in *Vhl*-competent RTE cells or in renal interstitial cells in response to *Vhl* inactivation in the RTE.

These studies highlight a gap in our current knowledge about the effects of *in vivo* *Vhl* inactivation on gene expression in the different types of cells of the RTE. Resolving this gap might provide crucial insight into the tissue-specific, direct, and time-resolved effects *VHL* inactivation has on cells and help to identify possible modes of oncogenesis and tumour suppression.

1.6 *PBRM1* functions and relationship with *VHL*

Polybromo-1 (*PBRM1*; also known as *BAF180*) is a 140 kbp protein coding gene on human chromosome 3 at 3p21.1 and codes for a 1,689 amino acid 193 kDa protein (<https://www.ncbi.nlm.nih.gov/gene/55193>) that contains six tandem bromodomains (BRD), two bromo-adjacent homology (BAH) domains, and a high-mobility group (HMG) domain^{158,159}. The *PBRM1* gene is partially conserved across eukaryotes and exhibits >90% sequence identity within mammals^{158,159}.

1.6.1 *PBRM1* is a subunit of the PBAF SWI/SNF chromatin remodelling complex

PBRM1 is best characterised as an accessory subunit of the PBAF chromatin remodelling complex^{158,160,161}. PBAF is a minor SWI/SNF chromatin remodelling complex that regulates chromatin accessibility by altering the position of nucleosomes in an ATP-dependent manner. PBAF complexes, defined by the presence of *PBRM1*, *BRD7* and *ARID2* subunits, exhibit distinct localisation on chromatin¹⁵⁹ and effects on transcriptional regulation¹⁶² compared to the other BAF or ncBAF SWI/SNF chromatin remodelling complexes in mammals. The selective actions of PBAF complexes are partially regulated by the properties of *PBRM1*¹⁵⁸. The six BRD domains in *PBRM1* each exhibit distinct affinities for acetylated lysine residues at positions 4, 9, 14, 18, and 27 of Histone H3. This suggests that *PBRM1* interactions with nucleosomes might be governed by the specific patterns of histone acetylation^{163,164}. Furthermore, the C-terminal domain of *PBRM1* mediates unique contacts between PBAF and the nucleosome that are not observed with BAF or ncBAF complexes¹⁶⁰.

Chromatin immunoprecipitation and sequencing (ChIP-seq) has revealed that *PBRM1*-containing PBAF complexes predominantly occupy gene promoters (between the +1 and +2 nucleosomes) and are correlated with H3K14Ac, K3K27Ac and H3K4me3 histone modifications¹⁶⁵. This localisation is distinct from that of BAF complexes which

predominantly occupy gene enhancer elements¹⁶⁵. Homozygous deletion of *PBRM1* in cultured cells prevents the incorporation of BRD7 into PBAF complexes¹⁶⁵. Interestingly, these PBRM1/BRD7-deficient PBAF complexes significantly lose their promoter-proximal location and gain occupancy at distal enhancer elements, indicating that PBRM1 regulates PBAF recruitment to promoter proximal sites on chromatin.

1.6.2 PBRM1 interacts with methylated microtubules

While PBRM1 is located in the nucleus during interphase, it colocalises with K40me3-methylated α -Tubulin situated at mitotic spindle poles during mitosis¹⁶⁶. Interestingly, this methylation is catalysed by SETD2 which is often inactivated in *VHL/PBRM1*-driven ccRCC¹⁶⁷⁻¹⁶⁹. Interactions between PBRM1 and methylated microtubules are mediated *via* the methyl group-recognising BAH domains on PBRM1 and not its acetyl group-recognising BRD domains, further supporting the role of methylation in this interaction. Interestingly, PBRM1 present on these microtubules continues to interact with members of the PBAF complex, and ARID2 and BRG1 (other components of PBAF) also colocalise with methylated microtubules at the mitotic spindle pole¹⁶⁶. This suggests that the PBAF complex has an extra-nuclear function in cytoskeletal organisation. The cell biological role of such PBAF activity has not been resolved.

1.6.3 Oncogenic effects of *PBRM1* inactivation in *VHL*-null cells

PBRM1 inactivation is the most common event to occur in cells after *VHL* mutation and 3p loss in the evolution of ccRCC. Mice that model biallelic *Vhl* and *Pbrm1* inactivation in the RTE develop renal cancer^{104,109,111}. Notably, those that model biallelic *Vhl* on its own, biallelic *Pbrm1* inactivation on its own, and biallelic *Vhl* inactivation together with monoallelic *Pbrm1* inactivation, do not develop renal cancer over 20 months after recombination is induced^{104,109,111}. One study has used an inducible *Pax8-CreERT2* allele to model concomitant *Vhl* and *Pbrm1* inactivation in cells of the RTE. In addition to developing renal tumours at 20 months after recombination is induced, kidneys from these mice exhibited

an age-dependent increase in the allelic frequency of recombined *Vhl*, suggesting an expansion of the *Vhl*-null cell population in these kidneys over time¹⁰⁹. Re-introduction of *PBRM1* in *PBRM1*-null *VHL*-deficient renal cancer cell lines decreases their ability to form colonies in a soft agar assay and to form xenograft tumours in immune-deficient mice^{111,165,166,170-172}, supporting the identification of *PBRM1* as a tumour suppressor in *VHL*-null cells. Interestingly, *PBRM1* inactivation in *VHL*-competent renal cell lines (HK2) has no effect on their growth or colony forming capacity, supporting *PBRM1*-driven tumour suppression in renal cells to be dependent on concomitant *VHL* inactivation¹⁶⁵. These observations suggest that specific alterations in *VHL*-null cells caused by additional *PBRM1* inactivation have oncogenic effects in the RTE.

As with *VHL* inactivation, only a few studies have focussed on the effects of *PBRM1* inactivation on *VHL*-null RTE cells *in vivo* or on non-neoplastic *VHL*-null renal cells in culture. Work performed on renal cancer cell lines, ccRCC biopsies, and mouse models of *Vhl* and *Pbrm1* inactivation have nonetheless suggested three possible modes of action for *PBRM1* – regulation of HIF-mediated gene expression, regulation of NFκB signalling, and regulation of genomic stability – that can be explored further in relevant tissue contexts.

1.6.3.1 Regulation of HIF-dependent gene expression

RNA-seq analysis of A704 (*VHL* and *PBRM1*-deficient) cells demonstrated modest decreases in expression of some HIF-target genes such as *ALDOC*, *CCND1*, *IGFBP3* but not others such as *GLUT1*, and *PDK1*¹⁷², in response to *PBRM1* re-introduction. Interestingly, many of the HIF-target genes not altered after *PBRM1* expression were those identified as conserved HIF targets across multiple cell lines¹⁴⁹. A similar analysis in 786-O cells (*VHL*-deficient but *PBRM1*-competent) demonstrated an increase in genes associated with hypoxia upon CRISPR-mediated deletion of *PBRM1*¹⁷². These changes did not reflect changes in protein levels of HIF1A or HIF2A. These studies thus suggested that *PBRM1* inactivation amplifies HIF-dependent gene expression in *VHL*-null cells. The findings were partially supported by RNA-seq analysis demonstrating an upregulation of genes known to be HIF

targets in the kidneys of 12-week old *Vhl*^{fl/fl}; *Pbrm1*^{fl/fl}; *Ksp-Cre* (*Vhl/Pbrm1* KO) mice compared to kidneys from *Vhl*^{fl/fl}; *Ksp-Cre* (*Vhl* KO) mice¹¹¹. Notably, these genes were not altered in kidneys of *Pbrm1*^{fl/fl}; *Ksp-Cre* mice when compared to wild-type mice, suggesting that the amplification of the HIF response is a specific effect of *Pbrm1* inactivation in *Vhl*-null cells.

These findings are in contrast with two other studies. siRNA-mediated silencing of *PBRM1* in 786-O, SN12C, or TK10 renal cancer cell lines (all derived from *VHL*-null but *PBRM1*-competent ccRCC), which increased their proliferation and colony forming ability, did not cause significant alterations in the expression of hypoxia-associated genes when assayed by microarray analysis⁹⁵. Moreover, RNA-seq analysis of Caki-2 cells (*VHL* and *PBRM1* deficient) demonstrated an increase in expression of genes associated with hypoxia upon *PBRM1* re-introduction in direct contrast with the studies described above¹⁷³. The discrepancies between these studies have not been explained.

1.6.3.2 Regulation of NFκB signalling

RNA-seq analysis of 786-O cells with and without CRISPR-mediated *PBRM1* KO as well as of NCC432 (*VHL* and *PBRM1*-deficient) cells with or without *PBRM1* re-introduction has demonstrated significant upregulation of genes associated with TNFα and NFκB-mediated signalling in *PBRM1*-deficient cells¹⁶⁵. This upregulation was associated with specific occupancy of PBRM1-deficient PBAF complexes at enhancer elements enriched for NFκB p65 subunit RELA binding sites¹⁶⁵.

Upregulation of NFκB and STAT3-mediated signalling was also observed in RNA-seq analysis of kidneys of *Vhl/Pbrm1* KO mice compared to those of *Vhl* KO mice^{111,165}, and was notably not observed in kidneys of *Pbrm1*^{fl/fl}; *Ksp-Cre* mice when compared to wild-type mice. This further supports NFκB signalling as a specific target of *Pbrm1* inactivation in *Vhl*-null cells. However, why NFκB signalling is altered after *PBRM1* inactivation only in *VHL*-null cells is not known.

1.6.3.3 Regulation of genomic stability

While *Vhl*-null MEFs grown at 5% pO₂ for 20 days exhibit increased levels of DNA damage markers γ H2AX and 53BP1, *Vhl/Pbrm1*-null MEFs do not exhibit such an increase when compared to *Vhl*-competent MEFs. This difference is independent of a change in the proliferative behaviour between *Vhl*-null and *Vhl/Pbrm1*-null MEFs¹⁰⁹. Furthermore, while *Vhl*-null MEFs exhibit significantly more replication fork stalling upon exposure to mitomycin C and aphidicolin, *Vhl/Pbrm1*-null MEFs do not exhibit such an increase when compared to *Vhl*-competent MEFs¹⁰⁹. These observations suggest that *Pbrm1* inactivation protects *Vhl*-null cells from replicative stress. This is supported by studies *in vivo* showing that kidneys of *Vhl*^{f/f}; *Pax8-CreERT2* mice exhibit significantly more γ H2AX-positive RTE cells than those of *Vhl*^{f/f}; *Pbrm1*^{f/f}; *Pax8-CreERT2* mice when treated with tamoxifen (to induce *Vhl* and *Vhl/Pbrm1* recombination respectively) and mitomycin C (to induce replication fork stalling)¹⁰⁹. It has been hypothesised that *Pbrm1* inactivation somehow alters γ H2AX-mediated signalling downstream of replication fork stalling and prevents cell cycle arrest in damaged cells; this allows cells with DNA damage to proliferate and incur further mutations and genomic rearrangements that promote oncogenesis. This hypothesis has not been tested.

No study has assessed the effects of *PBRM1* inactivation on the mitotic spindle in *VHL*-null cells. However, *PBRM1* inactivation results in the formation of chromosome bridges and micronuclei in *VHL*-competent HEK293T cells¹⁶⁵. Re-introduction of *PBRM1* mutants that could form the PBAF complex but not interact with α -Tubulin does not rescue the genomic instability phenotype¹⁶⁵, suggesting that this effect is specific to the interaction of *PBRM1* with methylated microtubules. Whether, and in what contexts, this phenotype occurs in *VHL*-null RTE cells *in vivo* will be of interest to examine.

1.7 The need for a novel experimental approach

As reviewed above, the molecular functions of VHL as an E3 ubiquitin ligase subunit or as an adaptor protein have been defined, and it has been shown that *VHL* inactivation results in HIF stabilisation, subsequent upregulation of HIF-target genes, and, in some contexts, dysregulated organisation of the cytoskeleton and extracellular matrix. However, most studies of *VHL* inactivation, with or without *PBRM1* inactivation, have been performed in contexts where neoplastic transformation has already taken place, or in mouse models where the phenotypes observed might not be directly relevant to those of *Vhl*-null cells in otherwise normal tissues. Collectively, these have revealed a gap in our current knowledge on the exact cellular phenotypes of *VHL*-null or *VHL/PBRM1*-null cells resident in morphologically normal tissues *in vivo*. Illuminating this gap is essential to furthering our understanding of ccRCC evolution. The following questions are of particular interest to examine:

1. Do *VHL*-null cells generated in tissues exhibit survival and proliferative phenotypes that associate with the known tissue-specificity of *VHL*-driven cancer?
2. How does *VHL* inactivation affect gene expression in cells *in vivo* and are these effects tissue- and cell-type specific? To what extent are these changes composed of HIF-dependent gene expression?
3. Does *VHL* inactivation have any immediate oncogenic effects on cells at all? Why are *VHL*-null cells in even cancer-associated tissues thought to require years-to-decades to form detectable tumours?
4. How does additional *PBRM1* loss affect the cellular phenotypes of *VHL*-null cells? What aspects of these effects are specific to the *VHL*-null condition? Are these effects also tissue- or cell type-specific?

Examination of these questions requires two key technological abilities – 1) the ability to accurately induce, identify and isolate *VHL*-null cells from otherwise *VHL*-

competent tissues *in vivo* without having to rely on detecting morphological abnormalities or on indirect or context-specific effects of *VHL* inactivation, and 2) the ability to assess cellular phenotypes *in situ* and *ex vivo* with cellular and temporal resolution. Both these abilities can be provided by “reporter” mouse models in which a genetic intervention or a specific cell lineage is ‘marked’ by the expression of a fluorescence or enzyme-based reporter protein¹⁷⁴⁻¹⁷⁸. However, accurate systems that report on *Vhl* inactivation in mice have not been developed. Recently single-cell sequencing technologies have been applied to the kidney and other organs, providing a wealth of data on gene expression patterns, on nephron heterogeneity and on cellular responses in non-neoplastic disease^{89,179-183}. These technologies, which furnish cellular data at magnitudes beyond what can be achieved with traditional histological analyses, have not yet been applied to the study of non-neoplastic *VHL*-null cells in the kidney and other organs.

A novel experimental approach is thus merited, that focusses on assaying cellular phenotypes of *Vhl*-null cells in morphologically normal tissues *in vivo* from the timepoint at which *Vhl* is inactivated and traces changes in cell behaviour and gene expression over time. This approach has been undertaken in my thesis.

The aims of this thesis are to:

1. Develop and validate a novel conditional reporter *Vhl* allele that accurately marks *Vhl* recombination and employs it in an experimental system that allows co-incident assessment of phenotypes induced specifically in *Vhl*-null *versus* *Vhl*-competent cells in different tissues (**Chapter 2**).
2. Assess whether cells display organ-specific survival and proliferative behaviours immediately after *Vhl* inactivation and if these relate to tissue-specific oncogenesis associated with *Vhl* inactivation (**Chapter 3**).
3. Assess how *Vhl* inactivation affects the long-term survival, proliferation, and morphological phenotypes of cells in different compartments of the kidneys, and

if these alter over time. Examine evidence for time-dependent tumour-suppressive effects of *Vhl* inactivation that might inform on its insufficiency and latency of oncogenesis (**Chapter 4**).

4. Assess how *Vhl* inactivation affects gene expression across the different cell types in the RTE, and if these alter over time. Examine contributions of HIF-dependent gene expression to these changes, evidence for time-dependent tumour-suppressive effects of *Vhl* inactivation, and evidence for particular oncogenic adaptations in rare renal cell populations that might otherwise be missed in histological or bulk examination (**Chapter 5**).
5. Assess the survival, proliferation, morphological and gene expression phenotypes entrained by concomitant *Vhl* and *Pbrml* inactivation in different renal compartments and cell types of the RTE. Look for differences that might provide insight into the sufficiency and acceleration of oncogenesis with additional *Pbrml* inactivation in *Vhl*-null cells (**Chapter 6**).

The caveats and implications of the experimental results presented are discussed at the end of each chapter. Salient findings and their wider implications on the investigation and understanding of *VHL*-associated cancer are discussed in **Chapter 7**.

Chapter 2: A Novel Lineage Tracing Mouse Model of *Vhl* Inactivation

2.1 Introduction

This thesis is centred around the study of *Vhl*-null cells *in vivo* in different tissues and organs. This requires an experimental system in which:

1. One or both copies of the *Vhl* gene is completely and irreversibly inactivated with no off-target effects, and in which such inactivation can be induced with spatiotemporal precision.
2. Cells in which such inactivation has occurred can be identified *in situ* and isolated for *ex vivo* analyses.

Cre-loxP systems are routinely used in biomedical research to induce gene inactivation in mice¹³⁵. Such systems have been used widely to inactivate *Vhl* in mice *in vivo* or in primary culture¹⁸⁴. There are two main *Vhl* alleles engineered for this purpose, *Vhl*^{tm1jae}¹⁸⁵ and *Vhl*^{tm1Lss}¹⁸⁶, that differ in their recombination design. In *Vhl*^{tm1jae}, loxP sites flank the *Vhl* promoter and exon 1; recombination between the loxP sites inactivates *Vhl* by preventing transcription and removing the coding region necessary to produce VHL protein¹⁸⁵. In *Vhl*^{tm1Lss}, loxP sites flank *Vhl* exons 2 and 3¹⁸⁶; recombination between the loxP sites removes regions of the *Vhl* gene that code for critical residues participating in interaction of VHL with its binding partners Elongins B and C, as well as its substrates like HIF1A and HIF2A^{23,34}. Notably, recombination of *Vhl*^{tm1Lss} functionally inactivates *Vhl* but does not affect either its promoter or exon 1, which can still be transcribed.

Use of either of these *Vhl* alleles and appropriate inducible Cre lines will satisfy the first condition set above for an experimental system. However, it will not confer any ability to identify recombined cells with direct means. The only means of identifying *Vhl*-null cells

with these alleles is to assay for absence of VHL, for HIF stabilisation or expression of known HIF targets. Identification by means of a negative result in staining is difficult, assaying for HIF stabilisation is technically demanding, while expression of HIF-target genes can often be cell-type specific. Furthermore, these methods cannot be utilised if cells with co-deletion of *Vhl* and any HIF genes are to be studied. ‘Reporter alleles’ have often been used in biomedical research to ‘mark’ cells that have undergone Cre-mediated recombination¹⁷⁴. These involve a fluorescent reporter, or an enzyme (such as β -Galactosidase) placed downstream of a “*lox-STOP-lox*” cassette. Cre-mediated recombination of this cassette excises the STOP codon and allows for the expression of the fluorescent reporter or enzyme. Recombined cells can then be visualised directly by fluorescence or revealed histochemically based on activity of the enzyme expressed *in situ*¹⁷⁴. It is possible to breed one of the existing *Vhl* alleles and a reporter allele together in a mouse and recombine both with the same Cre allele; a substantial proportion of cells would recombine both alleles and could thus be identified on the basis of expression of the reporter. However, recombination of *Vhl* will always be independent of the recombination of the reporter, and one could never guarantee that all cells expressing the reporter have also recombined the *Vhl* allele.

This has created the need to develop a novel *Vhl* allele fit for purpose. In this chapter, I describe a novel conditional *Vhl* allele that combines the design elements of the *Vhl*^{*tm1Lss*} allele and the recombination-dependent expression of a tdTomato fluorescence reporter into a single allele: *Vhl*^{*tm1.1b(tdTomato)Pjr*}. I then validate the fidelity of tdTomato expression with *Vhl* recombination. I further describe an experimental approach that utilises this allele to model marked monoallelic and biallelic *Vhl* inactivation and then optimise its use with two different inducible Cre lines to controllably induce marked *Vhl* recombination in the kidney or across multiple organs in the mouse.

2.2 Results

2.2.1 Allele Design and Recombination

2.2.1.1 Structure of *Vhl*^{tm1.1b(tdTomato)*Pj*r}

The floxed *Vhl*^{tm1.1b(tdTomato)*Pj*r} allele (*Vhl*^{*Pj*r:fl}) (**Figure 2.1**) contains the full length *Vhl* gene (three exons and two introns) followed by an anti-sense *tdTomato* cassette. The *tdTomato* cassette consists of a splice-acceptor site (SA), a porcine teschovirus 2A (P2A) self-cleaving peptide sequence, the *tdTomato* coding sequence, and a transcription termination and polyadenylation (pA) site. In addition, the allele contains two pairs of *lox* sites. One pair of inward facing *loxP* sites flanks the *tdTomato* cassette; another pair of *lox2272* sites faces outward, with one site within Intron 1, and the other downstream of the anti-sense *tdTomato*. Importantly, this downstream *lox2272* site is contained within the region flanked by the *loxP* sites. As all *lox* sites are within introns or away from splice sites, and as the *tdTomato* cassette is anti-sense to *Vhl* and lacks its own promoter, the unrecombined allele is expected to express full length *Vhl* whose gene product will be capable of interacting with its binding partners and substrates^{23,34} and is expected to not express *tdTomato* (**Figure 2.1**).

2.2.1.2 Two-step recombination of *Vhl*^{*Pj*r:fl}

Vhl^{*Pj*r:fl} is expected to undergo a two-step recombination to go from a ‘floxed’ allele to a knockout-form of the allele when exposed to Cre recombinase.

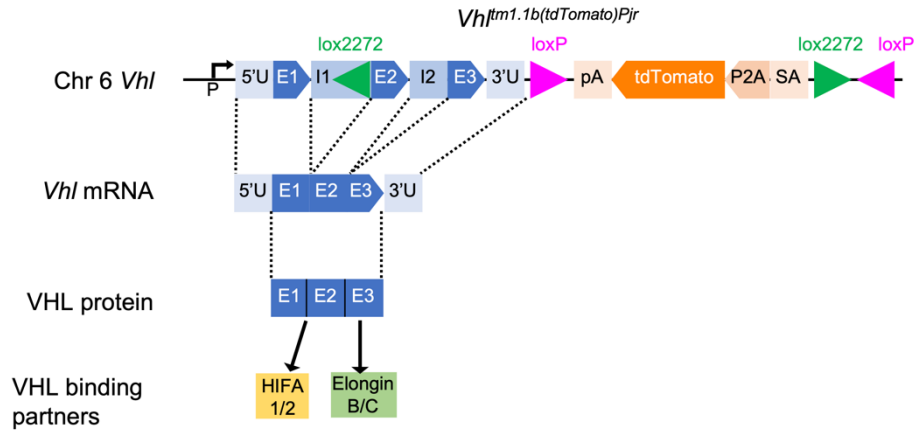


Figure 2.1: Structure of *Vhl*^{tm1.1b(tdTomato)Pjr}. Schematic design of *Vhl*^{tm1.1b(tdTomato)Pjr} at the endogenous *Vhl* locus on mouse chromosome 6. P – *Vhl* promoter; U – untranslated region; E – *Vhl* exon; I – *Vhl* intron; pA – polyadenylation site; P2A - porcine teschovirus 2A peptide; SA – Splice Acceptor. Dashed lines denote spliced and translated regions. Single arrows indicate interactions between different VHL regions and VHL binding partners.

The rate of Cre-mediated recombination is inversely proportional to the linear distance between *lox* sites¹⁸⁷. Thus, recombination between the *loxP* sites on *Vhl*^{pjr.fl} is expected to be preferred over the recombination between the *lox2272* sites. The anti-parallel orientation of the *loxP* sites facilitates a reversible Cre-mediated inversion of the flanked region¹⁸⁸; this places the *tdTomato* cassette sense to *Vhl* and flips the downstream *lox2272* site so that the two *lox2272* sites are now in a parallel configuration (**Figure 2.2**). *tdTomato* is not expected to be expressed from this “incompletely recombined” (*Vhl*^{pjr.inrec}) form as it is downstream of the *Vhl* 3'-UTR and does not have its own promoter. The parallelly-oriented *lox2272* sites then facilitate an irreversible Cre-mediated excision of the flanked region¹⁸⁸; this removes *Vhl* exons 2 and 3 and the *Vhl* 3'-UTR that carried its transcriptional termination site, thereby ‘knocking it out’. Additionally, it places *tdTomato* immediately downstream of *Vhl* exon 1, where it can be transcribed under the control of the *Vhl* promoter and spliced with *Vhl* exon 1 due to the placement of the splice-acceptor (SA) site. Thus, *tdTomato* is ‘knocked in’ (**Figure 2.2**). The irreversibility of this step arises from the fact that one copy

of the *loxP* site is also excised. It is worth noting that, were the *lox2272* sites in *Vhl^{lpr-fl}* to recombine before the *loxP* sites, the final outcome of recombination will remain the same.

In this knock-out form (*Vhl^{lpr-KO}*) of the allele, the co-transcribed *Vhl-exon-1-tdTomato* transcript will be co-translated, and the *VHL* exon 1 fragment (which lacks the residues that interact with VHL binding partners^{23,34,186}) is expected to be uncoupled from tdTomato protein by ribosome-skipping orchestrated by the P2A polypeptide sequence¹⁸⁹. Thus, Cre-mediated recombination of the novel allele is expected to mark the excision of *Vhl* by expression of a functional tdTomato protein.

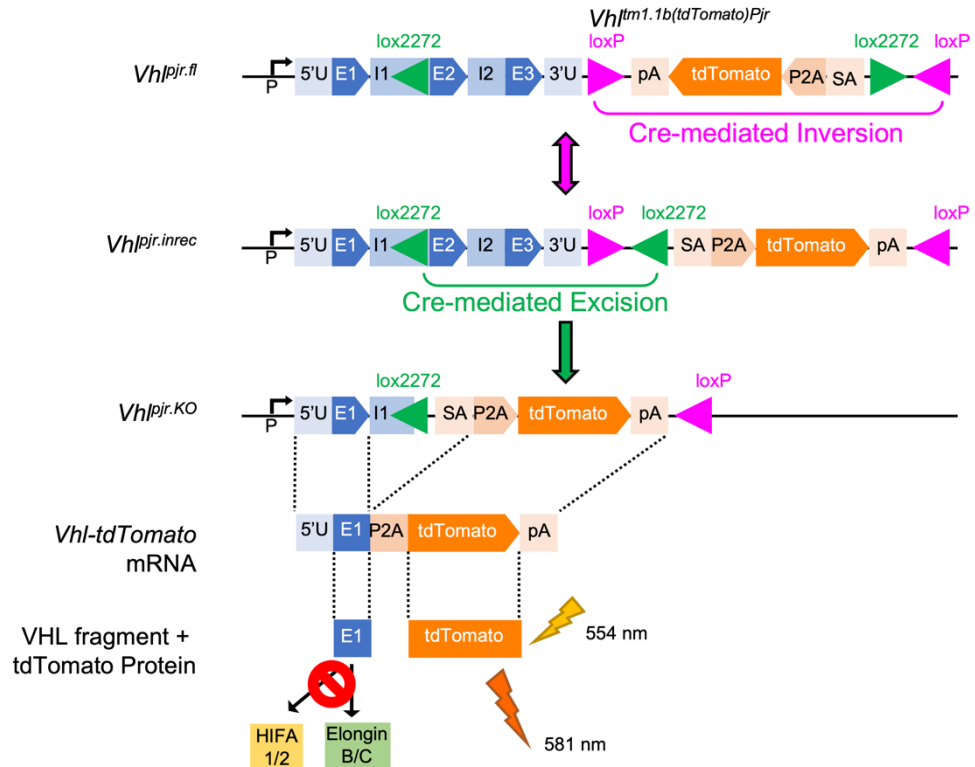


Figure 2.2: Two-step Cre-mediated recombination of *Vhl^{lpr-fl}*. Double arrow indicates reversible process. Single arrow indicates irreversible process. *Vhl^{lpr-fl}*, *Vhl^{lpr.inrec}*, and *Vhl^{lpr-KO}* refer to “floxed”, “incompletely recombined”, and “knockout” forms of the *Vhl^{lpr-fl}* allele. P – *Vhl* promoter; U – untranslated region; E – *Vhl* exon; I – *Vhl* intron; pA – polyadenylation site; P2A - porcine teschovirus 2A peptide; SA – Splice Acceptor. Dashed lines denote spliced and translated regions, Lightning symbols denote excitation and emission wavelengths for tdTomato fluorescence. Red stop sign indicates no interaction between VHL exon 1 fragment and HIFA-1/2 or Elongin B/C.

2.2.2 Recombining *Vhl^{pjr.fl}* with inducible Cre

One copy of this allele is sufficient to mark recombined cells. In this project, *Vhl^{pjr.fl}* has been coupled with either wild-type (*Vhl^{wt}*) or constitutively deleted *Vhl* alleles (*Vhl^{jae.KO}*) – both non-marking – to generate *Vhl^{wt/pjr.fl}* or *Vhl^{jae.KO/pjr.fl}* mice (see **Chapter 8**) in which monoallelic recombination of *Vhl^{pjr}* should result in marked *Vhl*-heterozygous or *Vhl*-null cells respectively. Recombination has been induced with the tamoxifen-inducible Cre-loxP system¹³⁵. *Ubc-CreERT2*, in which Cre expression is driven by the ubiquitously expressed *Ubc* promoter has been used to induce recombination across multiple organs in the mouse, or in mouse embryonic fibroblasts (MEFs) in culture¹⁹⁰. *Pax8-CreERT2*, in which Cre expression is driven by the (largely) renal tubular epithelium (RTE) specific *Pax8* promoter has been used to induce restricted recombination in the kidney¹³⁷.

2.2.2.1 *Vhl^{pjr}* recombines fully and only after Cre activation

The design and recombination of *Vhl^{pjr}* was tested by assaying for various forms of the allele in *Vhl^{wt/pjr.fl}*; *Ubc-CreERT2* MEFs with or without Cre activation. MEFs were treated for 24 hours with 4-hydroxytamoxifen (4-OHT; metabolically active form of tamoxifen)¹⁹¹ or with vehicle control (Veh). MEFs were harvested three days after treatment, and the presence of the different forms of *Vhl^{pjr}* was assayed by genomic PCR.

Vhl^{pjr.KO} was detected in MEFs only when they carried Cre and were treated with 4-OHT (**Figure 2.3A**). *Vhl^{pjr.inrec}* was detected in the same population of cells, but with a greatly reduced intensity (**Figure 2.3B**). *Vhl^{pjr}* allele thus recombines to completion after, and only after, Cre activation, suggesting that very few cells carry inverted *tdTomato* without *Vhl* knockout.

2.2.2.2 *tdTomato* can be detected after Cre activation

Having established that the allele recombines as expected, I tested ways by which expressed *tdTomato* could be detected in recombined cells *in situ*, *ex vivo* (to assay primary cultures), and in suspension (to isolate recombined cells). Recombination was induced *in vivo* in *Vhl^{wt/pjr.fl}*; *Pax8-CreERT2* mice by oral gavage of 5x 2 mg tamoxifen (TMX). Kidneys

from these mice were then either processed for histological analyses of tdTomato expression or enzymatically dissociated to create cell suspensions for tdTomato-based flow cytometry or primary renal culture.

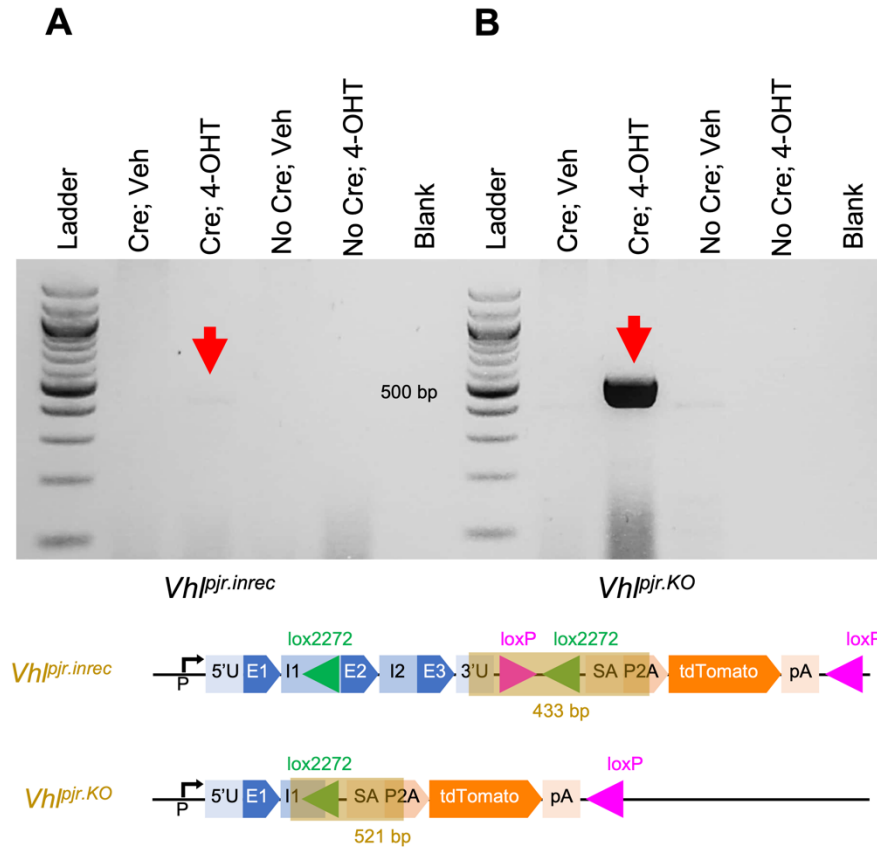


Figure 2.3: *Vhl^{pjr.fl}* recombines fully and only after Cre activation. Gel electrophoresis of genomic PCR products for (A) *Vhl^{pjr.inrec}* or (B) *Vhl^{pjr.KO}* forms of *Vhl^{pjr}* in *Vhl^{wt/pjr.fl}* MEFs with or without *Ubc-CreERT2* treated with 4-OHT or vehicle (Veh). Presence of relevant bands indicated with red arrows. Schematics indicating unique amplicons for *Vhl^{pjr.inrec}* or *Vhl^{pjr.KO}* PCR given below, with amplicons for each PCR highlighted in yellow-ochre. P – *Vhl* promoter; U – untranslated region; E – *Vhl* exon; I – *Vhl* intron; pA – polyadenylation site; P2A - porcine teschovirus 2A peptide; SA – Splice Acceptor.

Three methods for tdTomato detection were optimised – immunostaining on tissue sections (**Figure 2.4A**), flow cytometry of dissociated tissue (**Figure 2.4B**), and live-cell imaging of primary cultures (**Figure 2.4C**). tdTomato-fluorescence could not be detected in

kidney sections without signal amplification with an antibody. Immunostaining provided consistent visualisation of tdTomato-positive cells with a reasonable signal-to-noise ratio. Combined with the ability to sort tdTomato-positive cells for downstream analyses, these techniques allow study of recombined cells across tissues and timepoints.

2.2.2.3 All tdTomato-positive cells are recombined

I tested if tdTomato-positivity reported *Vhl* recombination faithfully. tdTomato-positive and tdTomato-negative cells were isolated from dissociated kidneys of *Vhl^{wt/pjr.fl}; Pax8-CreERT2* mice by fluorescence-activated cell sorting (FACS) and the different forms of *Vhl^{pjr}* carried within the sorted populations were assayed by genomic PCR. The *Vhl^{pjr.KO}* allele was detected in tdTomato-positive cells (**Figure 2.5A**) and was present at higher levels than in tdTomato-negative cells in relation to the *Vhl^{wt}* allele (**Figure 2.5B**). Little to no unrecombined *Vhl^{pjr.fl}* was detected in tdTomato-positive cells (**Figure 2.5A**), though it was detected in tdTomato-negative cells. tdTomato-positive cells carried the *Vhl^{pjr.KO}* allele, but little to none of the *Vhl^{pjr.fl}* allele (**Figure 2.5B**). Together, the data suggest that most if not all Tomato-positive cells have undergone *Vhl^{pjr}* recombination, and that a majority, but not all, tdTomato-negative cells are unrecombined. Thus, in this novel model, while tdTomato negativity does not completely exclude the possibility of *Vhl* recombination, tdTomato-positivity accurately marks *Vhl* recombination.

2.2.3 Control of *in vivo* recombination frequency by titration of TMX dose

Detection of tdTomato-positive cells in tissues after Cre-mediated recombination is dependent on several factors – TMX dose (effective dose being affected by regional variation in TMX distribution and uptake within each organ)¹⁹², expression levels of Cre in each cell, extent to which Cre is translocated to the nucleus upon 4-OHT binding, and finally, the complexity of *lox* sites that are recombined by the Cre^{188,193}. The behaviour of *Pax8-CreERT2* and *Ubc-CreERT2* with this novel allele and with different doses of TMX was tested empirically.

2.2.3.1 Recombination with *Pax8-CreERT2*

The *Pax8-CreERT2* allele was developed to induce restricted recombination in the RTE^{137,157}. However, other *Pax8*-driven Cre alleles have shown extra-renal activity in the uterus and cells of the Mullerian system^{194,195}. I tested tissue-restriction and precision of recombination in *Vhl*^{wt/pjr.fl}; *Pax8-CreERT2* mice given no TMX, 1 mg TMX, or five daily doses of 2 mg (5x 2 mg) TMX by oral gavage and harvested 1-3 weeks after the first dose. These doses have been used to activate inducible Cre systems by others¹⁹⁶.

Recombination was assayed in the kidney, liver, spleen, pancreas, adrenal glands, testis, uterus, gut, heart, lungs, and brain. No tdTomato-positive cells were detected in any assayed organ in the absence of tamoxifen administration. Extra-renal recombination was detected only in the epithelial lining of the uterus.

tdTomato-positive cells were detected in the cortex, outer medulla, inner medulla, and the papilla of the kidney at both doses of TMX (**Figure 2.6A**). tdTomato-positivity was restricted to tubular epithelial cells (including parietal cells of the Bowman's capsule), with no interstitial cells appearing to have recombined (**Figure 2.6B**). Frequency of recombination was assessed by quantifying the proportion of cells in each region of the kidney positive for tdTomato staining using HALO (see **Chapter 8**). At both doses, recombination was most frequent in the papilla, followed by the cortex, inner medulla, and outer medulla. The TMX dose significantly affected the extent of recombination in the cortex ($p = 0.03$) and the outer medulla ($p = 0.03$); recombination in the cortex increased from $5.7\% \pm 4.1\%$ (95% confidence interval (CI)) to $12.5\% \pm 2.0\%$ (95% CI) when the dose was increased from 1 mg to 5x 2 mg. Reduced recombination frequency with 1 mg TMX was accompanied by reduced precision, with the coefficient of variation for recombination in the cortex rising from 34.9% with 5x 2 mg TMX to 68.9% with 1 mg TMX. No difference ($p > 0.05$) in recombination frequencies was observed with the two TMX doses in the inner medulla and papilla, suggesting that 1 mg TMX might be saturating the activation of *Pax8-CreERT2* in these regions. Taken together,

5x 2 mg TMX generates precise and reproducible *Vhl*^{pir} recombination in the kidney suitable for marking and tracking cells *in situ* over time.

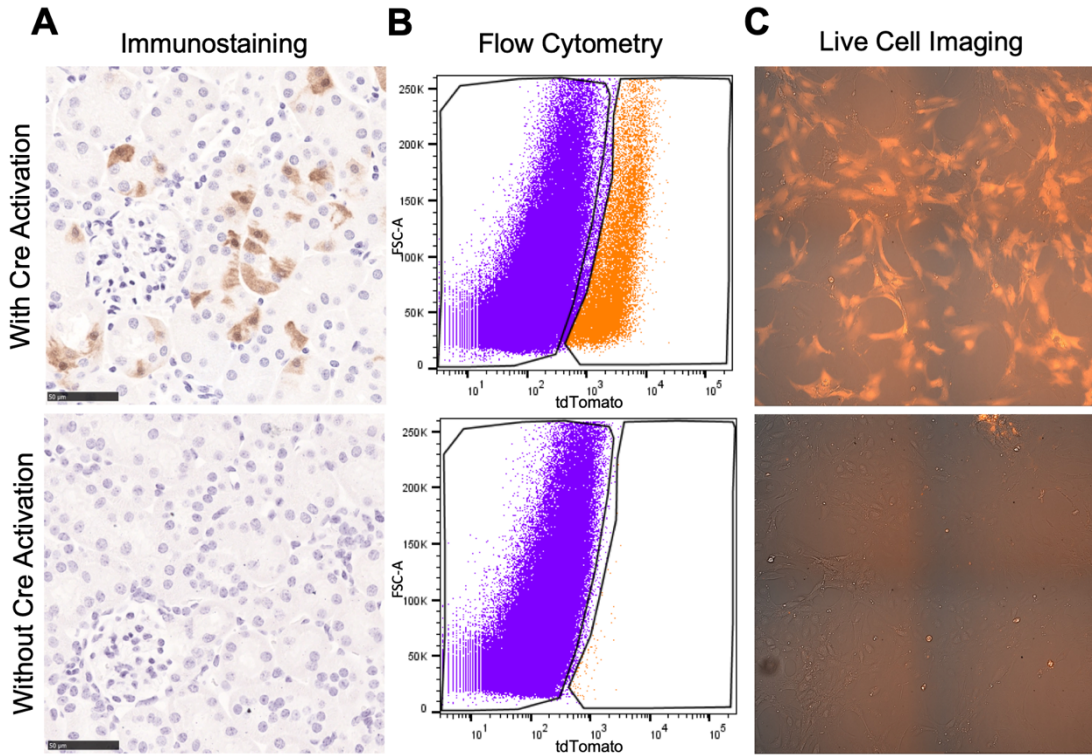


Figure 2.4: Detection of tdTomato *in situ* and *ex vivo* after Cre activation. (A) tdTomato immunohistochemistry in kidney sections and (B) tdTomato fluorescence-based FACS on renal cells from *Vhl*^{wt/pir.fl}; *Pax8-CreERT2* mice given 5x 2 mg TMX (top) or no TMX (bottom) and harvested one week after first dose. Scale bar denotes 50 μ m. FACS gates shown. (C) Live-cell imaging of primary culture of dissociated *Vhl*^{wt/pir.fl}; *Pax8-CreERT2* kidneys treated with 4-OHT (top) or vehicle (bottom) for 24 h and cultured for 4 days. Differential contrast image (grey) overlaid on orange tdTomato signal.

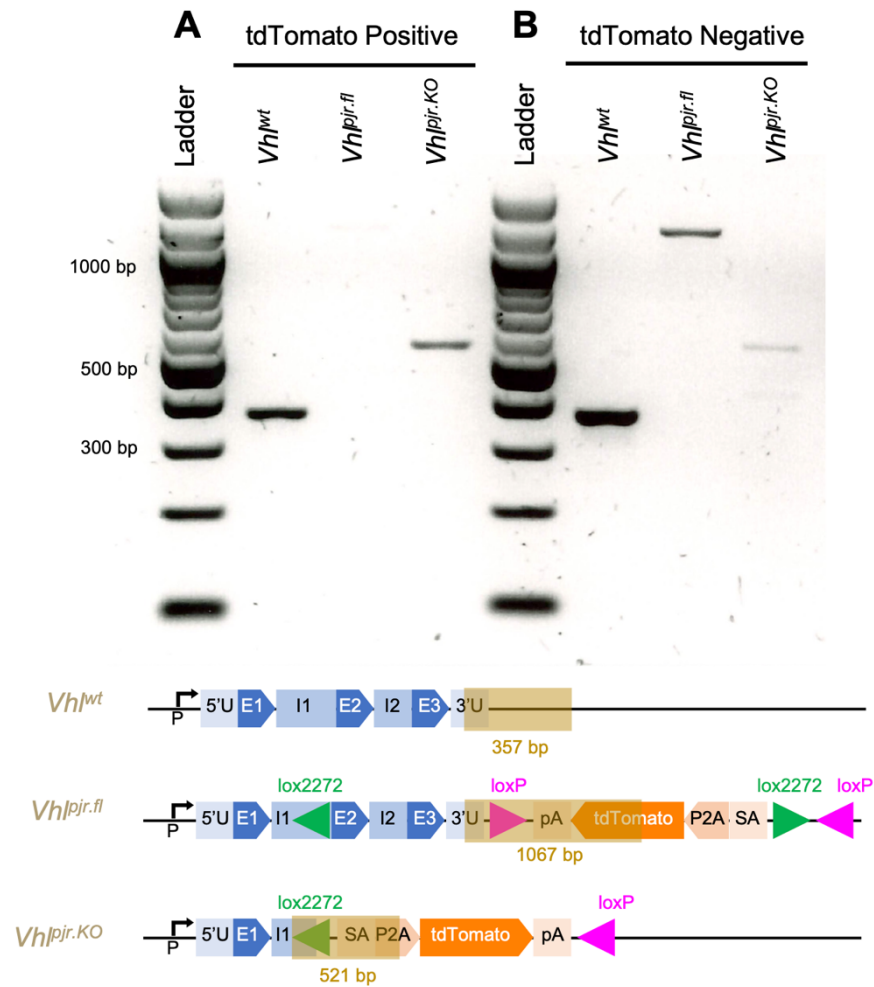


Figure 2.5: tdTomato-positive cells carry recombined *Vhl*^{lpr}. Gel electrophoresis of genomic PCR for *Vhl*^{wt}, *Vhl*^{lpr.fl}, and *Vhl*^{lpr.KO} alleles performed on FAC-sorted cells (**A**) tdTomato-positive cells, or (**B**) tdTomato-negative cells from kidneys of *Vhl*^{wt/lpr.fl}; *Pax8-CreERT2* mice given 5x 2 mg TMX and harvested one week after first dose. Schematics of unique amplicons for each allele given below (yellow-ochre). P – *Vhl* promoter; U – untranslated region; E – *Vhl* exon; I – *Vhl* intron; pA – polyadenylation site; P2A – porcine teschovirus 2A peptide; SA = Splice Acceptor.

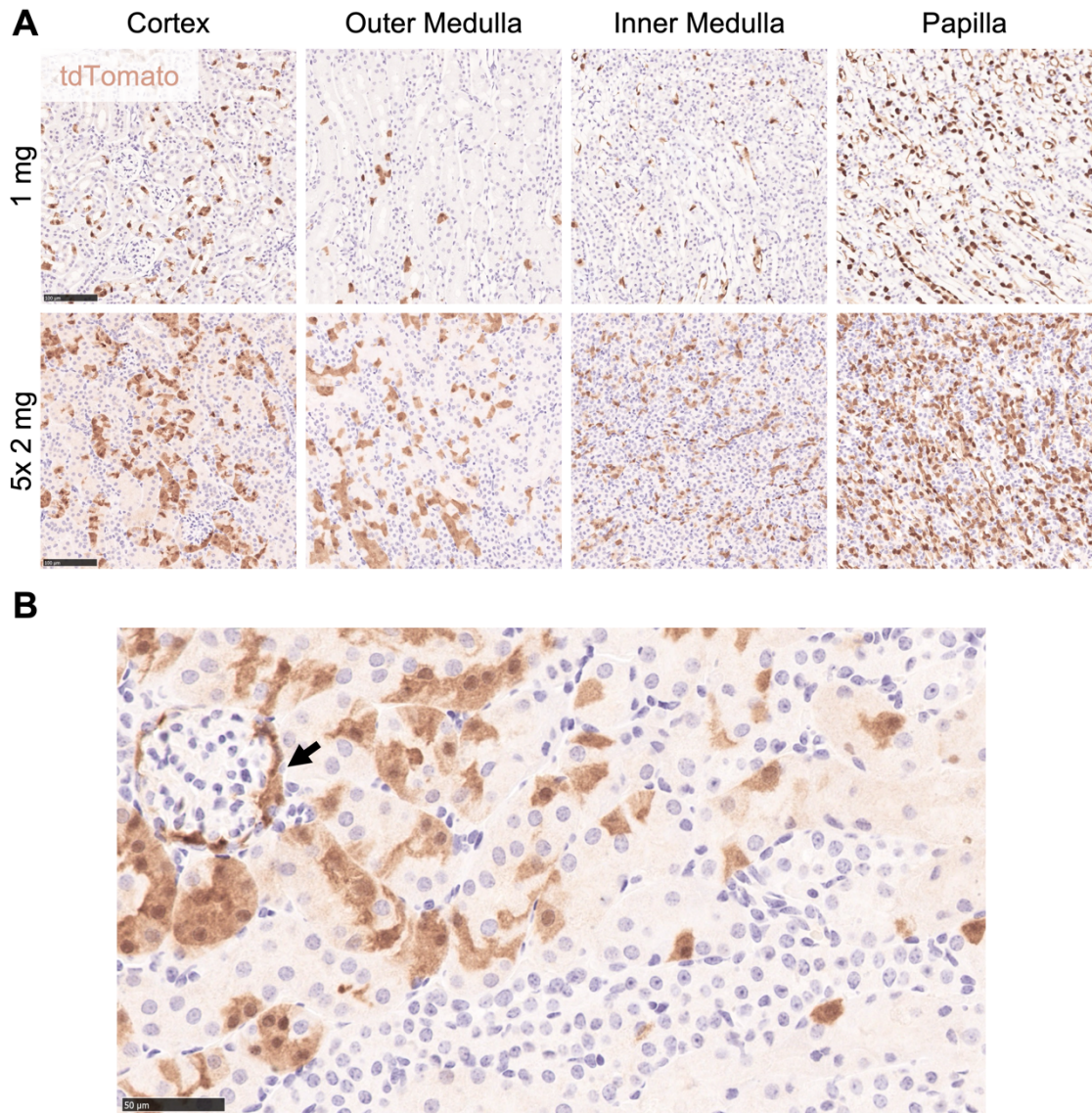


Figure 2.6: TMX-dependent recombination in kidney with *Pax8-CreERT2*. (A) Representative tdTomato immunohistochemistry (IHC) in different anatomical regions of kidneys of *Vhl*^{wt/pjr.fl}; *Pax8-CreERT2* mice given 1 mg or 5x 2 mg TMX harvested one week after first dose. Scale bar denotes 100 μm. (B) Higher magnification view of tdTomato IHC in renal cortex of *Vhl*^{wt/pjr.fl}; *Pax8-CreERT2* mice given 5x 2 mg TMX and harvested one week after first dose. Black arrow denotes tdTomato-positive parietal cells. Scale bar denotes 50 μm.

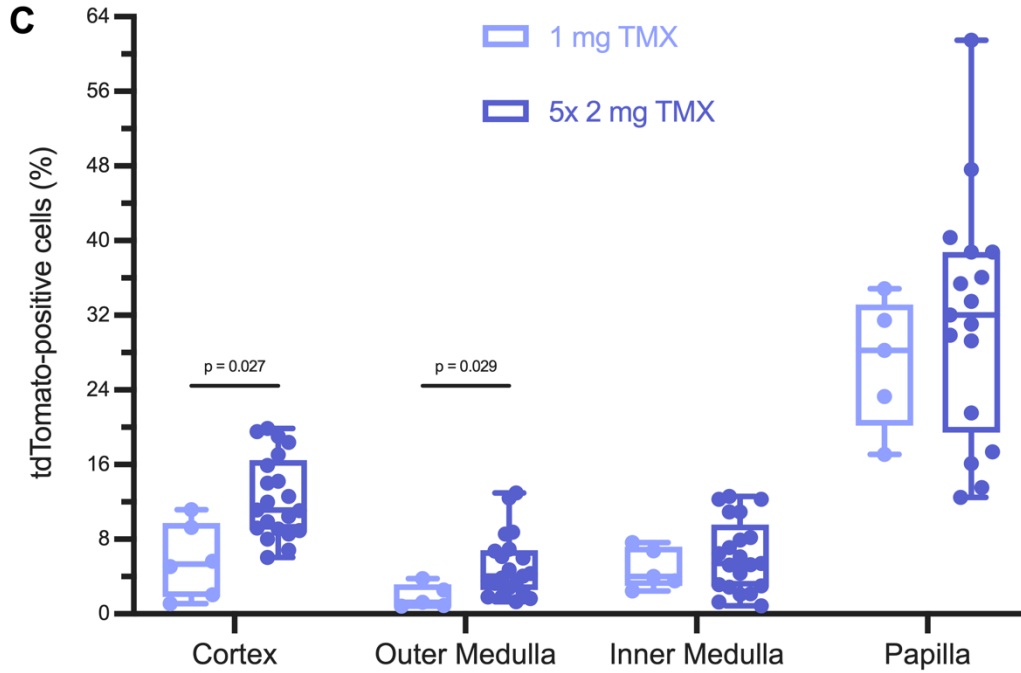


Figure 2.6 (contd.): TMX-dependent recombination in kidney with *Pax8-CreERT2*. (C) tdTomato-positive cells as a proportion of total cells in each renal region as quantified by tdTomato IHC in kidneys of *Vhl^{lwt/pjr.fl}; Pax8-CreERT2* mice given 1 mg (n = 6) or 5x 2 mg TMX (n = 21) and harvested 1-3 weeks after first dose. Median and inter-quartile range plotted. Pair-wise comparisons by multiple Mann-Whitney tests with Holm-Šidák correction. Only significant ($p < 0.05$) comparisons shown.

2.2.3.2 Recombination with *Ubc-CreERT2*

Induction of Cre activity in multiple organs simultaneously has been achieved widely using Cre recombinase alleles under the control of ubiquitously active promoters (e.g., β -actin) that are inserted in a universally accessible region of chromatin. The best characterised such locus is *ROSA26Sor*, but since this is located on the same chromosome as *Vhl* (Chromosome 6), it cannot be crossed with two transgenic *Vhl* alleles as used in the current experimental approach. Hence, in this project, I have induced recombination across multiple organs using a *Ubc-CreERT2* allele that is driven by the ubiquitously active *Ubiquitin C* promoter and is inserted at the *Ndor2* locus on Chromosome 2¹⁹⁰.

I expected this Cre line to be more sensitive to TMX dosing than *Pax8-CreERT2*, owing to its stronger promoter. 1 mg, 0.02 mg, or no TMX was administered to *Vhl^{wt/pjr.fl}; Ubc-CreERT2* mice (n = 8, 4, 3 respectively) by oral gavage and recombination at 1 week after dosing was assayed by tdTomato IHC in the kidney, liver, spleen, heart, brain, lungs, adrenal glands, pancreas, gut, uterus, and testes.

Analysis of mice given no TMX revealed sporadic spontaneous Cre activity in the lung and brain (**Figure 2.7**), as has been reported for *Ubc-CreERT2*¹⁹⁷. Such activity was not observed in the kidney, liver, spleen, adrenal glands, and the pancreas in the absence of tamoxifen dosing.

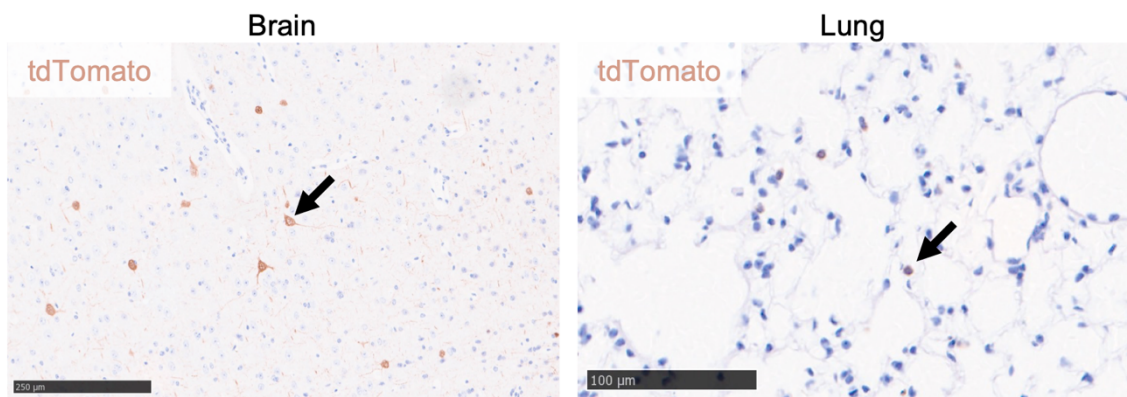


Figure 2.7: Spontaneous *Vhl^{pjr}* recombination by *Ubc-CreERT2* in the brain and lung. Representative tdTomato IHC in brains and lungs of *Vhl^{wt/pjr.fl}; Ubc-CreERT2* (n = 3) mice given no TMX. Black arrows denote tdTomato-positive cells. Scale bar denotes 250 μ m for brain, 100 μ m for lung.

The 1 mg TMX dose generated consistent and extensive recombination in the kidney, liver, and the spleen (**Figure 2.8A**). While most tdTomato-positive cells in the kidney were in the RTE, recombination was also evident in the glomerular cells, parietal cells, and renal interstitial cells (**Figure 2.8B**). tdTomato-positive cells were also detected in the adrenal cortex and medulla, and in the pancreatic islets and acini. However, the extent of recombination in these tissues was lower than was observed in the kidney, liver, or spleen.

Recombination induced with the 0.02 mg dose was much more restricted compared with the 1 mg dose. Analysis of tdTomato-positivity in one 4 μ m section from each of $n = 4$ *Vhl*^{wt/pjrr.fl}; *Ubc-CreERT2* mice given 0.02 mg TMX harvested one week after dosing revealed a total of 0, 0, and 4 tdTomato-positive cells in the spleen, adrenal gland, and pancreas respectively (**Figure 2.9A**). In the kidney, tdTomato-positive cells could be detected in all renal regions (**Figure 2.9B**), but with much reduced frequency than was observed with 1 mg TMX. For instance, in the cortex, 1 mg TMX induced recombination in $44.1\% \pm 4.8\%$ (95% CI) cells; in comparison, 0.02 mg TMX induced recombination only in $0.25\% \pm 0.25\%$ (95% CI) of cells. This amounted to an average of 175 tdTomato-positive cells among an average 80,535 total cortical cells counted per section (**Figure 2.9C**). Rare singlet tdTomato-positive hepatocytes could be observed in livers at this dose (**Figure 2.9D**). These made up $0.62\% \pm 0.37\%$ (95% CI) of all liver cells quantified, amounting to an average of 460 marked cells among 69,703 total liver cells in one section which were significantly ($p = 0.02$) fewer than with the 1 mg dose (**Figure 2.9E**). In all cases, recombination was observed to be consistent across 8 sections cut at 50 μ m intervals through the same tissue block, indicating that recombination was evenly induced across each organ. However, at this dose the recombination induced was much less precise than with 1 mg TMX between different animals, as reflected in the higher coefficients of variation in recombination frequency in the kidney at 0.02 mg than at 1 mg (**Figure 2.9F**).

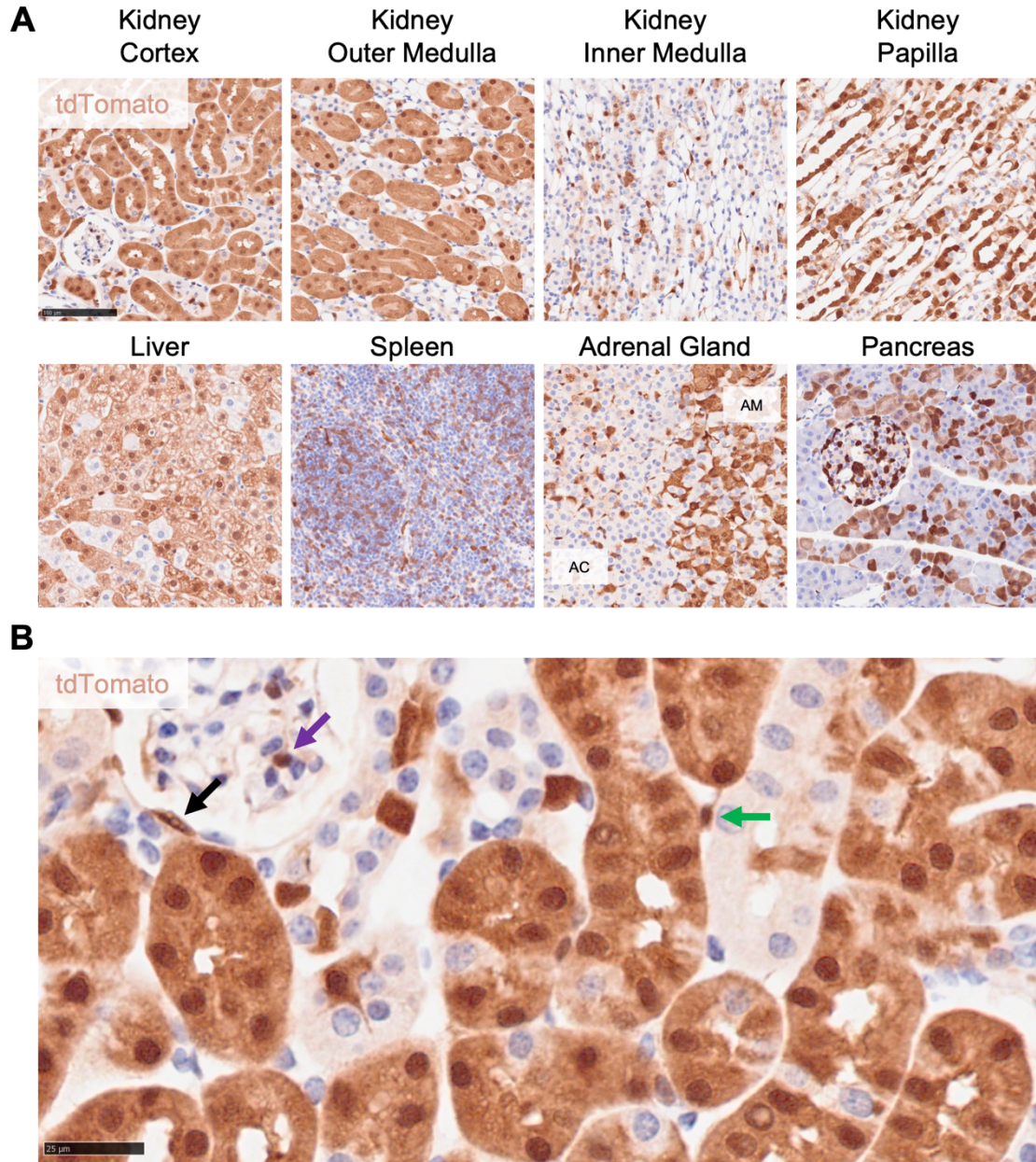


Figure 2.8: Simultaneous recombination across multiple organs with *Ubc-CreERT2* and 1 mg TMX dose. (A) Representative tdTomato IHC in kidneys, livers, spleens, adrenal glands, and pancreas of *Vhl^{wt/pjr.fl}; Ubc-CreERT2* mice (n = 8; n = 6 for papilla) given 1 mg TMX and harvested one week after dosing. AM – adrenal medulla; AC – adrenal cortex. Scale bar denotes 100 μ m. (B) Higher magnification view of tdTomato IHC in renal cortex of *Vhl^{wt/pjr.fl}; Ubc-CreERT2* mice (n = 8) given 1 mg TMX and harvested one week after dosing. tdTomato-positive parietal cells (black arrow), glomerular cell (purple arrow), and renal interstitial cell (green arrow). Scale bar denotes 25 μ m.

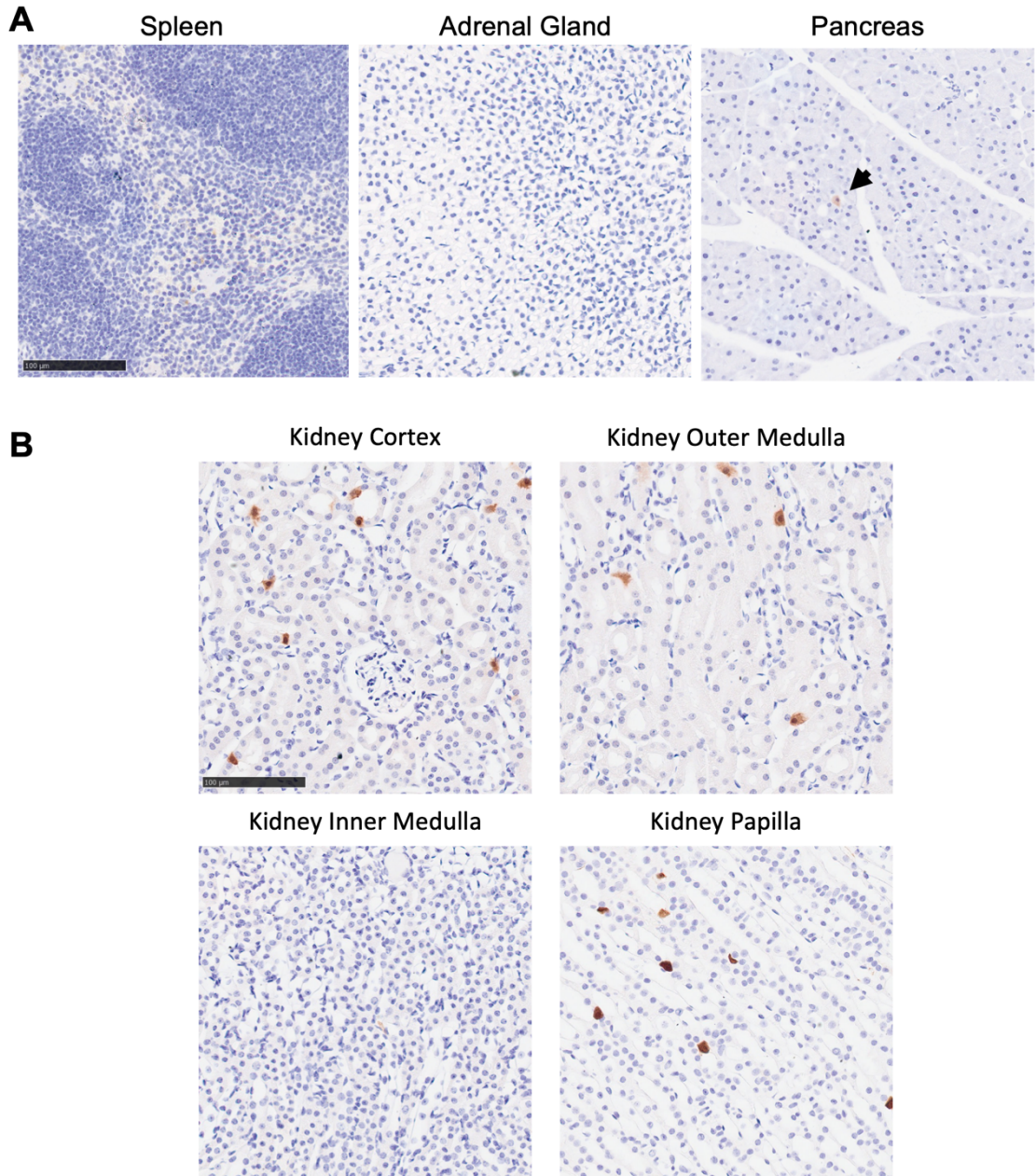


Figure 2.9: Simultaneous recombination across multiple organs with *Ubc-CreERT2* and 0.02 mg TMX dose. (A) Representative tdTomato IHC in spleens, adrenal glands, and pancreas of *Vhl^{wt/pjr.fl}; Ubc-CreERT2* mice given 0.02 mg TMX and harvested one week after first dose (n = 4). tdTomato-positive cell denoted by black arrow. Scale bar denotes 100 μm. (B) Representative tdTomato IHC in different anatomical regions of kidneys of *Vhl^{wt/pjr.fl}; Ubc-CreERT2* mice given 0.02 mg TMX and harvested one week after first dose (n = 4; n = 2 for papilla). Scale bar denotes 100 μm.

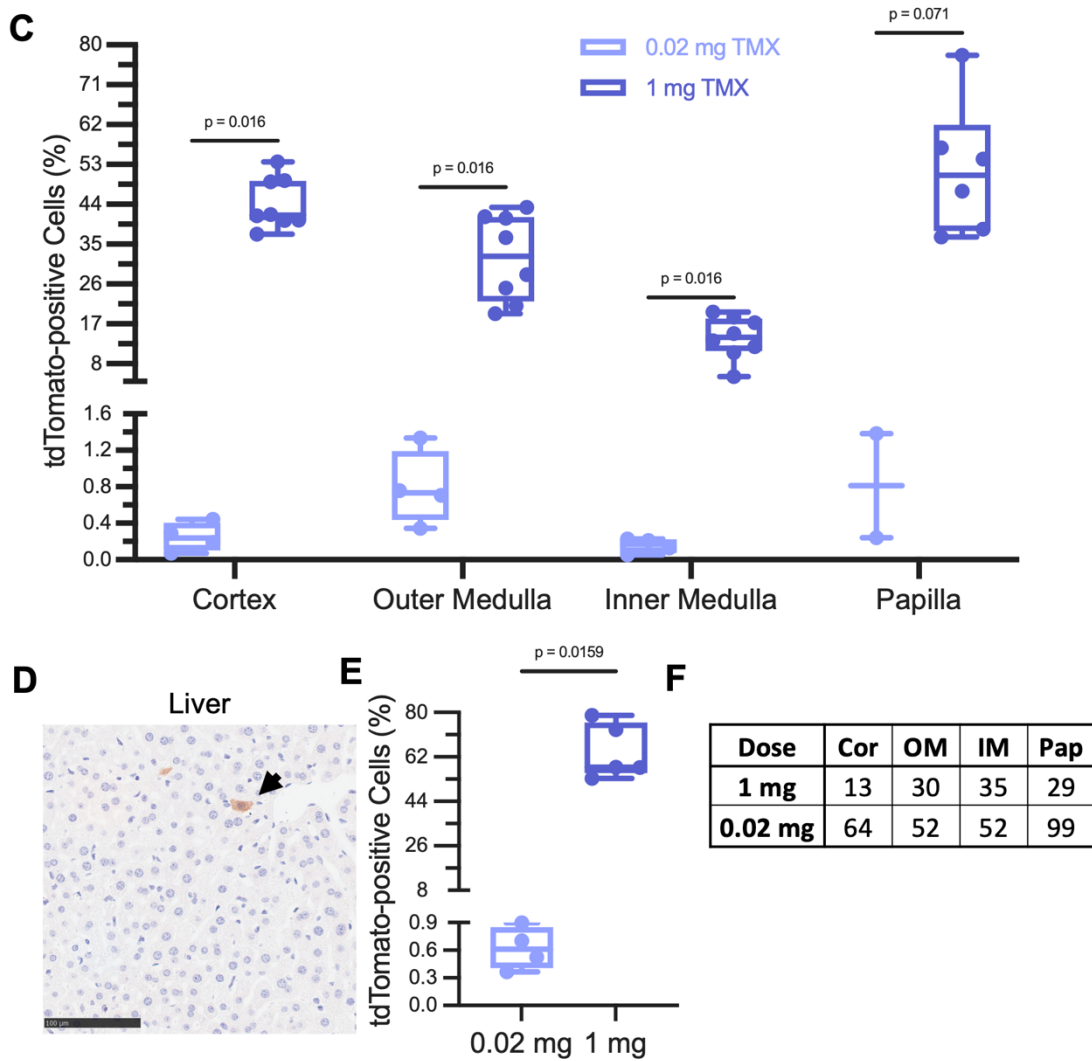


Figure 2.9 (contd.): Simultaneous recombination across multiple organs with *Ubc-CreERT2* and 0.02 mg TMX dose. (C) tdTomato-positive cells as a proportion of total cells in each renal region calculated by quantification of tdTomato IHC in kidneys of *Vhl*^{wt/pjr.fl}; *Ubc-CreERT2* mice given 0.02 mg (n = 4; n = 2 for papilla) or 1 mg TMX (n = 8; n = 6 for papilla) and harvested 1 week after dosing. Median and inter-quartile range plotted. Pairwise comparisons by multiple Mann-Whitney tests with Holm-Šidák correction. (D) Representative tdTomato IHC in livers of *Vhl*^{wt/pjr.fl}; *Ubc-CreERT2* mice given 0.02 mg TMX and harvested 1 week after dosing. Scale bar denotes 100 μm. Black arrow denotes a tdTomato-positive cell. (E) tdTomato-positive cells as a proportion of total cells in livers of *Vhl*^{wt/pjr.fl}; *Ubc-CreERT2* mice given 0.02 mg (n = 4) or 1 mg (n = 5) TMX and harvested 1 week after dosing. Median and inter-quartile range plotted. (F) Coefficient of Variation (%) of recombination frequency quantified in different renal regions in (C). Cor = Cortex, OM = Outer Medulla, IM = Inner Medulla, Pap = Papilla.

Taken together, the 1 mg TMX was considered appropriate for inducing *Vhl*^{pjr} recombination simultaneously and precisely in multiple organs. The 0.02 mg TMX appeared suitable for inducing very rare instances of recombination that better mimic mutagenic profiles in cancer. However, the high coefficients of variation observed might underpower direct quantitative comparisons of tdTomato-positivity between conditions.

2.2.4 Marked biallelic *Vhl* inactivation and marked 'control'

The extent and pattern of recombination induced can differ significantly between tissues, or regions within the same tissue, even at the same tamoxifen dose. This has been demonstrated above with data from *Vhl*^{wt/pjr.fl} animals. Any study on the survival and proliferation of *Vhl*-null cells across contexts and conditions will have to account for these baseline differences in the induction of recombination, as well as in the behaviour of distinct cell types and cell states that are targeted by Cre recombinase alleles expressed under different promoters and activated by different doses of TMX. I have coupled the *Vhl*^{pjr.fl} allele with a constitutively knocked-out, unmarked *Vhl*^{jae.KO} allele (see **Chapter 8**). Recombination in these *Vhl*^{jae.KO/pjr.fl} mice is expected to produce marked *Vhl*-null cells with the same frequency and pattern as seen with *Vhl*^{wt/pjr.fl} mice (**Figure 2.10**). Comparison between marked cells, induced in the same tissue with the same Cre alleles activated by the same TMX dose, in tissues from *Vhl*^{jae.KO/pjr.fl} and *Vhl*^{wt/pjr.fl} mice can thus shed insight on the specific effects of *Vhl* inactivation in particular contexts.

I validated this experimental approach first by establishing that equal recombination is induced in both genotypes with the same Cre and TMX dose; **Figure 2.11A** exemplifies this for the kidney with *Pax8-CreERT2* and 5x 2 mg TMX harvested one week after the first dose. tdTomato-positive and -negative cells were then isolated by FACS from these kidneys and assayed for HIF1A and HIF2A stabilisation by immunoblotting. tdTomato protein was detected in both genotypes but only in cells sorted as tdTomato-positive. HIF1A and HIF2A were detected only in tdTomato-positive cells from *Vhl*^{jae.KO/pjr.fl}; *Pax8-CreERT2* mice

(Figure 2.11B). This confirmed that marked cells in *Vhl^{jae.KO/pjr.fl}; Pax8-CreERT2* mice were indeed *Vhl*-null, and that the equivalent population in *Vhl^{wt/pjr.fl}; Pax8-CreERT2* mice was *Vhl*-competent.

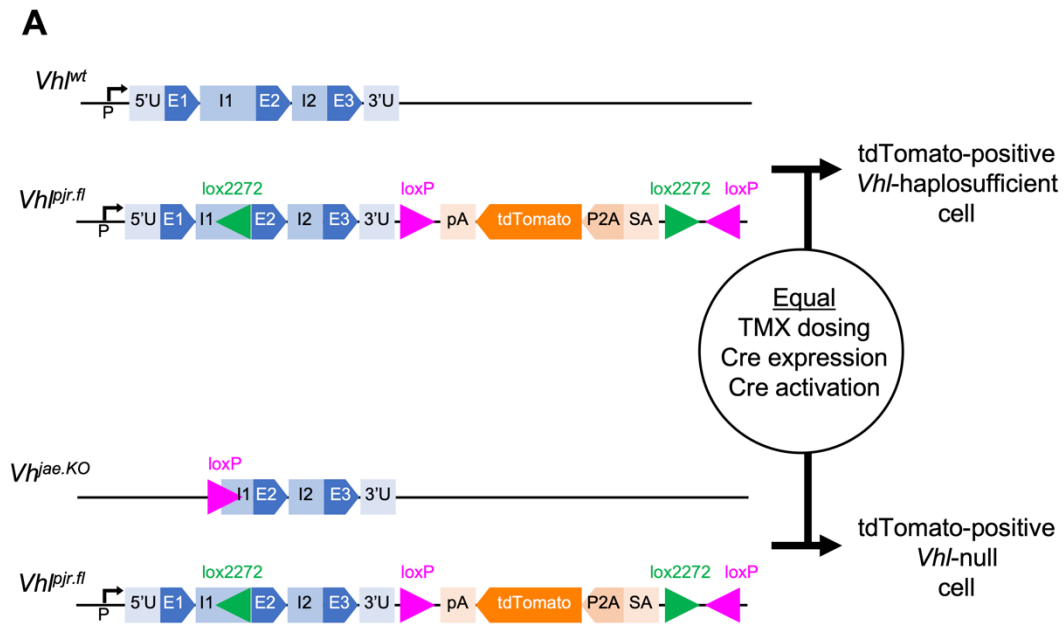


Figure 2.10: Marked biallelic *Vhl* inactivation with marked ‘control’. (A) Schematic depicting coupling of *Vhl^{pjr.fl}* with *Vhl^{wt}* or *Vhl^{jae.KO}* alleles to generate tdTomato-positive *Vhl*-haplosufficient or *Vhl*-null cells respectively. P – *Vhl* promoter; U – untranslated region; E – *Vhl* exon; I – *Vhl* intron; pA – polyadenylation site; P2A – porcine teschovirus 2A peptide; SA – Splice Acceptor.

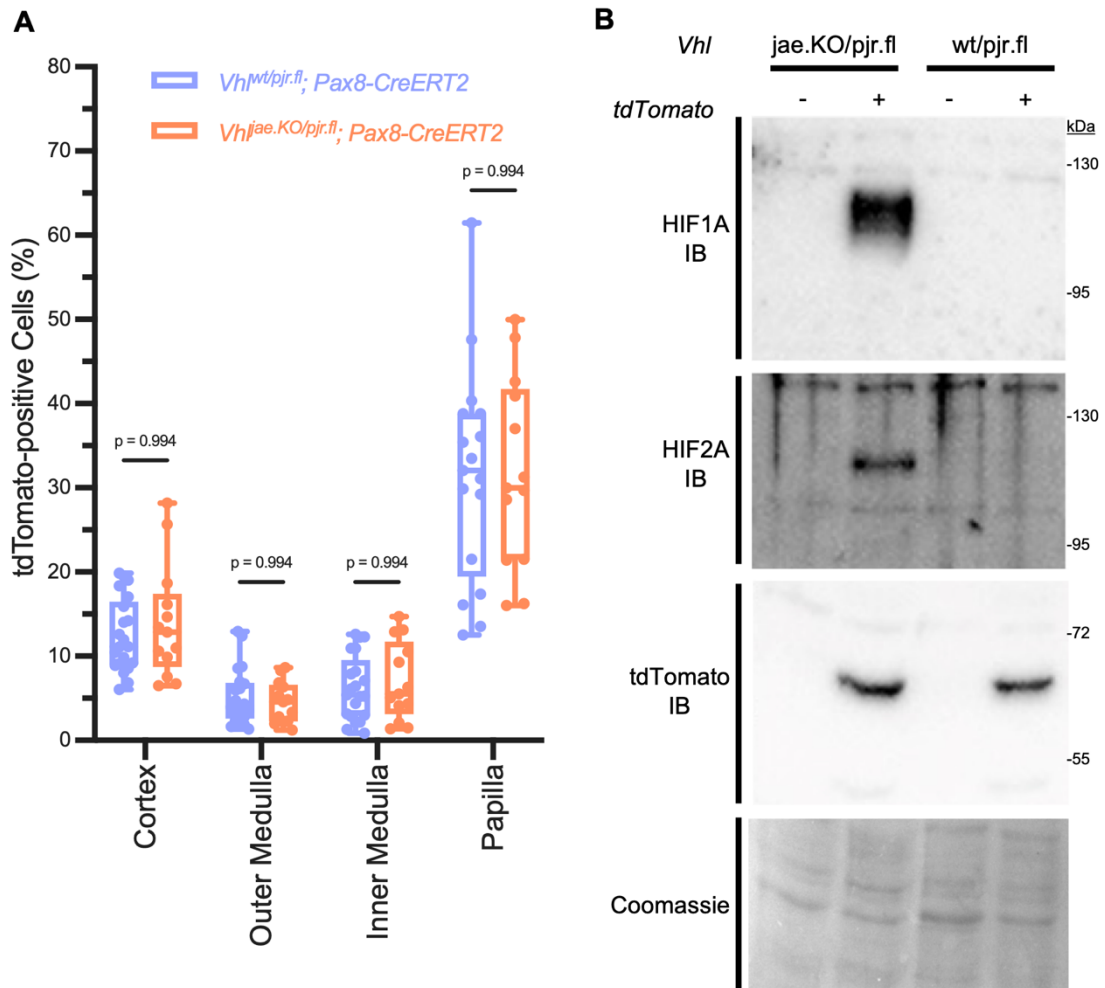


Figure 2.11: tdTomato-positive cells in *Vhl*^{jae.KO/pjr.fl} mice are *Vhl*-null. (A) tdTomato-positive cells as a proportion of total cells in each renal region calculated by quantification of tdTomato IHC in kidneys of *Vhl*^{wt/pjr.fl}; *Pax8-CreERT2* (n = 21) or *Vhl*^{jae.KO/pjr.fl}; *Pax8-CreERT2* (n = 14) mice given 5x 2 mg TMX and harvested 1-3 weeks after first dose. Median and inter-quartile range plotted. Pair-wise comparisons by multiple Mann-Whitney tests with Holm-Šídák correction. (B) Representative immunoblots for HIF1A, HIF2A, or tdTomato protein in tdTomato-positive (+) or tdTomato-negative (-) cells sorted by flow cytometry from dissociated kidneys of *Vhl*^{jae.KO/pjr.fl}; *Pax8-CreERT2* or *Vhl*^{wt/pjr.fl}; *Pax8-CreERT2* mice given 5x 2 mg TMX and harvested one week after first dose. Immunoblotting performed by Dr Norma Masson.

2.3 Discussion

Here, I have described a new *Vhl* allele in which Cre-mediated excision of *Vhl* is faithfully coupled to the insertion of a tdTomato fluorescent reporter gene (**Figures 2.1-2.2**). I have validated the design and function of this allele by genomic PCR, immunostaining, and flow cytometry (**Figures 2.3-2.5**). I have then described the utility of this allele to controllably induce recombination and assay recombined cells across multiple organs simultaneously, or specifically in the kidney using inducible Cre recombinase alleles activated by TMX dosing by oral gavage (**Figures 2.6-2.9**). Finally, I have established an approach by which the effects of *Vhl* inactivation, or fates of *Vhl*-null cells can be assayed against baseline differences in the numbers and types of cells capable of recombination with a given Cre and TMX dose in various tissue contexts (**Figures 2.10 and 2.11**). The ability to accurately mark recombined cells has yielded insights into the efficiency of inducible Cre systems, as well as provided a novel framework for studying *Vhl* inactivation accurately *in vivo*.

The development of this model was guided by two specific aims – 1) cells with *Vhl* inactivation be accurately and comprehensively identified in tissue, and 2) such *Vhl* inactivation be induced with total precision in space and time. The data presented in this chapter indicates that my model achieves both aims but with certain caveats.

2.3.1 Accurate reporting of *Vhl* inactivation

Mouse models in which recombination of a gene of interest is ‘reported’ by the expression of a fluorescent marker have been developed and described in the literature¹⁷⁴. Almost all of these employ a ‘lox-STOP-lox’ reporter allele. In such alleles, a β -galactosidase or fluorescent reporter gene is placed downstream of a floxed ‘stop’ codon; the reporter can be translated only after Cre-mediated excision of the ‘STOP’ codon. These alleles are in essence, reporters of Cre-activity in any given cell, and not specifically of recombination of any gene of interest.

The *Vhl^{pjr}* allele, on the other hand, is a true reporter of *Vhl* recombination. This is because it not only carries the ‘reporter’ and ‘gene of interest’ elements *in cis*, but also links *Vhl* knock-out and tdTomato knock-in in the structural mechanism of its recombination (**Figure 2.2**). The ability to mark *Vhl*-recombined cells using a single allele is also what allows marked *Vhl*-null or *Vhl*-het cells to be produced simply by coupling *Vhl^{pjr}* with unmarked *Vhl^{wt}* or *Vhl^{jae.KO}* alleles (**Figure 2.10**).

Thus, by design, all tdTomato-positive cells arising from *Vhl^{pjr}* recombination must have knocked-out that copy of *Vhl*. This is supported at the genomic level (**Figure 2.5**) and indirectly on the basis of HIF stabilisation (**Figure 2.10**). However, the converse is not necessarily true – not all tdTomato-negative FAC-sorted cells had an intact copy of *Vhl*. This is most likely a result of inaccurate FAC-sorting – weak tdTomato expression driven by the endogenous *Vhl* promoter possibly allows some recombined cells to not pass gating thresholds for tdTomato fluorescence intensity set during flow cytometry. Thus, while lineage marking in my model is accurate, it is possibly not comprehensive.

2.3.2 Spatiotemporal precision in recombination

TMX-inducible Cre recombinases allow for recombination to be induced at a defined timepoint and events following recombination to be assayed in real time. The utility of these inducible Cre-recombinase systems can be confounded by their spontaneous activation in tissue in the absence of TMX administration¹⁹⁷. Such activity was not detected in any assayed tissues with *Pax8-CreERT2*. However, I observed tdTomato-positive cells in the brains and lungs of *Vhl^{wt/pjr.fl}; Ubc-CreERT2* mice in the absence of TMX dosing, indicating that the *Ubc-CreERT2* had facilitated *Vhl^{pjr}* recombination in some cells of these tissues spontaneously (**Figure 2.7**). These tissues are thus best excluded from any temporal analysis of marked cells with *Ubc-CreERT2*.

For both Cre lines, I tested a ‘high’ and a ‘low’ tamoxifen dose. The ‘high’ doses corresponded to dosing regimens used for the Cre lines in literature^{109,198}. The ‘low’ doses

were tested to see if recombination can be induced in only a few cells without affecting the *Vhl* status in most cells of the tissue. Such rare induction of recombination might better mimic the relatively low frequencies of cells harbouring somatic mutations in normal tissues^{14,70}. As expected, I found that different tissues exhibited different levels of recombination in response to activation of the same Cre recombinase with a given of TMX, suggesting tissue-dependent expression of Cre recombinase or distribution of TMX (or its active form 4-OHT). The ‘high’ dose tested for both Cre recombinase alleles generated recombination with relatively low variation between organisms and genotypes, making it suitable for further analyses (**Figures 2.6, 2.8 and 2.9**). Recombination with the ‘low’ doses was more complicated. With *Pax8-CreERT2*, lowering TMX dose by 90% had surprisingly little effect on total recombination frequencies in the kidney; furthermore, any such reduction was accompanied by increased variation between animals. However, most tdTomato-positive cells in the cortex with the low dose were ‘singlets’, unlike with the high dose, where they were often surrounded by tdTomato-positive neighbours. This distinction might provide a means to study paracrine signalling effects of *Vhl* inactivation. With *Ubc-CreERT2*, there was a dramatic difference in recombination induced between the high and low doses. The high dose makes simultaneous study of the kidney, liver, spleen, pancreas, and adrenal gland possible, but is susceptible to systemic effects of *Vhl* inactivation caused by widespread recombination that might impact fates of marked cells. The low dose induces ‘scattered’ recombination in the kidney and liver in a manner better resembling what might be expected by ‘random’ somatic mutagenesis in pre-cancerous tissues. However, it fails to recombine *Vhl^{ppir}* in other organs. The very low levels of recombination also present a practical challenge in isolating marked cells. My protocol for flow cytometry (see **Chapter 8**) allows me to isolate ~100,000 tdTomato-positive cells from kidneys with 10%-20% of dissociated cells being tdTomato-positive in 20-30 minutes. Doing so for kidneys with <0.5% tdTomato-positive cells will take several hours, which might stress or kill the cells before analysis.

Qualitative and quantitative assessment of tdTomato-positivity performed in the chapter has demonstrated that recombination with inducible Cre systems is non-uniform and incongruent across Cre lines and TMX doses. Studies that rely on histological or bulk analysis of tissues subjected to such recombination are confounded by this non-uniformity, especially if recombined cells cannot be identified or isolated. The lineage-marking approach developed in this project provides a solution to this problem.

Altogether, the mouse model developed, and the dosing regimens tested here provide a way of assessing fates of *Vhl*-null cells over time and examining the effects of *Vhl* inactivation against baseline cell-type specific differences *in situ* and *ex vivo*.

Chapter 3: Assessing Cell Fates

Following *Vhl* Inactivation in Multiple Organs

3.1 Introduction

Tumorigenesis associated with biallelic *VHL* inactivation is highly tissue-specific. Inheritance of one defective *VHL* allele in VHL disease is associated with increased susceptibility to clear cell renal cell carcinoma (ccRCC) and CNS haemangioblastoma but very rarely other malignancies⁶⁴. Conversely, genomic sequencing across common sporadic cancer types has shown *VHL* to be mutated specifically in ccRCC compared to other cancers (<https://portal.gdc.cancer.gov/genes/ENSG00000134086>; accessed on 09 August 2023)⁷⁵. VHL is best characterised as the recognition component of a ubiquitin E3 ligase complex that targets hypoxia inducible transcription factors (HIF1A and HIF2A) for conditional oxygen-dependent degradation³⁴; consequently, *VHL* loss stabilises HIF and is expected to entrain HIF-driven gene expression in cells¹⁴⁵. The function of VHL is conserved across cell types and species^{199,200}, which makes tissue-specificity of *VHL*-associated tumorigenesis intriguing.

Missense and nonsense mutations in oncogenes and tumour suppressor genes respectively are detected in normal tissues at higher rates than is expected from random mutagenesis caused by errors in DNA replication or exposure to environmental and chemical mutagens²⁰¹, indicating that cells with these mutations are specifically over-represented in tissues. This ‘positive selection’ of oncogenic mutations is thought to arise from survival advantages conferred on cells by these mutations that results in the expansion of their population in tissue²⁰¹. Such expansion amplifies the probability that these cells incur successive mutations necessary for transformation and oncogenesis^{14,202}. It has been

hypothesised that tissue-specific tumorigenesis might arise because survival advantages imparted on cells in normal tissue by mutations are tissue-specific – biallelic *VHL* mutation may confer a survival advantage to cells in the renal cortex (where ccRCC occurs), but not in the liver where *VHL*-associated cancers are not found. Negative selection, wherein cells incurring a cancer-associated mutation are removed from normal tissues, may also contribute to tissue-specific tumorigenesis. In such cases, a mutation might negatively impact cell survival in tissues that do not exhibit cancers associated with that mutation; the population of mutated cells would then be lost from those tissues, thereby preventing oncogenesis. However, underrepresentation of mutations is difficult to detect, especially compared to detecting overrepresentation if the total number of naturally-occurring mutations is in itself quite low¹⁴. Whether positive or negative cell selection impacts on the tissue-specific survival and expansion of cells harbouring *VHL* inactivation remains to be elucidated.

The novel reporter model, described in **Chapter 2**, allows cells that have undergone *Vhl* recombination to be identified and quantified *in situ* based on the expression of a tdTomato reporter. Recombined cells are either *Vhl*-null or *Vhl*-competent, depending on whether mice are *Vhl*^{fl^{ae}.KO/pjr.fl} or *Vhl*^{wt/pjr.fl} respectively. If a given Cre recombinase allele activated by a given tamoxifen (TMX) dose induces recombination equally (supported by data in **Chapter 2**), comparison of the number of tdTomato-positive cells in tissues from mice of the two genotypes should inform on cell fates entrained specifically by biallelic *Vhl* inactivation.

In this chapter, I test and validate this premise to explore tissue-specific survival phenotypes entrained by biallelic *Vhl* inactivation in multiple organs using the reporter mouse model. *Ubc-CreERT2* is used to generate recombined cells in multiple organs of *Vhl*^{fl^{ae}.KO/pjr.fl}; *Ubc-CreERT2* (“KO”) and *Vhl*^{wt/pjr.fl}; *Ubc-CreERT2* (“Control”) mice. The numbers and fates of marked cells in KO mice (that are *Vhl*-null) are compared to those from Control mice (that are *Vhl*-competent) and selective processes inferred. These analyses are corroborated by

orthogonal approaches to infer the presence of cell death and proliferative processes active in different tissues. Systemic pathologies associated with widespread *Vhl* inactivation are described and their impact on the utility of this experimental system discussed. Finally, evidence that the cellular outcomes of *Vhl* inactivation may depend on the extent of recombination induced is examined.

3.2 Results

3.2.1 Systemic effects of widespread *Vhl* inactivation in mice

Vhl^{wt/pjr.fl}; *Ubc-CreERT2* (“Control”) and *Vhl*^{jae.KO/pjr.fl}; *Ubc-CreERT2* (“KO”) mice were given 1 mg TMX (dose optimised in **Chapter 2**) with the intention of harvesting tissues either 1 week after dosing (for an “early” timepoint) or at intervals spanning 4 weeks to 12 months after dosing (for “late” timepoints) to compare time- and genotype-dependent changes in the number of tdTomato-positive cells in different organs. However, all KO mice assigned for the late timepoints developed skin erythema between the 2nd and 3rd week after dosing (n = 15) which was noticeable on the limbs, ears, and snout (**Figure 3.1A**). KO mice also developed splenomegaly (**Figure 3.1B**) starting at 1 week after dosing (n = 8 at 1-week; 15 at 3-5 weeks) and suffered from polycythaemia that exacerbated between 1 week and 3-5 weeks after TMX dosing (**Figure 3.1C**). Associated with these pathologies, KO mice developed a hunched posture and a ruffled coat. Consequently, KO mice had to be culled by 5 weeks after dosing to comply with the conditions of the mouse procedural licence. These findings highlight the limitations of conducting studies with widespread *Vhl* inactivation. Notwithstanding these pathologies, examination of tissues from these mice has revealed insights into both, the effects of *Vhl* inactivation on the survival of cells in tissues and the ability to analyse these using my experimental system and are described below.

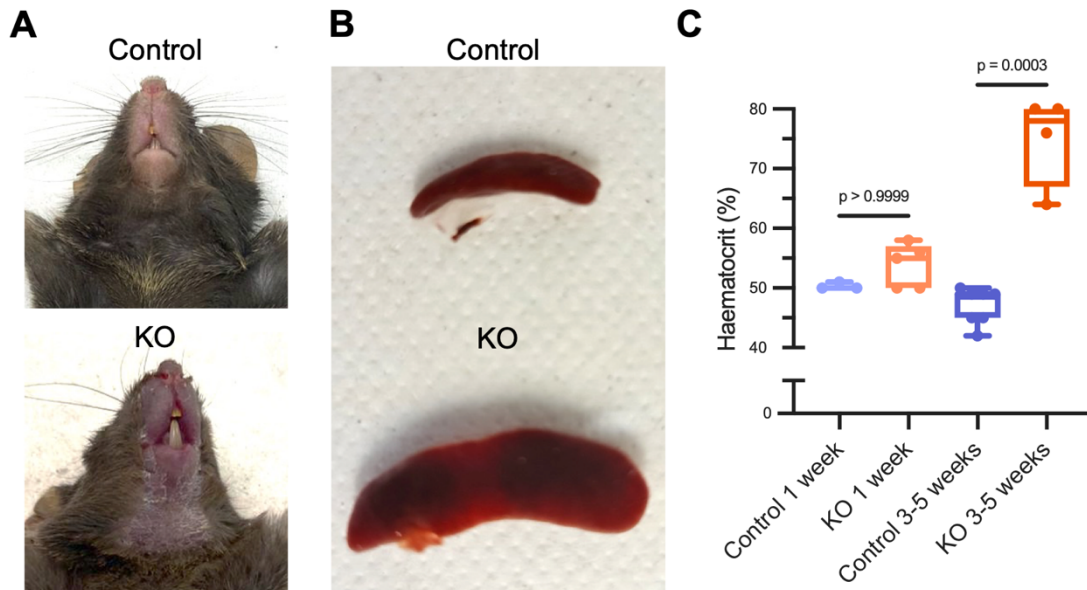


Figure 3.1: Systemic effects of widespread multi-organ *Vhl* inactivation. (A) Representative photographs depicting skin erythema in snouts of KO mice given 1 mg TMX and harvested 3-5 weeks after dosing compared to snouts of Control mice. (B) Representative photographs depicting enlarged spleens of KO mice compared to those from Control mice given 1 mg TMX harvested 3-5 weeks after dosing. (C) Haematocrits of Control or KO mice given 1 mg TMX and harvested 1 or 3-5 weeks after dosing (n = 3, 5, 7, 4 for Control 1 week, KO 1 week, Control 3-5 weeks and KO 3-5 weeks respectively). Median and inter-quartile range plotted. Pairwise comparisons by Kruskal-Wallis test with Dunn's correction.

3.2.2 *Vhl*-null cells are lost from multiple organs over 5 weeks following recombination

3.2.2.1 Time-dependent decrease in numbers of tdTomato-positive cells in organs from KO but not Control mice

To test if biallelic *Vhl* inactivation had an effect on the survival of cells in tissues, I compared by tdTomato IHC the number and distribution of tdTomato-positive cells in kidneys, livers, pancreas, spleens, and adrenal glands of Control and KO mice given 1 mg TMX and harvested 3-5 weeks after dosing. The four renal regions of the kidney – cortex, outer medulla, inner medulla, and papilla – were analysed separately as they had shown region-specific induction of recombination in response to the same TMX dose (**Chapter 2**).

Remarkably fewer tdTomato-positive cells were observed in all assayed organs from KO mice ($n = 9, 7, 3, 3, 5$ for kidney (all regions), liver, pancreas, adrenal glands, spleen respectively) compared to those from Control mice ($n = 9, 11, 3, 3, 5$ for kidney (all regions), liver, pancreas, adrenal glands, spleen respectively) (**Figure 3.2**). This reduction was observed in all regions of the kidney, though it was less prominent in the papilla compared to the cortex (**Figure 3.2**). Both, the adrenal medulla, and adrenal cortex exhibited this reduction (**Figure 3.2**) as did both pancreatic islets and acinar cells.

To confirm that the lower number of tdTomato-positive cells in organs from KO mice was significant, and that it did not arise from differences in initial recombination, I quantified the proportion of cells in a given tissue that stained positive for tdTomato by IHC using HALO (see **Chapter 8**; used in **Chapter 2**) in Control and KO mice given 1 mg TMX and harvested at different intervals between 1 and 5 weeks after dosing. This and subsequent analyses were focussed on the kidney and liver as they exemplified the phenotype seen across all organs and exhibited extensive and consistent levels of recombination across animals. At the 1-week timepoint, neither the liver nor any region of the kidney exhibited a significant difference ($p = 0.23, 0.14, 0.22, 0.81, \text{ and } 0.27$ for cortex, outer medulla, inner medulla, papilla, and liver respectively) in the proportion of cells positive for tdTomato between Control and KO mice (**Figure 3.3**). Tissues from KO mice contained progressively fewer tdTomato-positive cells over five weeks after TMX dosing (**Figure 3.3**); this reduction was statistically significant in the cortex ($p < 0.001$), outer medulla ($p = 0.001$), inner medulla ($p < 0.02$), and the liver ($p = 0.03$) but not in the papilla ($p = 0.23$) (**Figure 3.3**). Such a decrease was not observed in any tissue from Control mice (**Figure 3.3**).

Taken together, these observations indicate that the number of tdTomato-positive cells detected by IHC decreases over time in multiple organs of KO mice but not in Control mice.

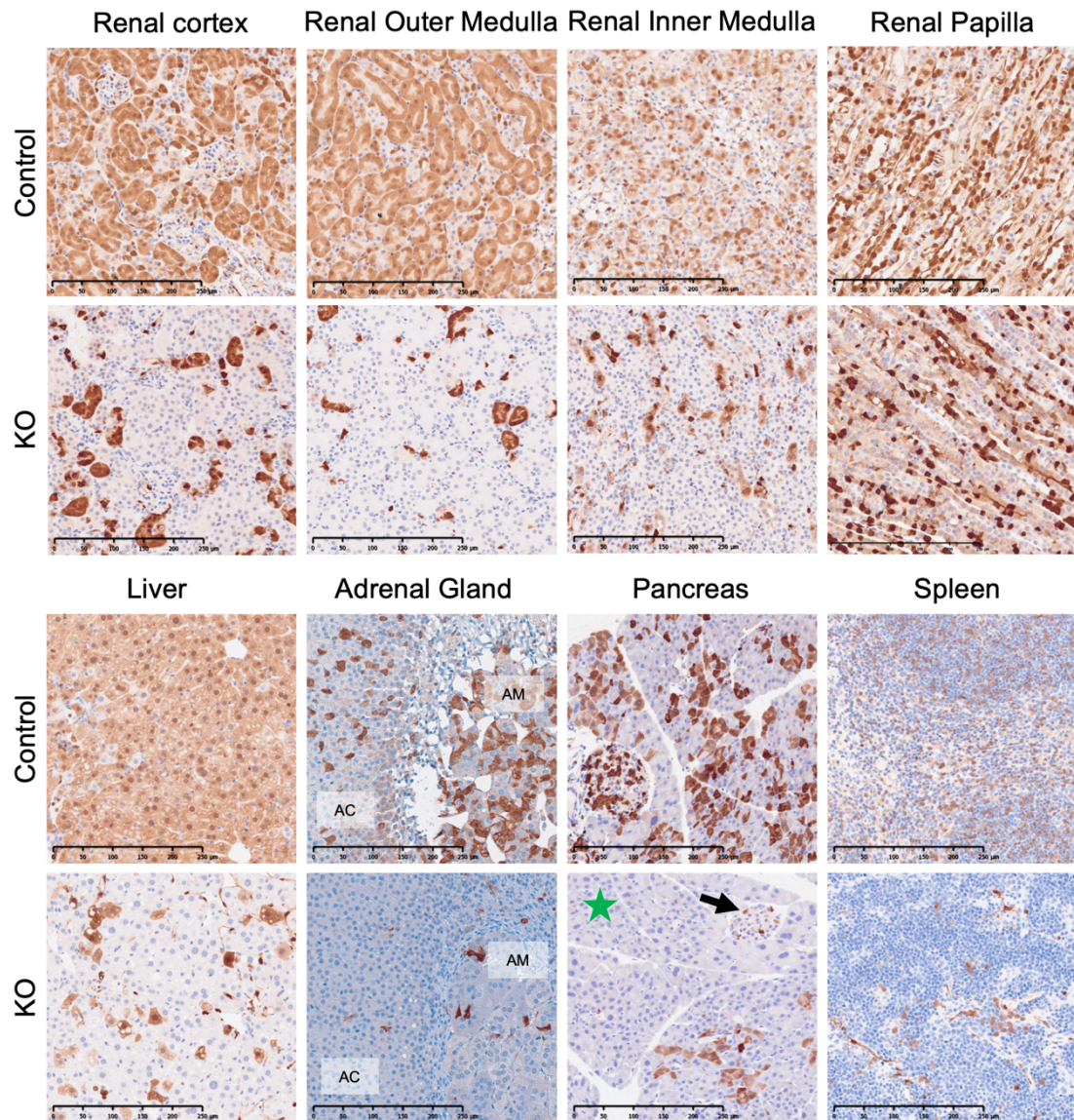


Figure 3.2: Reduced numbers of tdTomato-positive cells in KO kidneys 3-5 weeks after recombination. Representative tdTomato IHC in kidneys (different anatomical regions shown), livers, adrenal glands (medulla and cortex), pancreas (including pancreatic islet), and spleens of Control or KO mice given 1 mg TMX and harvested 3-5 weeks after dosing ($n = 9, 11, 3, 3, 5$ and $n = 9, 7, 3, 3, 5$ for liver, adrenal gland, pancreas, and spleen in Control and KO respectively). Scale bar denotes 250 μm . AC – adrenal cortex, AM – adrenal medulla. Pancreatic acinar cells, and pancreatic islet denoted by green star and black arrow respectively. tdTomato IHC performed in collaboration with Dr Joanna Lima.

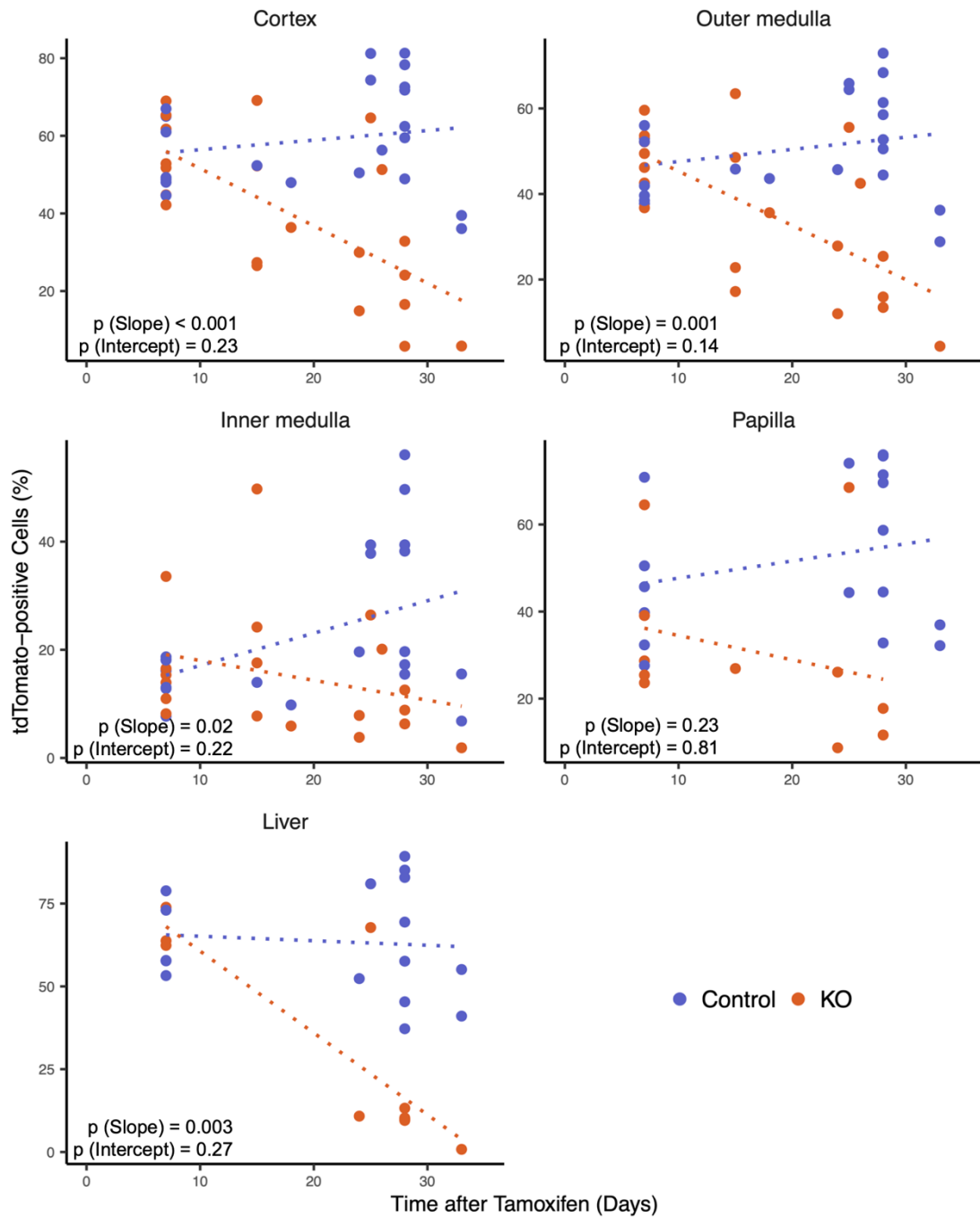


Figure 3.3: Proportion of tdTomato-positive cells decrease in organs from KO mice over time. Proportion of total cells that are tdTomato-positive in the liver and different regions of the kidney in Control or KO mice given 1 mg TMX and harvested at different intervals between 1 and 5 weeks after dosing ($n = 22, 21, 21, 17, 16$ and $n = 21, 20, 20, 12, 10$ for cortex, outer medulla, inner medulla, papilla and liver respectively in Control and KO respectively), as quantified by tdTomato IHC. Dotted lines denote linear regression. Slopes and intercepts of linear regression models compared by *t*-test with Bonferroni correction.

3.2.2.2 Loss of tdTomato-positive cells associates with decreased HIF stabilisation in kidneys from KO mice

The reduced numbers of tdTomato-positive cells likely represented early elimination of cells following biallelic *Vhl* inactivation. However, I wanted to gain further evidence that recombined cells had indeed been lost and that the result did not arise because tdTomato expression was somehow silenced in *Vhl*-null cells present in tissue.

HIF1A and HIF2A, stabilised in cells as a consequence of biallelic *Vhl* inactivation³⁴, were detected by immunoblotting in tdTomato-positive cells, but not in tdTomato-negative cells from kidneys of KO mice (**Chapter 2**). If *Vhl*-null cells were indeed being eliminated from kidneys of KO mice, then the lower levels of tdTomato in these kidneys should have reflected lower levels of stabilised HIF1A and HIF2A proteins. Conversely, a reduction in tdTomato levels without corresponding changes in HIF levels should have indicated tdTomato silencing. This principle was employed to test by immunoblotting whether the reduction in the number of tdTomato-positive cells reflected a reduction in the number of *Vhl*-null cells or was caused by silencing of in kidneys from KO mice.

Immunoblotting for tdTomato, HIF1A, and HIF2A was performed on protein extracts prepared from kidneys from Cre-negative *Vhl*^{wt/pjr.fl} (“Control no Cre”) and *Vhl*^{flae.KO/pjr.fl} (“KO no Cre”) mice, Cre-positive Control (*Vhl*^{wt/pjr.fl}; *Ubc-CreERT2*) mice, and Cre-positive KO (*Vhl*^{flae.KO/pjr.fl}; *Ubc-CreERT2*) mice that exhibited either similar or lower proportions of tdTomato-positive cells compared to kidneys from Control mice as assayed by IHC. As expected, tdTomato was detected in extracts from Control and KO mice, but not from Cre-negative mice of either genotype (**Figure 3.4**). Notably, the intensity of the tdTomato signal by immunoblot closely associated with the number of tdTomato-positive cells detected by IHC performed on the contralateral kidney from the same mice (**Figure 3.4**). Neither isoform of HIF was detected in Control or Cre-negative kidneys by immunoblot. However, HIF1A and HIF2A were detected in kidneys from KO mice. Crucially, the intensities of the HIF signals in extracts were much lower in extracts of kidneys from KO mice exhibiting a lower

amount of tdTomato protein and fewer tdTomato-positive cells than from the KO mouse with high levels of tdTomato and high numbers of tdTomato-positive cells (**Figure 3.4**).

These results demonstrate that the levels of HIFA proteins stabilised are proportional to the total levels of, and the proportions of cells positive for tdTomato in those kidneys. This supports the hypothesis that the loss of tdTomato-positive cells reflects a loss of *Vhl*-null cells from kidneys of KO mice over time.

3.2.2.3 Loss of tdTomato-positivity associates with decreased levels of recombined *Vhl*^{pjr.KO} in kidneys and livers from KO mice

As a further test that the reduction in staining for tdTomato genuinely reflected an elimination of cells, I assayed for the presence of *Vhl*-null cells in tissues from KO mice by genomic analyses. *Vhl* recombination in my experimental system converts the *Vhl*^{pjr.fl} allele to *Vhl*^{pjr.KO} and does not involve the second (*Vhl*^{wt} or *Vhl*^{jae.KO}) *Vhl* allele. As all cells must carry one copy of the ‘second’ *Vhl* allele, but only recombined cells carry one copy of the *Vhl*^{pjr.KO} allele, the amount of *Vhl*^{pjr.KO} alleles relative to the amount of ‘second’ *Vhl* alleles present in a tissue must reflect the proportion of surviving cells that have undergone *Vhl* recombination in that tissue. If *Vhl*-null cells were indeed being eliminated from organs of KO mice, then for the same amount of *Vhl*^{jae.KO} or *Vhl*^{wt} alleles, organs of KO mice should carry less *Vhl*^{pjr.KO} than organs of Control mice. This principle was employed to test by genomic PCR if tissues with fewer tdTomato-positive cells as detected by IHC carried fewer *Vhl*-null cells as assayed by relative abundance of recombined *Vhl* alleles.

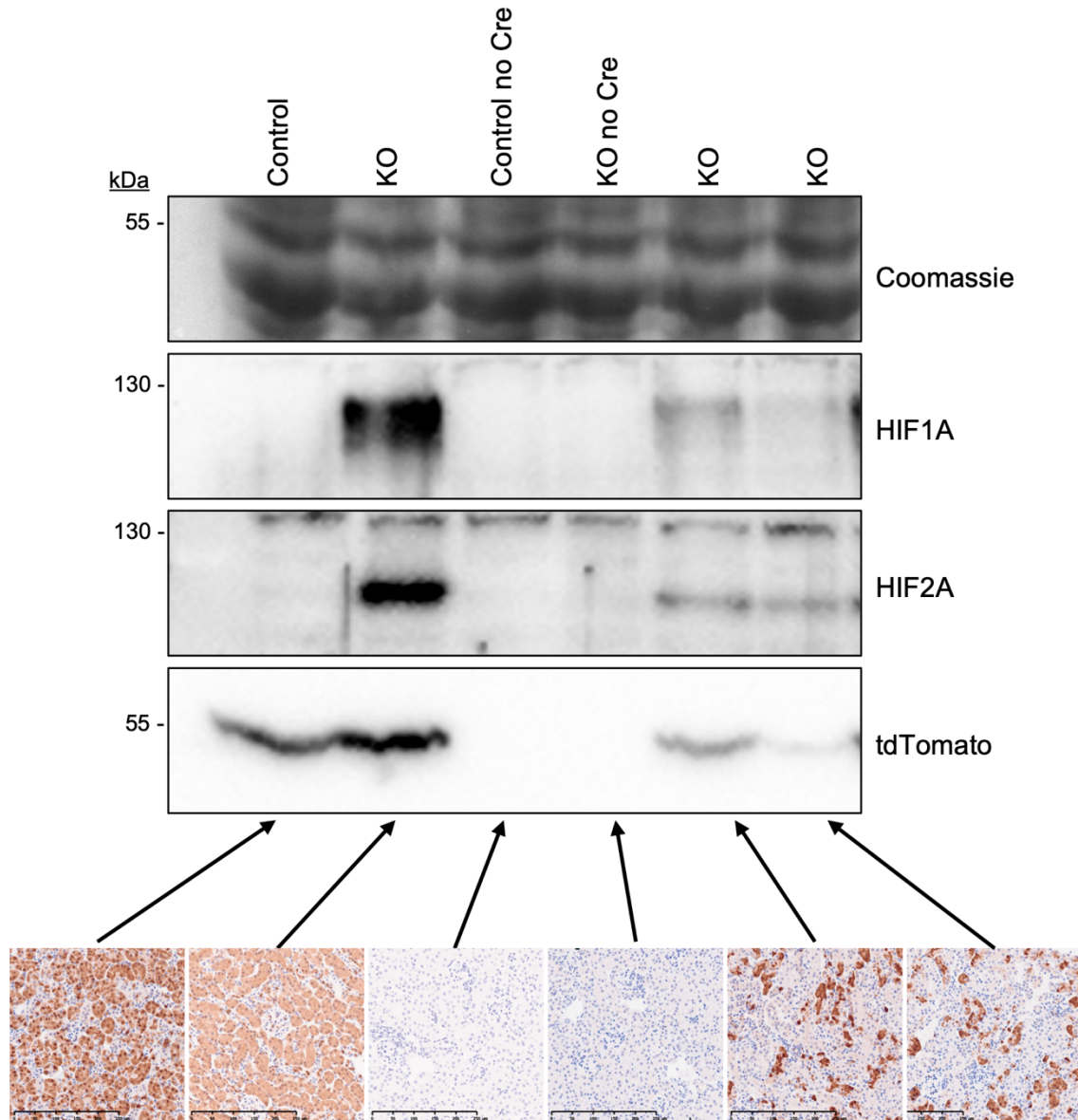


Figure 3.4: Loss of tdTomato is matched by loss of stabilised HIF in kidneys of KO animals. Immunoblots for tdTomato, HIF1A, and HIF2A performed on protein extracts of whole kidneys of *Vhl*^{wt/pjr.fl}; *Ubc-CreERT2* (Control), *Vhl*^{lae.KO/pjr.fl}; *Ubc-CreERT2* (KO), *Vhl*^{wt/pjr.fl} (Control no Cre), or *Vhl*^{lae.KO/pjr.fl} (KO no Cre) mice (top). tdTomato IHC performed on contralateral kidneys from same mice as used for immunoblotting (below) matched by black arrows. Immunoblotting performed by Dr Norma Masson.

Formalin-fixed paraffin-embedded (FFPE) sections were cut from kidneys and livers of Control and KO mice (n = 2) given 1 mg harvested 4 weeks after dosing; tdTomato IHC was performed to confirm that tissues from the KO mice contained fewer tdTomato-positive

cells than those from Control mice. Genomic DNA was then extracted from tissue sections and the presence and abundance of *Vhl*^{wt}, *Vhl*^{jae.KO}, and *Vhl*^{pjr.KO} alleles was assayed by PCR.

Similar band intensities were observed for amplicons corresponding to the ‘second’ *Vhl* allele (*Vhl*^{wt} for Control, *Vhl*^{jae.KO} for KO) from all tissues assayed (**Figure 3.5**), indicating that similar amounts of genomic DNA were recovered and amplified. However, band intensities for the *Vhl*^{pjr.KO} amplicons in tissues from KO mice were lower than those from Control mice (**Figure 3.5**). This indicates that for the same amount of total genomic DNA, tissues from KO mice that exhibit loss of tdTomato-positive cells by IHC also contain less recombined *Vhl*^{pjr.KO} allele. Therefore, tissues from KO mice carry fewer recombined cells than tissues from Control mice and supports the hypothesis that cells in tissues from KO tissues are lost after *Vhl* inactivation.

Collectively, these results indicate that widespread *Vhl* inactivation induced by *Ubc-CreERT2* leads to the time-dependent loss of *Vhl*-null cells in multiple organs over a period of 5 weeks after dosing. This loss is corroborated by a reduction in the levels of HIF1A and HIF2A stabilised and a reduction in the amount of recombined *Vhl*^{pjr.KO} allele present in organs from KO mice. These findings indicate that widespread *in vivo* *Vhl* inactivation as induced in this system confers a strong negative survival disadvantage to cells in multiple organs.

3.2.3 Mode of cell loss following widespread *Vhl* inactivation in the kidney and liver

Next, I sought to identify the modalities by which *Vhl*-null cells were lost from tissues, and thus how survival disadvantages might have been entrained.

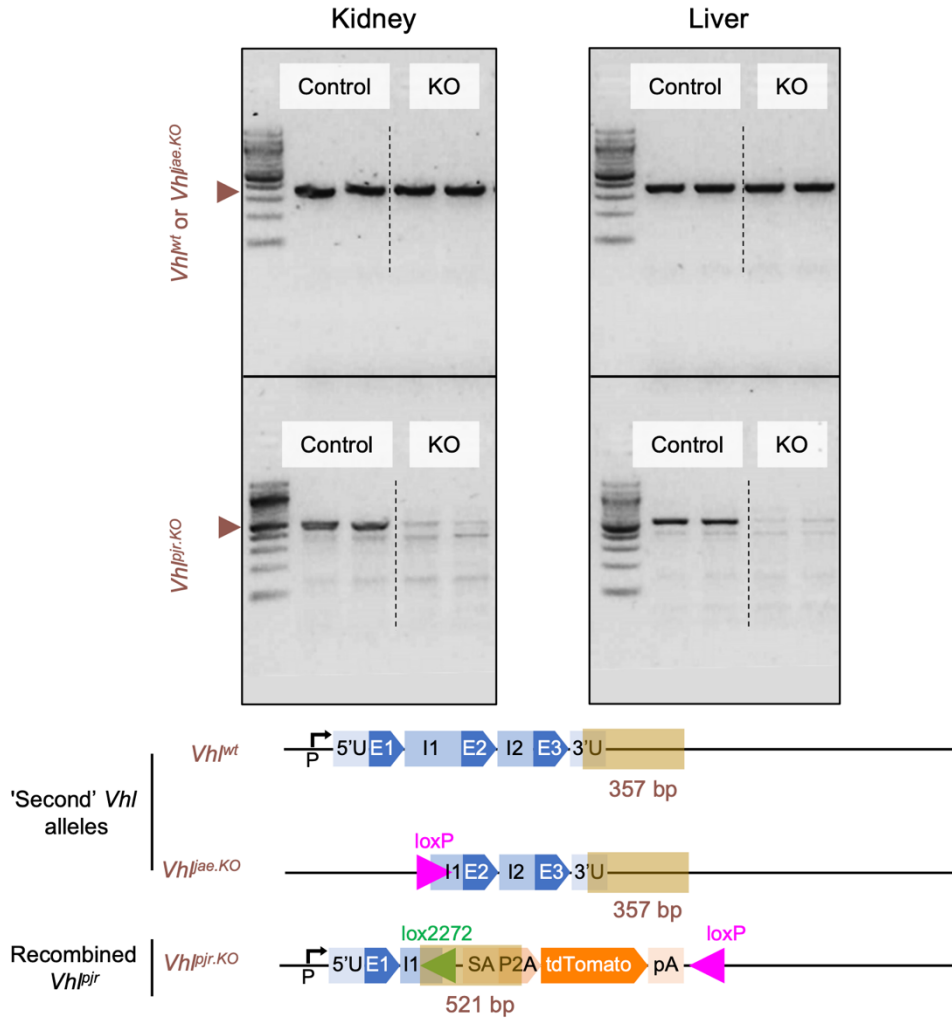


Figure 3.5: Reduced levels of recombined *Vhl*^{l^{pir}.KO} allele in organs of KO animals at 4 weeks after dosing. Gel electrophoresis for *Vhl*^{wt}, *Vhl*^{l^{ae}.KO}, or *Vhl*^{l^{pir}.KO} alleles amplified by PCR from genomic DNA extracted from kidneys or livers of Control (n = 2) or KO (n = 2) animals given 1 mg TMX and harvested 4 weeks after dosing. Schematics for amplicons for each PCR reaction depicted. Brown arrows indicate expected positions of amplicons as indicated in the schematic.

3.2.3.1 *Vhl* inactivation is associated with increased apoptosis in the liver but not in the kidney

Kidneys and livers from KO and Control mice given 1 mg TMX and harvested 1 week after dosing were assessed for signs of apoptotic cell death by TUNEL assay²⁰³. Livers from KO mice (n = 4) exhibited increased numbers ($p = 0.03$) of TUNEL-positive hepatocytes compared to those from Control (n = 3) mice at the 1-week timepoint (**Figure**

3.6A and **3.6B**). This subsided to levels seen in Control mice at 4 weeks after dosing ($n = 2$) (**Figure 3.6B**), by which time *Vhl*-null cells were known to be lost from tissues.

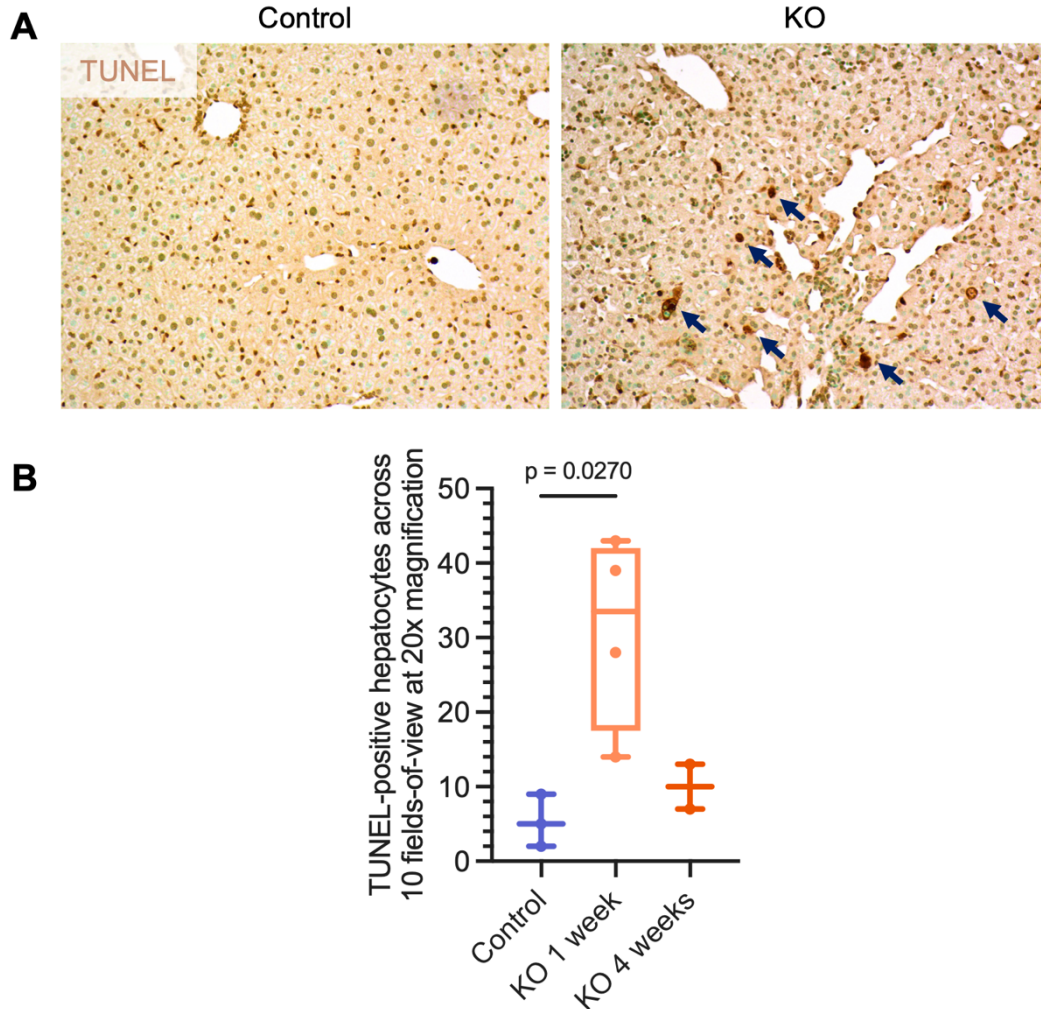


Figure 3.6: Transient increase in numbers of apoptotic hepatocytes in livers from KO mice. **(A)** Representative TUNEL assay in livers from Control ($n = 3$) and KO ($n = 4$) mice given 1 mg TMX and harvested 1 week after dosing. TUNEL-positive hepatocytes denoted by blue arrows. **(B)** Number of TUNEL-positive hepatocytes quantified in ten fields-of-view at 20x magnification in livers of Control ($n = 3$) mice given 1 mg TMX and harvested 1 week after dosing and KO mice given 1 mg TMX and harvested 1 week ($n = 4$) or 4 weeks ($n = 2$) after dosing. Median and inter-quartile range plotted. Pairwise comparisons by Kruskal-Wallis test with Dunn's correction. Only significant ($p < 0.05$) comparisons shown.

While tdTomato-positivity of the TUNEL-positive cells was not confirmed, a majority of TUNEL-positive cells were expected to be tdTomato-positive owing to the high (60%-80%) levels of recombination as seen in livers at this timepoint by tdTomato IHC (**Figure 3.2**). Expression of the cleaved form of the apoptotic protein²⁰³ Caspase-3 was also detected in livers from KO, but not Control mice at the 1-week timepoint (**Figure 3.7**). Together, these results suggest that apoptosis in hepatocytes contributes to ‘cell loss’ after *Vhl* inactivation.

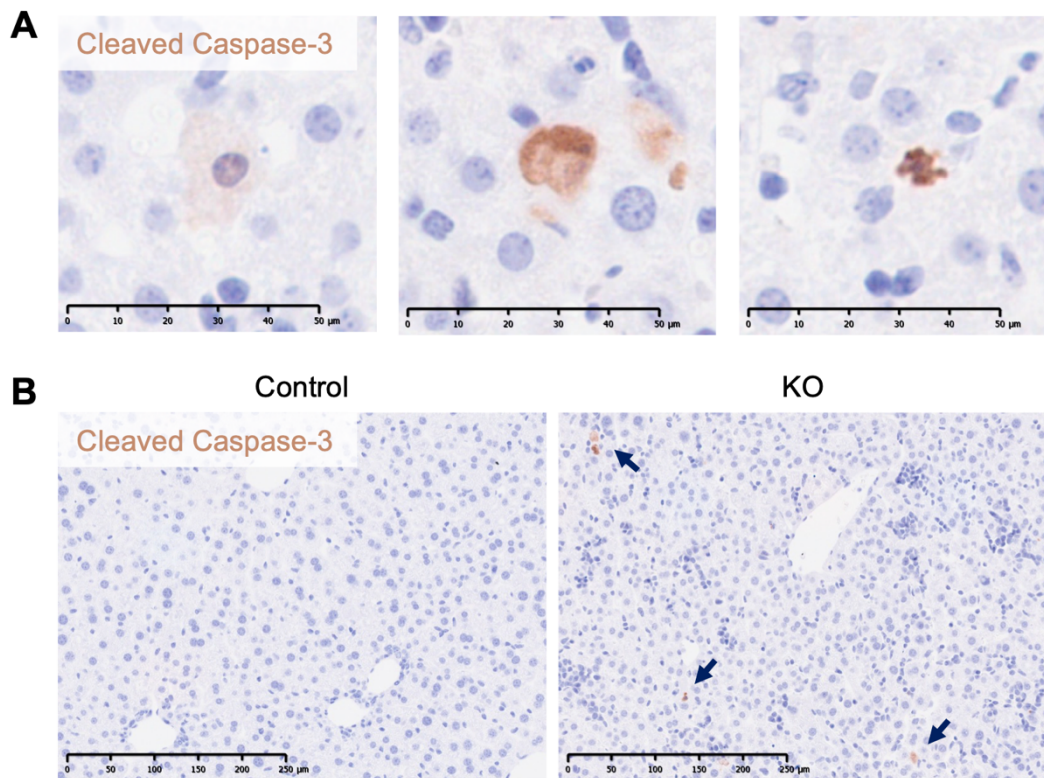


Figure 3.7: Increased apoptosis in hepatocytes following *Vhl* inactivation. (A) Representative cleaved Caspase-3 IHC in livers depicting positively stained cells in different stages of cell death. Scale bar denotes 50 µm. (B) Representative cleaved Caspase-3 IHC in livers of Control (n = 3) and KO (n = 4) mice given 1 mg TMX and harvested 1 week after dosing. Cleaved Caspase-3-positive cells denoted by blue arrows. Scale bar denotes 250 µm.

These experiments could not be replicated in the kidney. TUNEL assays – chromogenic assays performed on formalin-fixed paraffin embedded (FFPE) sections and fluorescence-based assays performed on frozen sections – revealed several TUNEL-positive cells in kidneys from Control mice (**Figure 3.8**) which, on the basis of their normal morphology and high frequency, were considered to be falsely positive for the TUNEL signal.

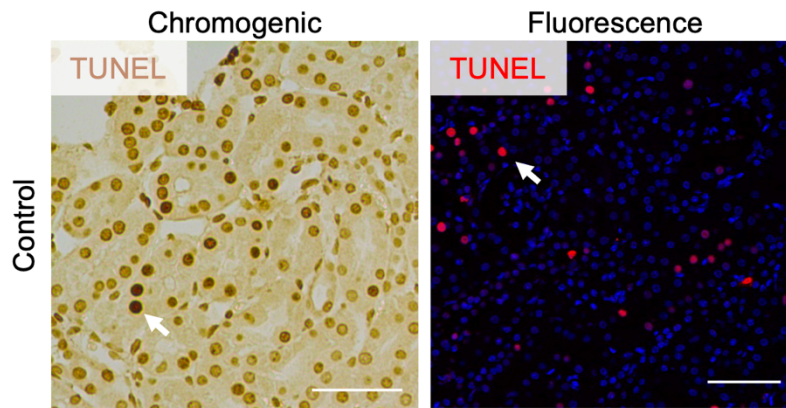


Figure 3.8: High levels of background TUNEL-positivity in kidneys. Representative TUNEL assay (chromogenic – left, and fluorescence – right) in kidneys from Control (n = 3) mice given 1 mg TMX harvested 1 week after dosing. TUNEL-positive RTE cells denoted by white arrows. Scale bar denotes 100 μ m.

IHC for cleaved Caspase-3 did not reveal any signs of apoptosis nor any genotype-specific differences (**Figure 3.9**). An effect of *Vhl* inactivation on cell death in the kidney could not be inferred.

3.2.3.2 *Vhl* inactivation is associated with expansion of resident immune cells in the liver but not the kidney

Certain forms of cell death results in the recruitment of immune cells that clear cell debris. Expression of “kill me” or “eat me” signals on cell surfaces also results in the recruitment of cytotoxic immune cells that kill or phagocytose cells in tissue²⁰⁴⁻²⁰⁶. I hence assayed for immune infiltration in tissues from Control and KO mice.

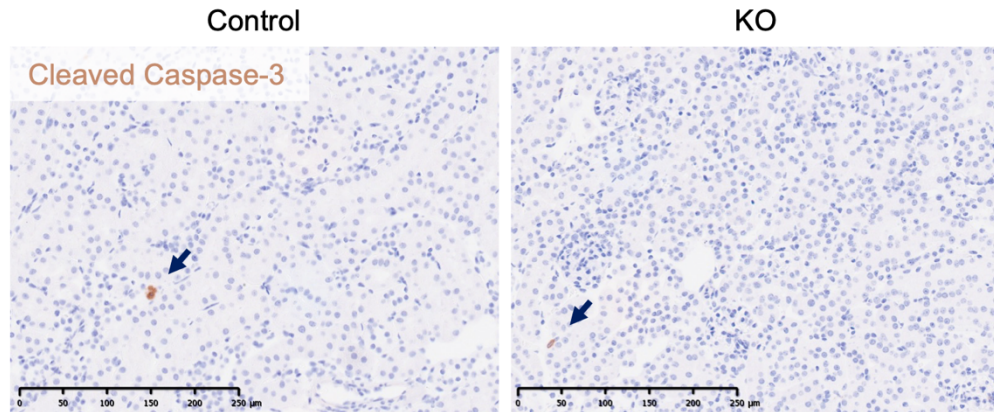


Figure 3.9: No signs of apoptosis in the kidney following *Vhl* inactivation. (A) Representative cleaved Caspase-3 IHC in kidneys from Control (n = 3) and KO (n = 4) mice given 1 mg TMX and harvested 1 week after dosing. Cleaved Caspase-3-positive cells denoted by blue arrows. Scale bar denotes 250 μ m.

I found that livers from KO (n = 4) mice given 1 mg TMX and harvested 1 week after dosing contained a greater number of resident macrophages (identified by expression of macrophage marker²⁰⁷ F4/80 by IHC) than livers from Control (n = 3) mice at the same timepoint (**Figure 3.10A**), indicating expansion of hepatic macrophages associated with *Vhl* inactivation and entrained apoptosis. The liver contains a specialised resident macrophage population called Kupffer cells that has known roles in liver homeostasis and response to injury. F4/80 expression in livers from KO mice co-localised with expression of the Kupffer cell marker²⁰⁸ CLEC4F (**Figure 3.10B**), confirming that these macrophages are Kupffer cells. These cells were tdTomato-negative (**Figure 3.10C**), indicating that they had not undergone *Vhl* inactivation themselves. These results suggest that *Vhl*-competent Kupffer cells are activated after *Vhl* inactivation in the liver parenchyma and may be involved in removing/clearing *Vhl*-null cells from the liver over time.

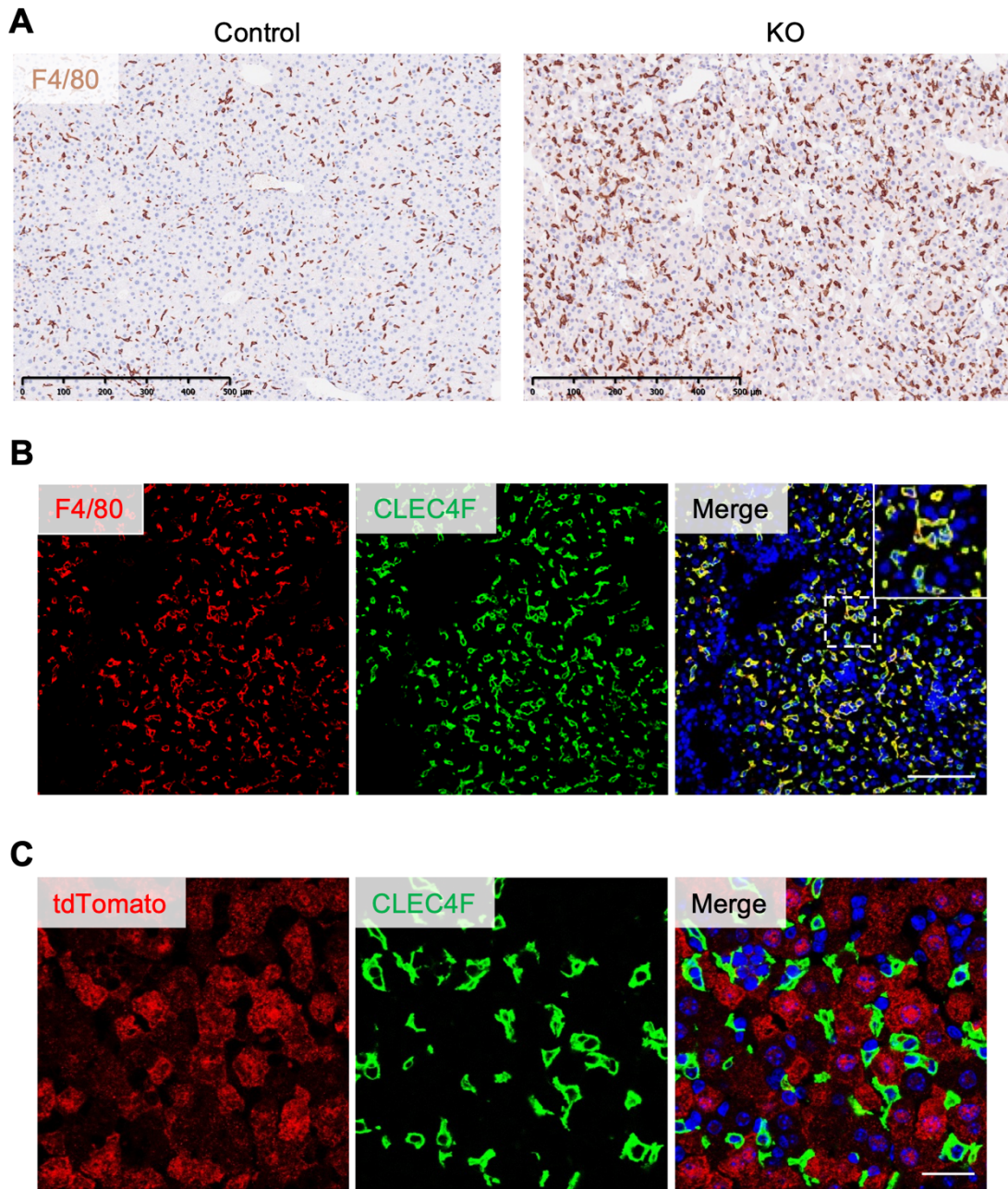


Figure 3.10: Increased numbers of Kupffer cells in livers of KO mice. (A) Representative F4/80 IHC in livers of Control (n = 3) and KO (n = 4) mice given 1 mg TMX and harvested 1 week after dosing. Scale bar denotes 500 μm . (B) Representative immunofluorescence staining (IF) depicting F4/80 (red), CLEC4F (green) and DAPI (blue) staining in livers of KO (n = 4) mice given 1 mg TMX and harvested 1 week after dosing. Scale bar denotes 100 μm . Area magnified in inset in the merged image highlighted by white square. (C) Representative IF depicting tdTomato (red), CLEC4F (green) and DAPI (blue) staining in livers of KO (n = 4) mice given 1 mg TMX and harvested 1 week after dosing. Scale bar denotes 25 μm .

Surprisingly, no differences were observed in the number or morphology of renal macrophages in kidneys of the same Control (n = 3) and KO (n = 4) mice at this timepoint (**Figure 3.11**). Furthermore, IHC for the pan-leukocyte marker²⁰⁹ CD45 did not exhibit any genotype-specific differences within the same kidneys (**Figure 3.11**). This indicated that *Vhl* inactivation did not affect renal immune cell populations at least as assayed by IHC 1 week after recombination was induced.

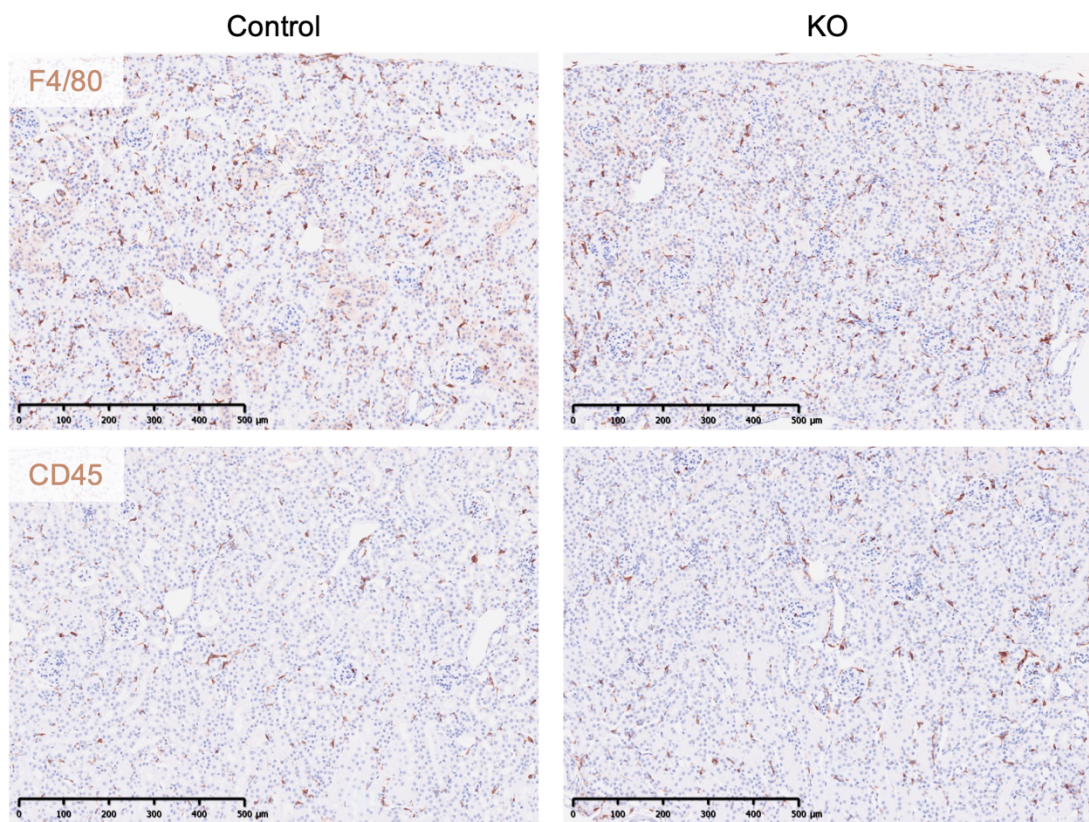


Figure 3.11: No signs of renal immune cell activation at 1 week after *Vhl* inactivation. Representative F4/80 (top) and CD45 (bottom) IHC in kidneys from Control (n = 3) and KO (n = 4) mice given 1 mg TMX and harvested 1 week after dosing. Scale bar denotes 500 μ m. CD45 IHC performed in collaboration with Dr Joanna Lima.

3.2.4 Tissue homeostasis in response to the loss of *Vhl*-null cells

Next, I sought to assess if the *Vhl*-competent population in the kidney and liver responded to the loss of *Vhl*-null cells and underwent proliferation to maintain homeostasis

in the tissues. Changes in relative organ masses in association with loss of *Vhl*-null cells were also assessed.

3.2.4.1 Compensatory proliferation of *Vhl*-competent cells in association with loss of *Vhl*-null cells from tissues

To assay proliferation of cells in tissues following *Vhl* inactivation, I analysed the incorporation of the deoxythymidine analogue 5-bromo-2'-deoxyuridine (BrdU)²¹⁰ provided continuously for 4 weeks in livers and kidneys of Control (n = 3) and KO (n = 3) mice given 1 mg TMX and harvested 4 weeks after TMX dosing, by which time tissues from all 3 KO mice were known to have exhibited the loss of *Vhl*-null cells.

The livers of KO mice exhibited greater BrdU incorporation than those from Control mice (**Figure 3.12A**). Notably, most (but not all) BrdU-labelled hepatocytes were tdTomato-negative (**Figure 3.12B**) and appeared in oligocellular juxtaposition in the tissue section. These observations suggest that *Vhl*-competent hepatocytes proliferate in the liver over the same timeframe that *Vhl*-null cells are lost from the tissue.

Increased BrdU incorporation was also seen in kidneys of KO mice compared to those from Control mice (**Figure 3.12C**). Surprisingly, a major proportion of this increase appeared to arise from tdTomato-positive RTE cells (**Figure 3.12D**), which contrasted with findings in the liver where most BrdU-labelled cells were tdTomato-negative. These observations do not provide evidence of compensatory proliferation in the kidney in association of loss of *Vhl*-null cells, and paradoxically suggest that *Vhl*-null RTE cells proliferate in the same timeframe that they are lost from the kidney.

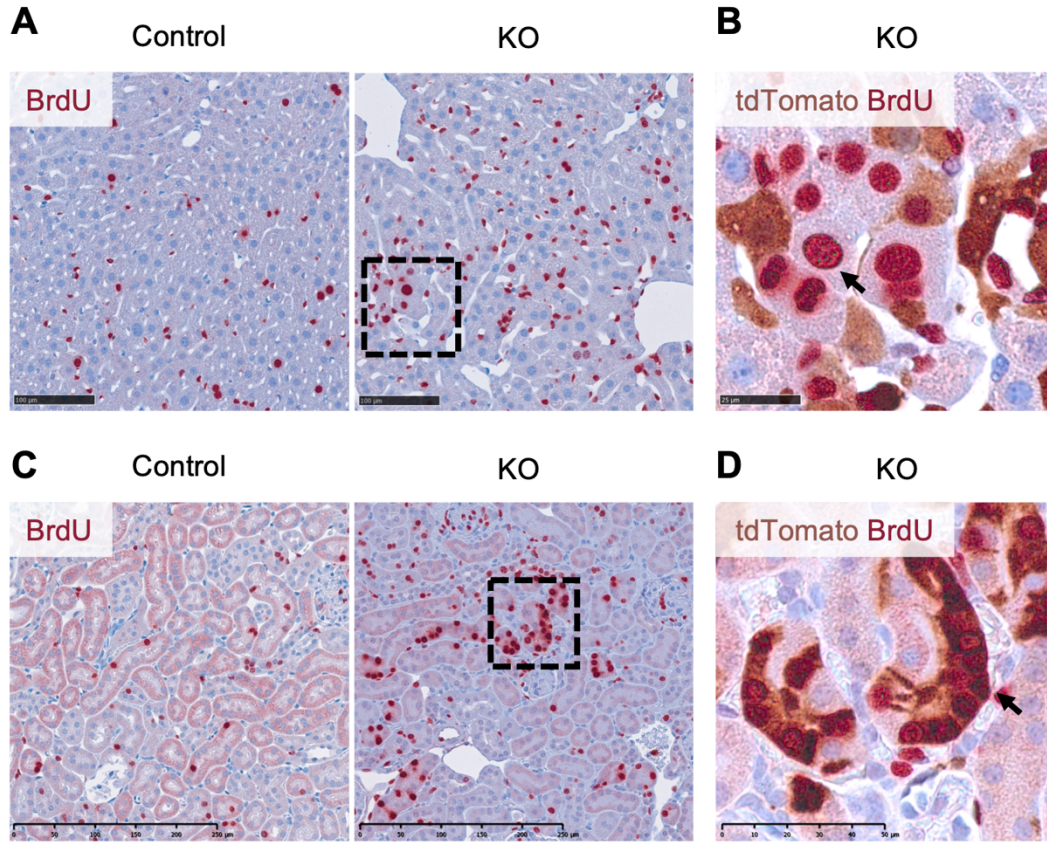


Figure 3.12: Proliferation associated with cell loss following widespread *Vhl* inactivation in the kidney and liver. (A) Representative BrdU IHC in livers from Control (n = 3) and KO (n = 3) mice given 1 mg TMX and thereafter continuously administered BrdU and harvested 4 weeks after TMX dosing. (B) tdTomato (brown) BrdU (red) dual IHC performed on a serial section depicting the region denoted by black rectangle in (A). tdTomato-negative BrdU-positive hepatocyte denoted by black arrow. Scale bars denote 100 μm (A) and 25 μm (B). (C) Representative BrdU IHC in kidneys from Control (n = 3) and KO (n = 3) mice given 1 mg TMX and thereafter continuously administered BrdU harvested 4 weeks after TMX dosing. (D) tdTomato (brown) BrdU (red) dual IHC performed on a serial section depicting the region denoted by black rectangle in (C). tdTomato-positive BrdU-positive RTE cells denoted by black arrow. Scale bars denote 250 μm (C) and 50 μm (D). tdTomato/BrdU dual IHC performed in collaboration with Dr Joanna Lima.

3.2.4.2 Loss of renal mass following widespread *Vhl* inactivation

Elimination of *Vhl*-null cells with little compensatory proliferation of *Vhl*-competent cells must result in a loss of renal mass in KO mice. I sought to test if kidneys that had lost

tdTomato-positive cells had also lost renal mass. Wild-type C57BL6/J mice have a well characterised renal mass to body mass ratio of $0.51\% \pm 0.07\%$ ²¹¹. If a Control and KO mouse given 1 mg TMX and harvested at the same timepoint exhibited similar changes in body weights over the course of the experiment and if kidneys from the KO mouse had fewer tdTomato-positive cells than those from the Control mouse, then a deviation of the renal mass to body mass ratio from the wild-type value must indicate a change in renal mass caused by *Vhl* inactivation. Based on this premise, Control and KO mice were weighed prior to dosing, given 1 mg TMX and harvested 4 weeks after dosing; final body weight and weight of the kidney and liver were measured at the time of harvest. The proportion of tdTomato-positive cells was assessed by tdTomato IHC.

Data from three pairs of Control and KO mice were analysed. These mice exhibited similar starting and final body weights, with a change in weights of -0.5%, 2.6%, and 3.3% for Control and -0.36%, 1.78% and 2.36% for KO mice over the 4 weeks since dosing. Control mice exhibited a renal weight to body weight ratio of 0.44%, 0.48%, and 0.56%, values which were consistent with those reported from wild-type C57B6/J mice. However, KO mice exhibited lower renal weight to body weight ratios of 0.22%, 0.33%, and 0.35% than the Control mice. Notably, relative renal weights decreased proportionally to the number of tdTomato-positive cells in kidneys from KO mice as assayed by IHC (**Figure 3.13A**). These results support a loss of renal mass in association with elimination of *Vhl*-null cells from the kidney.

Interestingly, the opposite was observed in livers from the same animals, with livers harbouring the fewest tdTomato-positive cells exhibiting the highest liver weight to body weight ratios (**Figure 3.13B**). This is possibly a consequence of compensatory proliferation by *Vhl*-competent hepatocytes or of inflammation as observed in **Figure 3.12** and **3.10** respectively.

Collectively, while these results do not identify clear modes of cell loss following *Vhl* inactivation, they are consistent with the loss of *Vhl*-null cells from the kidney and liver of KO mice over the course of five weeks after dosing. In the liver, loss of cells is supported by increased apoptosis, immune expansion, and compensatory proliferation of *Vhl*-competent cells. In the kidney, loss of cells is supported by the reduction in renal mass.

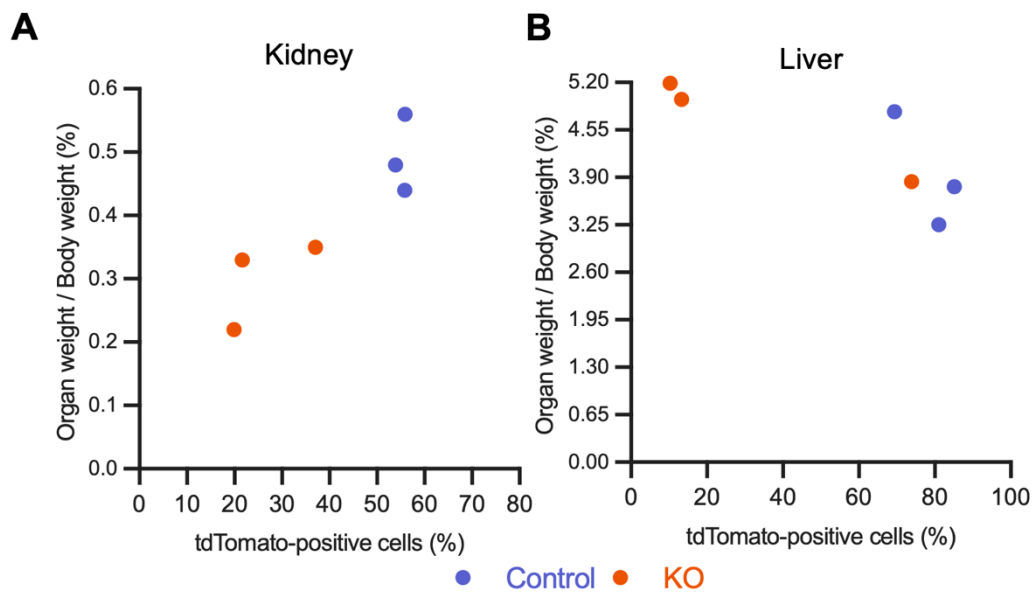


Figure 3.13: Changes in relative organ weights after cell loss associated with widespread *Vhl* inactivation. (A, B) Weights of kidney (A), and liver (B), as a proportion of total body weight plotted against the proportion of cells positive for tdTomato by IHC in Control (n = 3) and KO (n = 3) mice given 1 mg TMX and harvested 2-3 weeks after dosing.

3.2.5 Cell fates following *Vhl* inactivation with a low dose of TMX

Data presented so far has indicated that extensive recombination is induced by *Ubc-CreERT2* with 1 mg TMX across multiple organs, which is then followed by the death, clearance, or loss of a substantial proportion of recombined *Vhl*-null cells from tissues in a matter of 3-5 weeks after dosing. However, the several pathologies associated with such extensive *Vhl* inactivation prevented long-term analysis of cell fate because the mice became very sick. I thus tested if inducing recombination with a lower dose of TMX prevented the

development of pathologies associated with widespread *Vhl* inactivation and thus provided a better platform to study the cellular effects of *Vhl* inactivation in native tissue context.

KO mice (n = 4) given 0.1 mg TMX (instead of 1 mg TMX) also developed skin erythema and polycythaemia within 3 weeks of *Vhl* inactivation (data not shown), suggesting that even this dose was unlikely to be tolerated long-term. However, KO mice (n = 4) given 0.02 mg TMX did not develop skin erythema, nor did they exhibit hunched posture and ruffled coats for at least 8 months after dosing. KO mice did not exhibit increased haematocrits at the 8-month timepoint compared to Control (n = 4) (**Figure 3.14**), confirming the absence of polycythaemia induced with this TMX dose. Thus, this TMX dose generated *Vhl* inactivation without adversely and acutely impacting the health of the mouse.

Recombination was previously detected in kidneys and livers of Control mice given 0.02 mg TMX and harvested at 1 week after dosing (**Chapter 2**). To determine the effects of *Vhl* inactivation with this dose on the long-term survival of cells in these organs, Control and KO mice given 0.02 mg TMX were harvested at 8 months after dosing. Scattered tdTomato-positive cells were present in all renal regions and in the liver of Control and KO mice, suggesting that at least some *Vhl*-null were capable of long-term survival in their native tissue context over 8 months after *Vhl* inactivation. The median proportion of tdTomato-positive cells was lower for KO compared to Control mice in the inner medulla, papilla, and the liver; however, this decrease was not statistically significant, possibly owing to the high variability in quantification between mice (**Figure 3.15A**).

Interestingly, one of the KO mice exhibited a particularly high proportion of tdTomato-positive cells in the renal cortex (2.6% compared to 0.3%) but did not do so for other renal regions or the liver (**Figure 3.15A**). tdTomato-positive cells in that cortex were present as clusters of tdTomato-positive neighbours in the same renal tubule (**Figure 3.15B**), which was not seen in cortices from any other mouse with this dose at any timepoint. Other renal regions and the liver from the same mouse did not exhibit such a distribution of

tdTomato-positive cells, suggesting that it was a cortex-specific phenotype (**Figure 3.15B**). These observations open a possibility that at this recombination frequency, *Vhl*-null cells in the renal cortex enjoy a tissue-specific survival advantage and may survive and expand in the tissue over time.

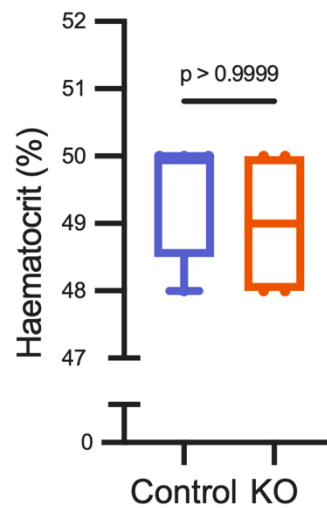


Figure 3.14: No polycythaemia associated with *Vhl* inactivation with 0.02 mg TMX. Haematocrits of Control or KO mice given 0.02 mg TMX and harvested 8 months after dosing (n = 4 per genotype). Median and inter-quartile range plotted. Pairwise comparisons by Mann-Whitney test.

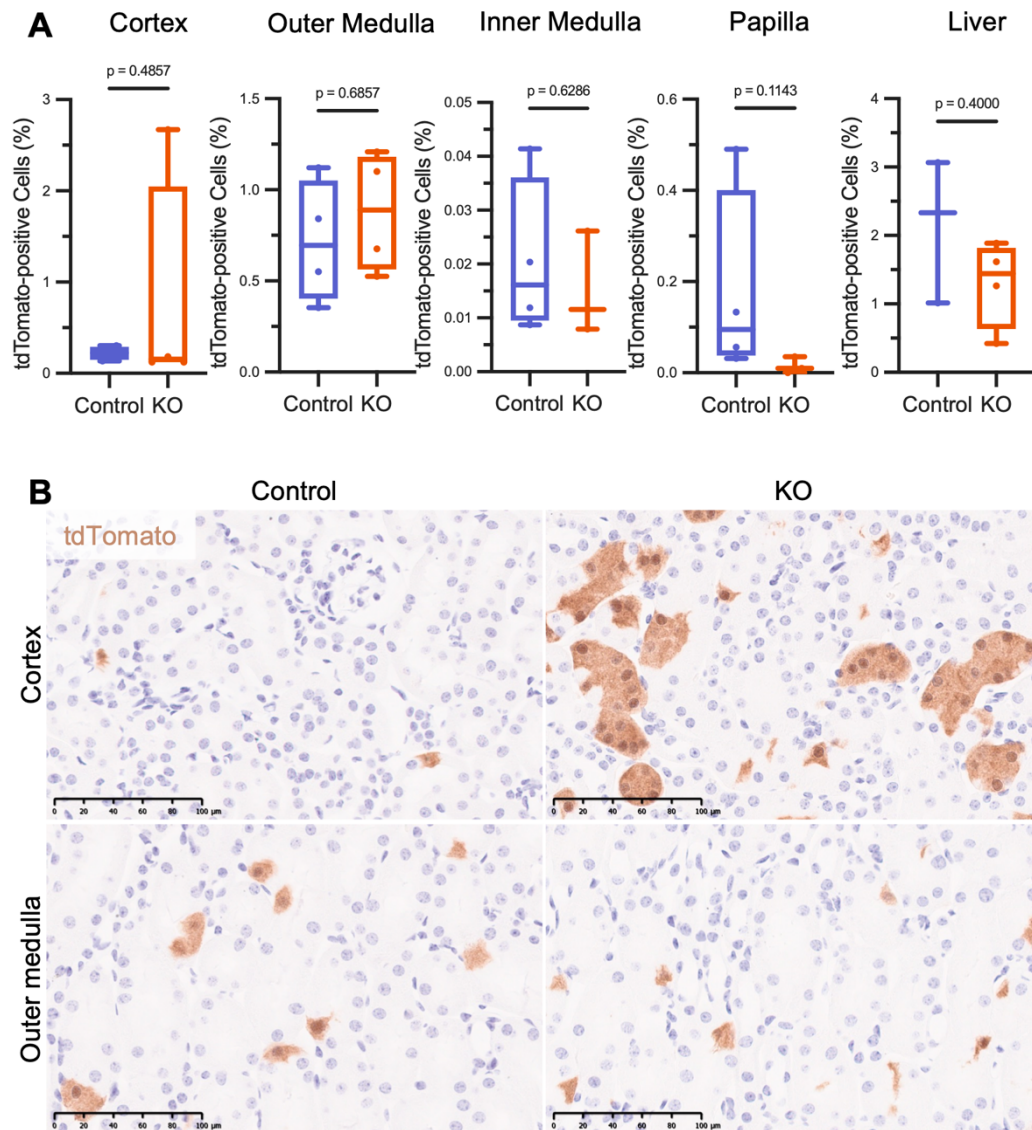


Figure 3.15: Long term retention of tdTomato-positive cells after sparse induction of recombination with 0.02 mg TMX. (A) Proportion of total cells that are tdTomato-positive in different renal regions and livers as quantified by tdTomato IHC from Control and KO mice given 0.02 mg TMX and harvested 8 months after dosing (n = 4, 4, 4, 4, 3 (Control) and n = 4, 4, 4, 3, 4 (KO) for cortex, outer medulla, inner medulla, papilla, and liver respectively). Median and inter-quartile range plotted. Pairwise comparisons by Mann-Whitney test. (B) tdTomato IHC from cortex (top) and outer medulla (bottom) from KO mouse exhibiting high cortical tdTomato-positivity and a representative Control given 0.02 mg TMX and harvested 8 months after dosing. Scale bar denotes 50 μ m.

3.3 Discussion

In this chapter, I employed the novel model system described in **Chapter 2** to test the hypothesis that *Vhl* inactivation exerts selection pressures on cells that are tissue-specific – that survival and proliferation of *Vhl*-null cells are supported in the renal cortex but not in tissues that do not exhibit *VHL*-associated cancer. *Ubc-CreERT2* was used to generate marked cells that were either *Vhl*-null or *Vhl*-haplosufficient in the kidney, liver, pancreas, adrenal glands and spleen of KO and Control mice respectively, and cellular effects of biallelic *Vhl* loss were inferred by comparing the retention of marked cells in tissues KO *versus* Control mice over time.

3.3.1 Loss of cells from tissues is an early consequence of biallelic *Vhl* inactivation

3.3.1.1 The tdTomato reporter allows survival of *Vhl*-null cells to be traced over time

The 1 mg dose of TMX was used in this experiment primarily, as it was shown to induce consistent recombination in multiple organs of Control mice (**Chapter 2**). Unexpectedly, a striking reduction in the number of tdTomato-positive cells was observed over the course of 3-5 weeks in all assayed organs of KO mice, but not of Control mice (**Figure 3.2** and **3.3**). This arose from a reduction in the number of *Vhl*-null cells and not because tdTomato was not detected in *Vhl*-null cells and was shown by two approaches. First, a correlation was demonstrated between the number of tdTomato-positive cells in tissue as assayed by IHC, levels of tdTomato protein in the tissue as assayed by immunoblotting, and the levels of HIF1A and HIF2A protein detected in the same tissue by immunoblotting (**Figure 3.4**). This provided evidence against the presence of *Vhl*-null (and thus HIF-positive) cells that did not express tdTomato in these tissues. Second, tissues from KO mice that exhibited a decrease in tdTomato-positive cells were shown to also carry less of the recombined *Vhl*^{ppir.KO} compared to Control tissues with high levels of tdTomato-positivity

(**Figure 3.5**). This provided evidence against the presence of cells in KO tissues that had undergone *Vhl* recombination, but not expressed tdTomato.

3.3.1.2 Tissue-specific modes of cell elimination from the liver and kidney

This widespread *Vhl* inactivation induced apoptosis in hepatocytes at the 1-week timepoint, which subsided to baseline levels by the 4-week timepoint by which time cell loss had occurred (**Figure 3.6** and **3.7**). The initial apoptotic burst was associated with the expansion of the *Vhl*-competent resident Kupffer cell population (**Figure 3.10**). Furthermore, loss of *Vhl*-null hepatocytes was matched by accumulated proliferation of *Vhl*-competent hepatocytes (**Figure 3.12**) that served to maintain relative hepatic mass in the mouse (**Figure 3.13**). These observations point to a possible mechanism wherein *Vhl* inactivation in hepatocytes activates apoptotic pathways and which are then cleared by the resident Kupffer cells. Signals from either the dying hepatocytes or the activated Kupffer cells may then induce proliferation in the surviving hepatocytes which then preserves hepatic mass.

Apoptosis could not be reliably detected with either TUNEL assays or IHC for apoptotic proteins such as cleaved Caspase-3 in the kidney (**Figure 3.8** and **3.9**), and no differences were observed in the number or morphologies of renal macrophages or pan-leukocytes populations 1 week after *Vhl* inactivation (**Figure 3.11**). However, a reduction in the proportion of tdTomato-positive (and by extension, *Vhl*-null) cells in kidneys of KO mice was associated with a reduction in the relative mass of the kidney (**Figure 3.13**). This reduction provides support for the loss of *Vhl*-null cells from the kidney though a mode for cell elimination could not be determined. Recently, a phenomenon named “epithelial defence against cancer” has been described to be active in the intestinal epithelium, wherein cells harbouring oncogenic mutations are pushed into the intestinal lumen by mechanical forces imposed on them by their genetically normal neighbouring cells^{212,213}. Notably, this mode of cell elimination occurs without the activation of apoptotic pathways or immune infiltration in the gut^{212,213}. It is possible that the loss of *Vhl*-null cells in the kidney occurs by such a process wherein *Vhl*-null RTE cells are pushed into the tubular lumen and eliminated *via* the

urine. This could be tested by assaying for increased presence of tdTomato-positive cells in the urine of KO compared to Control mice at timepoints between 1 and 3 weeks after dosing when the loss of *Vhl*-null cells is expected to occur.

Taken together, these results demonstrate that *Vhl*-null cells generated by widespread *Vhl* inactivation in multiple organs are indeed lost over time and that such decreases are evident from a comparison of the numbers of tdTomato-positive cells in tissues from KO *versus* Control mice at late timepoints. Surprisingly, this extensive cell elimination occurred in both cancer-prone (e.g., kidney and pancreas) and non-prone (e.g., liver and spleen) organs, suggesting that the hypothesis of tissue-specific selection of *Vhl*-null cells might not be true. However, these findings are confounded by the limitations of using a high dose of TMX with *Ubc-CreERT2* as described below.

3.3.2 Limitations of assessing *Vhl*-associated phenotypes following widespread *Vhl* inactivation in mice

Extensive *Vhl* inactivation across multiple tissues was associated with severe adverse effects on mouse health that included splenomegaly and polycythaemia. Both these pathologies have been described previously in studies of system-wide *Vhl* inactivation in which *Ubc-CreERT2* was activated by dietary administration of TMX through food for 10 days¹⁹⁸. The experimental system thus posed three problems. First, KO mice given 1 mg TMX could not be reliably maintained for longer than 3-5 weeks. Second, the effects of *Vhl* inactivation in specific cells could not be distinguished from the response of *Vhl*-null cells to systemic effects such as polycythaemia. Third, that such extensive *Vhl* inactivation meant that *Vhl*-null cells were almost always surrounded by other *Vhl*-null cells in the tissue parenchyma and interstitium. The behaviour of these *Vhl*-null cells may not reflect the behaviour of *Vhl*-null cells surrounded by other *Vhl*-competent cells; such context-specific behaviour has been demonstrated for cells bearing other oncogenic mutations *in vivo* and in cell culture^{131,133}.

Despite the limitations described above, this experimental system did help validate the utility of the novel reporter model in elucidating fates specific to biallelic *Vhl* inactivation *in vivo*. Crucially, it could be used to demonstrate that tdTomato-positivity continued to accurately report *Vhl* recombination and that a change in the numbers of tdTomato-positive cells in tissues from KO mice directly reported a change in the numbers of *Vhl*-null cells in the same tissues. This principle can be employed to assess cell fates in experimental systems using other Cre recombinase alleles or TMX doses. Furthermore, while cell loss was not found to be tissue-specific, *Vhl*-null RTE cells but not *Vhl*-null hepatocytes were observed to have proliferated early after *Vhl* inactivation. This observations points towards cells in the cancer-prone RTE having a pro-proliferative effect following *Vhl* inactivation and merits further investigation.

3.3.3 Assessing cell fates following *Vhl* inactivation in the absence of systemic pathologies

Considering the limitations discussed above, I re-focussed most of my work to the study of *Vhl*-null cells in experimental systems that did not adversely affect mouse health, and which could be maintained for months or over a year after *Vhl* recombination. These included the use of *Pax8-CreERT2* to restrict *Vhl* recombination in the kidney and study fates in the cancer-associated (e.g., renal cortex) *versus* non-associated (e.g., renal papilla) renal tissues (described in **Chapters 4-6**) or using very low doses of TMX to generate sparse *Vhl* recombination in the kidney and liver using *Ubc-CreERT2* with 0.02 mg TMX (**Figure 3.14**).

The limitations of using a 0.02 mg TMX dose were that recombination was not detected in any organ other than the kidney and liver (**Chapter 2**) and that the recombination induced was not as consistent across mice dosed as is observed for higher TMX doses. This experimental system thus needs larger cohort sizes to confer enough statistical power to the analyses. Nonetheless, preliminary investigations with this dose have revealed interesting phenomena. Loss of *Vhl*-null cells was hinted to be tissue-specific – loss of cells from the

renal inner medulla, papilla, and liver but retention of *Vhl*-null cells in the renal cortex and outer medulla. However, the fact that *Vhl*-null cells were still present in these tissues even 8 months after *Vhl* inactivation (**Figure 3.15**) indicates that not all *Vhl*-null cells are eliminated from tissues over time and that the acute negative cellular outcomes entrained by *Vhl* inactivation may depend on the frequency of recombination and/or systemic effects induced. A particularly interesting observation was of the possible expansion of *Vhl*-null cells over 8 months in the renal cortex of one of the KO mice. The renal cortex is the tissue-of-origin for ccRCC, and such an expansion may point to a tissue-specific survival advantage gained by *Vhl*-null tubular epithelial cells in other ‘normal’ tissues (**Figure 3.15**).

Altogether, data presented in this chapter leads me to the following conclusions:

1. *Vhl* inactivation can lead to the loss of cells from tissue which is accurately reflected by a reduction in the number of tdTomato-positive cells over time. This can be inferred by the low proportions of tdTomato-positive cells in organs from KO mice compared to those from Control mice.
2. Extensive recombination induced by *Ubc-CreERT2* with 1 mg TMX, while allowing the study of *Vhl* inactivation in multiple tissues, causes deleterious effects to the health of the mouse and thus confounds interpretation of cellular data.
3. Long term fates of *Vhl*-null cells may be better studied with low levels of recombination. However, doing so with *Ubc-CreERT2* and 0.02 mg TMX requires bigger sample sizes than obtained in this study to overcome mouse-to-mouse variation in recombination.
4. The renal cortex may support the expansion of *Vhl*-null cells. Considering the limitations of using *Ubc-CreERT2*, this hypothesis may be better resolved by comparisons of different cells residing in different regions of the kidney using *Pax8-CreERT2*.

These conclusions have informed the rest of the work contributing to this thesis. In the ensuing chapters, tissue-specific cell fates, associated survival advantages and tissue-specific gene expression changes entrained by *Vhl* inactivation over time have been studied in the context of the different cell types and anatomical regions of the kidney by coupling the novel reporter model with *Pax8-CreERT2*.

Chapter 4:

Cell Fates Following *Vhl* inactivation in the Renal Tubular Epithelium

4.1 Introduction

Biallelic inactivation of *VHL* is the truncal genomic event in the evolution of ccRCC but is thought to occur 5-40 years before the development of clinical disease^{70,214}. The evolution of ccRCC further involves loss of genes such as *PBRM1*, *SETD2*, and *BAP1* that lie on the same chromosome as *VHL*. Based on knowledge gained from recent genomic profiling of ccRCC in patients and the insufficiency of *Vhl* inactivation on its own in driving ccRCC in mouse models, recent research has focussed primarily on elucidating the oncogenic co-operation of *VHL* inactivation with these secondary mutations. However, little attention has been given to the impact, if any, of *VHL* inactivation alone on the behaviour or oncogenic capacity of cells in the renal tubule. Such impacts have been hinted at from biopsies from ccRCC patients. These found that *VHL*-null cells are present in morphologically normal renal tubules^{114,115} and that the frequency with which such cells are detected differs between different segments of the nephron¹¹⁴. These observations raise the possibility that *VHL* inactivation has nephron segment-specific consequences in the renal tubular epithelium (RTE) that impact on long-term survival and retention of *VHL*-null cells in tissue. It was hypothesised that studying if, and how, such consequences arise should provide new perspectives on the contributions of *VHL* inactivation to tissue-specificity and latency of ccRCC genesis.

Undertaking such a study requires a novel approach to assay *VHL*-null cells in the RTE. Cell culture has been used extensively to characterise the biochemical and cell

biological functions of *VHL* but cannot inform on immediate or long-term cell fates entrained by *Vhl* inactivation *in vivo*²¹⁵⁻²¹⁷. Mouse models of *Vhl* inactivation have so far demonstrated its insufficiency for tumorigenesis or dysplasia in the absence of further genetic or chemical perturbations^{39,184,218,219}, but have not been able to assay fully cell-specific and -autonomous phenotypes because:

1. *Vhl*-null cells could not be distinguished from *Vhl*-competent cells and therefore phenotypes arising directly in *Vhl*-null cells could not be distinguished from those arising in *Vhl*-competent cells in response to *Vhl* inactivation in their neighbours.
2. *Vhl*-dependent phenotypes in rare cell populations could not be discerned in analyses of bulk tissue or entire tissue sections.
3. Time-dependent changes in number and behaviour of *Vhl*-null cells could not be distinguished from those that naturally occur with age.

As such, a systematic study of these effects has not been performed across renal regions and over time with these mouse models, possibly because no overt oncogenic phenotypes were observed.

The model developed in this thesis and described in **Chapter 2** can effectively address these limitations. It does that by 1) providing a means to identify recombined cells accurately, and 2) using the *Vhl*^{wt/pjr.fl} mouse as a comparator to define the subset of cells targeted by a Cre allele at a given TMX dose and timepoint against which phenotypes in *Vhl*^{j^{lae}.KO/pjr.fl} mice can be assayed.

In this chapter, I employ my model to test the hypothesis that *Vhl* inactivation has nephron-segment specific effects on the long-term behaviour of cells in the RTE and provide the first systematic description of the survival and proliferative phenotypes of *Vhl*-null cells in the RTE at early (1-3 weeks) or late (4-12 months) timepoints after *Vhl* recombination. Retention and expansion of *Vhl*-null cells in the renal cortex, outer medulla, inner medulla, papilla and in the Bowman's capsule are assessed over time. The proliferative capacity and

proliferative history of *Vhl*-null cells is described. Finally, co-incident changes in renal morphology and in the *Vhl*-competent renal immune cell population are assayed. Together, these findings demonstrate tissue-specific phenotypes associated with *Vhl* inactivation and provide clues towards tumour suppression.

4.2 Results

4.2.1 Renal cortex, but not the papilla, supports the long-term survival of *Vhl*-null cells

To test how biallelic *Vhl* inactivation impacts on the long-term survival and behaviour of cells in the kidney, I compared the number and distribution of tdTomato-positive cells in four regions - cortex, outer medulla, inner medulla, and papilla (see **Chapter 8**) - from *Vhl*^{wt/pjr.fl}; *Pax8-CreERT2* (“Control”) and *Vhl*^{lae.KO/pjr.fl}; *Pax8-CreERT2* (“KO”) animals (at least n = 20 for each genotype and region) given 5x 2 mg TMX and harvested at two timepoints designated “early” (1-3 weeks) or “late” (4-12 months) after first dose.

tdTomato-positive cells were detected in all regions of the kidneys in both genotypes early after recombination (**Figure 4.1A**). No significant difference was observed in numbers of tdTomato positive cells between Control and KO animals at the early timepoint. This confirmed results reported in **Chapter 2** and indicated that recombination is not affected by the status of the WT or KO second *Vhl* allele. The proportion of cells in each region positive for tdTomato remained constant in kidneys from Control mice over the entire course of the experiment, indicating that monoallelic *Vhl* inactivation conferred neither advantage nor disadvantage to cells in the RTE.

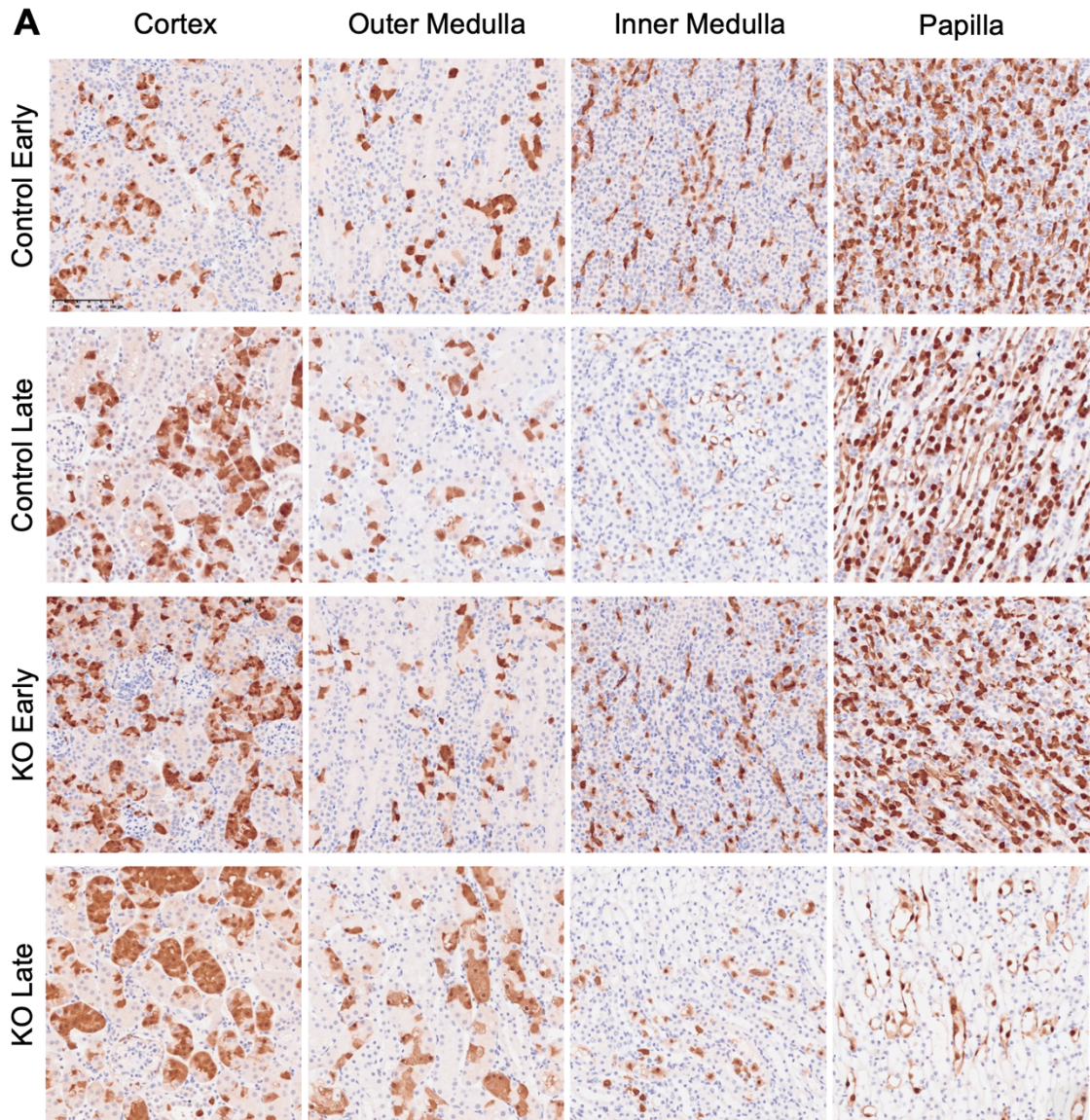


Figure 4.1: Region-specific retention of *Vhl*-null cells in the kidney. (A) Representative tdTomato IHC in different renal anatomical regions of Control or KO mice given 5x 2 mg TMX and harvested at the early or late timepoints. Scale bar denotes 100 μ m.

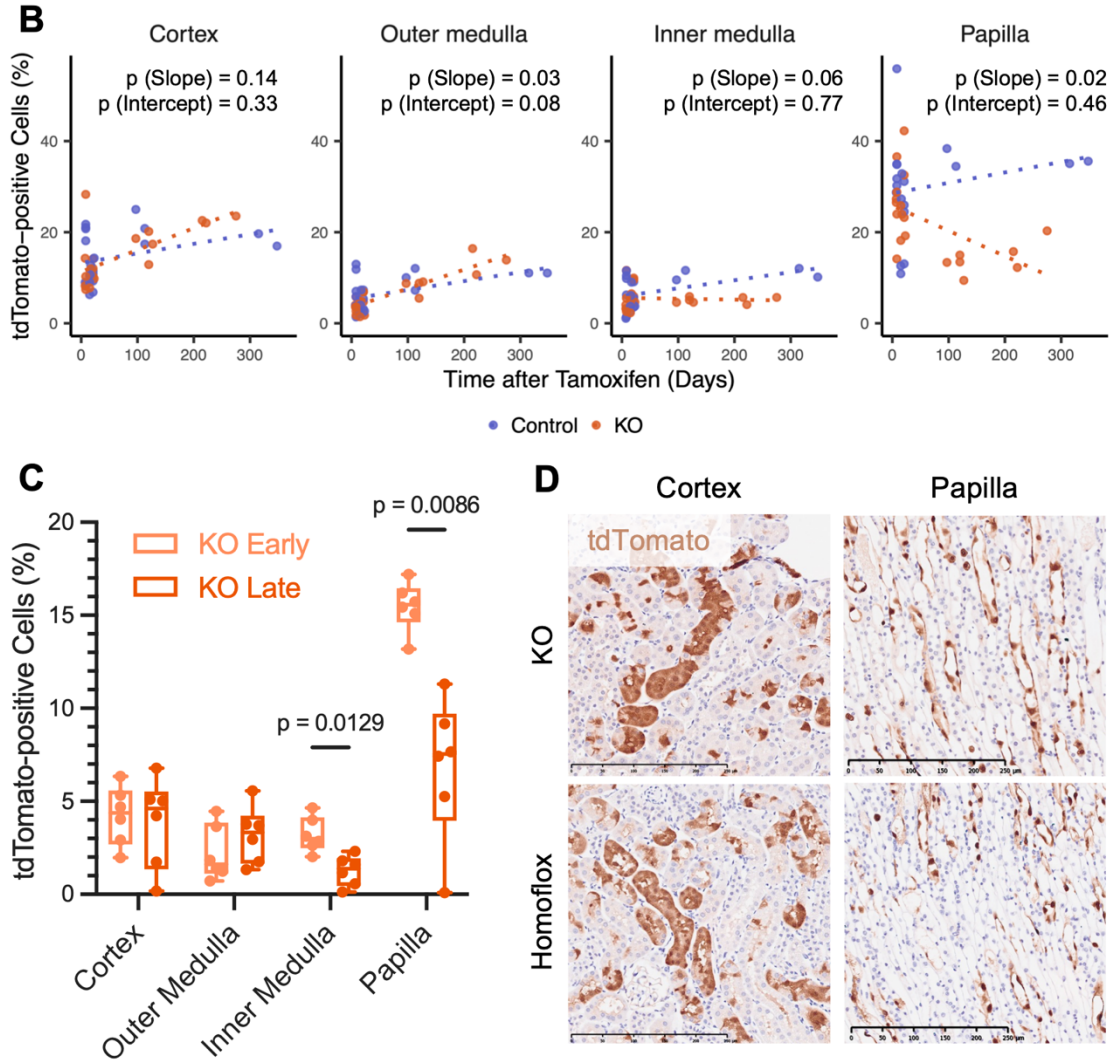


Figure 4.1 (contd.): Region-specific retention of *Vhl*-null cells in the kidney. (B) Proportion of total cells that are tdTomato-positive as quantified by tdTomato IHC in different regions of the kidney of Control or KO mice given 5x 2 mg TMX and harvested at different intervals after recombination ($n = 20$ for KO; $n = 25, 24, 25, 22$ for cortex, outer medulla, inner medulla, and papilla respectively for Control). Dotted lines denote linear regression. Slopes and intercepts of linear regression models compared by t -test with Bonferroni correction. **(C)** Proportion of total cells that are tdTomato-positive as quantified by tdTomato IHC in different regions of the kidney of KO mice given 1 mg TMX and harvested at early or late timepoints ($n = 6$). Median and inter-quartile range plotted. Pairwise comparisons by multiple Mann-Whitney tests with Holm-Šídák correction. Only significant ($p < 0.05$) comparisons shown. **(D)** Representative tdTomato IHC in cortex (left) and papilla (right) of kidneys of KO (top), and Homoflox (bottom) mice given 5x 2 mg TMX and harvested at the late timepoint. Scale bar denotes 250 μ m.

However, this proportion decreased significantly ($p = 0.02$) over time in the papilla and showed a downward trend ($p = 0.06$) in the inner medulla of kidneys from KO mice (**Figure 4.1B**). By contrast, the proportion increased significantly in the outer medulla ($p = 0.03$) and exhibited an upward trend ($p = 0.14$) in the cortex. This suggested that the renal cortex and outer medulla support the long-term retention of *Vhl*-null cells more than do the inner medulla and papilla.

These results were reproduced when mice were dosed with 1 mg TMX instead of 5x 2 mg TMX; comparison between KO mice at the early *versus* late timepoints showed a significant time-dependent decrease in the number of tdTomato-positive cells in the papilla and inner medulla ($p = 0.01$), but not in the cortex and outer medulla (**Figure 4.1C**).

In KO animals, one inactivated *Vhl* allele is inherited and the other is inactivated by targeted recombination by Cre in adulthood; *Vhl*-null cells are thus generated in a *Vhl*-haplosufficient tissue. This differs from most other studies investigating *Vhl* inactivation in the RTE²¹⁹; in these both *Vhl* alleles are recombined simultaneously (either *in utero* or induced in adulthood) and thus *Vhl*-null cells are generated in a *Vhl*-wild type tissue. I tested if the region-specific survival phenotype observed above were dependent on the inheritance of one inactive *Vhl* allele by comparing the numbers of tdTomato-positive cells in *Vhl*^{pjr.fl/pjr.fl}; *Pax8-CreERT2* mice (“Homoflox”) ($n = 4$) given 5x 2 mg TMX harvested at the late timepoint to those observed in Control and KO animals. All Homoflox mice phenocopied the KO mice (**Figure 4.1D**).

The slight increase in cell number in the cortex and outer medulla (**Figure 4.1B**) was associated with a change in distribution of tdTomato-positive cells in these regions. At the early timepoint, tdTomato-positive cells in the cortex and outer medulla of both genotypes often appeared as single cells or in small groups of 2-3 cells in the tubules. This was also seen for Control mice at the late timepoint. However, in the cortex and outer medulla of KO mice at the late timepoint, tdTomato-positive cells were often present in oligocellular juxtaposition

and encompassed the entire circumference of the tubule as seen in the tissue section (**Figures 4.1A** and **4.2**). This suggests that the initial population of *Vhl*-null cells generated in the cortex and outer medulla had expanded over time. Collectively, data from experiments with both doses of TMX indicate that biallelic *Vhl* inactivation confers a survival disadvantage to RTE cells in the renal papilla and inner medulla, but an advantage to those in the cortex and outer medulla.

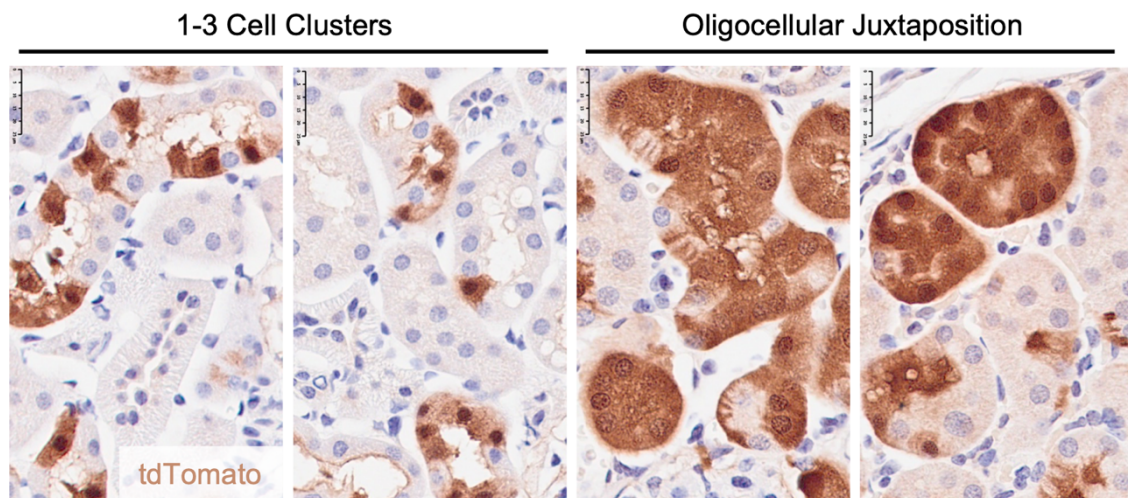


Figure 4.2: Different clustering patterns of tdTomato-positive cells in renal cortex. (A) Representative instances of tdTomato-positive cells appearing as clusters of 1-3 cells (left) in tubules and appearing in oligocellular juxtaposition encompassing entire profiles of the tubule (right) as assayed by tdTomato IHC in cortex of Control or KO mice given 5x 2 mg TMX and harvested at the late timepoint. Scale bar denotes 50 μm .

4.2.2 Effect of *Vhl* inactivation on cells of the Bowman's capsule

The tubular epithelium of the nephron originates with a collection of cells called the Bowman's capsule, which envelopes the glomerulus and acts as a permeability barrier for glomerular filtrate²²⁰. The outer or 'parietal' layer of the Bowman's capsule is continuous with cells of the proximal convoluted tubule. A study that compared the incidence of renal cancer in mice when *Vhl* and *Pbrm1* or *Bap1* were inactivated in different segments of the nephron has proposed that these tumours might arise from cells of the Bowman's capsule¹⁰⁴.

This claim contrasts with the generally held view of the proximal tubular origin of ccRCCs^{88,90}. As the original study used a Cre recombinase allele driven by the same *Pax8* promoter used here, I tested if cells of the Bowman's capsule were targeted by *Pax8-CreERT2* at the 5x 2 mg TMX dose, and if they entrained any survival advantage following *Vhl* inactivation.

The proportion of Bowman's capsules that contained tdTomato-positive cells was analysed by tdTomato IHC in kidneys of Control or KO mice (n = 6 per genotype) given 5x 2 mg TMX and harvested at early or late timepoints. At least 100 Bowman's capsules (spread across 1-3 sections) were counted manually and annotated for each mouse. tdTomato-positive cells were detected in Bowman's capsules in the kidneys of both Control and KO mice at the early timepoint (**Figure 4.3A**), confirming that my experimental system was capable of inducing *Vhl* recombination in these cells in both genotypes. The proportion of capsules containing tdTomato-positive cells did not change with time for either genotype (**Figure 4.3A**), indicating that *Vhl*-null cells were retained in the Bowman's capsule over time. Additionally, in 3 of 6 KO kidneys analysed at the late timepoint (notably all male), 3.3%-3.4% of Bowman's capsules were composed entirely of tdTomato-positive cells; this was not seen in any other condition where only a minority of cells in the capsule were tdTomato-positive (**Figure 4.3B**). This was reminiscent of the phenotype observed in cortical and outer medullary tubules (**Figure 4.1A**) and suggested that *Vhl*-null parietal cells may have divided over the time course of the experiment.

The parietal cell layer of the Bowman's capsule is known to have two morphologies in normal adult mammalian kidneys – a “low-cell” morphology in which the parietal cells have a flat appearance and transition abruptly to the columnar epithelium of the proximal tubule; and a “high-cell” morphology in which the parietal cells appear columnar and exhibit a smooth transition to the proximal tubule²²¹. The relative frequencies of these morphologies in the kidney have been shown to be affected by the sex and age of the mouse²²², as well as

pathologies including alcoholic liver disease, hypertension²²³, and diabetes²²⁴. I tested if *Vhl* inactivation specifically associated with a change in Bowman's capsule morphology. As expected, capsules of both cellular morphologies were detected in kidneys from Control mice (**Figure 4.3C**) with the proportion of "high-cell" capsules not significantly altered either over time or between genotypes (**Figure 4.3D**); this was seen for mice of both sexes (data not shown). Furthermore, no significant difference was observed between genotypes at the late timepoint even when Bowman's capsules containing tdTomato-positive cells were analysed separately (**Figure 4.3E**), indicating that neither the inheritance of an inactive *Vhl* allele nor the presence of *Vhl*-null cells affected parietal cell morphology over the time course of this experiment.

It is worth noting that there was a high degree of variation in this data which is possibly underpowering comparisons between conditions. Additionally, without dual-labelling with cell type specific markers, it is difficult to confidently distinguish 'high-cell' parietal cells from proximal tubular cells that might appear to envelop glomeruli because of the plane of sectioning. Collectively, these results demonstrate that *Vhl*-null cells in the Bowman's capsule are retained and possibly proliferate over time. However, such proliferation is not associated with a change in morphology that can be linked specifically with *Vhl* inactivation.

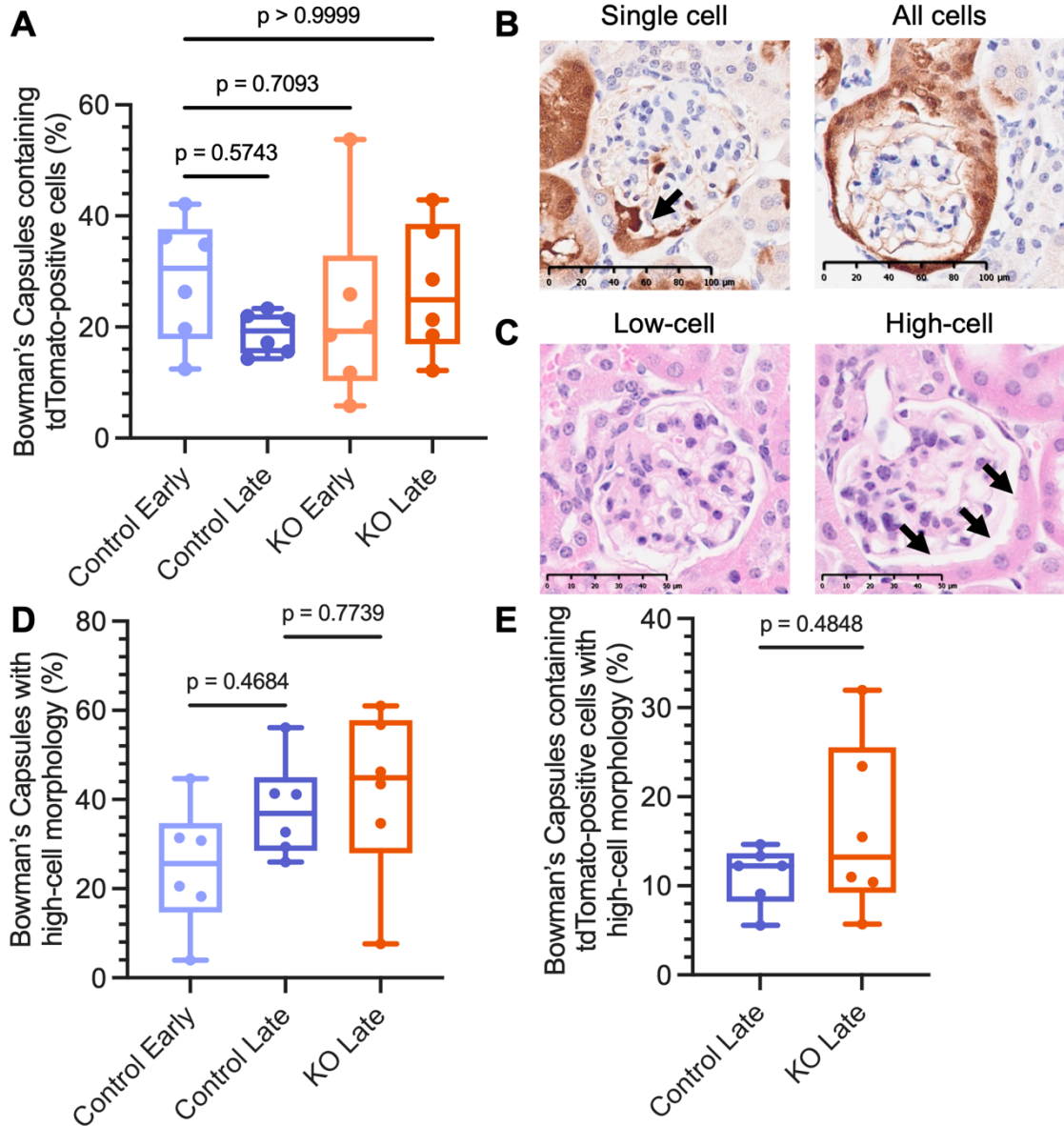


Figure 4.3: Effect of *Vhl* inactivation on the Bowman's capsule. (A) Proportion of Bowman's capsules containing at least one tdTomato-positive cell. (B) Representative Bowman's capsules with single (left) or entirely consisting (right) of tdTomato-positive cells as assayed by tdTomato IHC. Scale bar denotes 100 μm . (C) Representative Bowman's capsules with low-cell (left) or high-cell (right) morphology assayed by H&E staining. Columnar appearance of parietal cells denoted by black arrows. Scale bar denotes 50 μm . (D, E) Proportion of Bowman's capsules with high-cell morphology in total (D) or specifically within those containing tdTomato-positive cells (E). Median and inter-quartile range plotted. Pairwise comparisons by Kruskal-Wallis test with Dunn's correction (A, D) or Mann-Whitney test (E). Data from kidneys from Control or KO mice given 5x 2 mg TMX and harvested at early or late timepoints ($n = 6$).

4.2.3 *Vhl*-null cells exhibit a transient proliferative phenotype

The increased oligocellular juxtaposition of tdTomato-positive cells in kidneys from KO mice over time might arise from three phenomena – 1) cell death or removal of tdTomato-negative cells interleaving tdTomato-positive cells in the tubule, 2) proliferation of tdTomato-positive cells, and 3) migration of tdTomato-positive cells toward one another within the tubule. Unfortunately, migration of cells within the tubules cannot be assayed with my experimental system mainly because kidneys cannot be subjected to tdTomato real-time imaging at multiple timepoints. Additionally, as seen in **Chapter 3**, cell death processes in the kidney could not reliably be assayed by TUNEL assay and c-Caspase-3 IHC for any genotype or timepoint. This left open the hypothesis that *Vhl*-null cells undergo cell division within cortical and medullary tubules early after recombination.

That hypothesis was tested by assaying for the cell proliferation marker²²⁵ Ki67 in tdTomato-positive or -negative cells in kidneys from Control and KO mice at the early and late timepoints using dual-IHC (at least $n = 4$ for each genotype and timepoint). The proportion of tdTomato-positive or -negative cells that were positive for Ki67 was quantified in the renal cortex for both genotypes and timepoints. Double-labelled tdTomato- and Ki67-positive cells in all stages of cell division could be detected in the renal cortex and outer medulla by this assay (**Figure 4.4A**); few if any such cells were detected in inner medulla or papilla. Double-labelled cells were rarely detected in the Bowman's capsule and thus could not be reliably compared across conditions.

Quantification revealed that compared to those from Control kidneys, a significantly greater ($p = 0.01$) proportion of tdTomato-positive cells were Ki67-positive in the cortex of KO mice at the early timepoint (**Figure 4.4B**). Notably, neither Control nor KO mice at the late timepoint exhibited an increased proportion of cells that were both Ki67- and tdTomato-

positive compared to Control at the early timepoint, suggesting that only *Vhl*-null cells at the early timepoint exhibit increased proportion of cells in cycle.

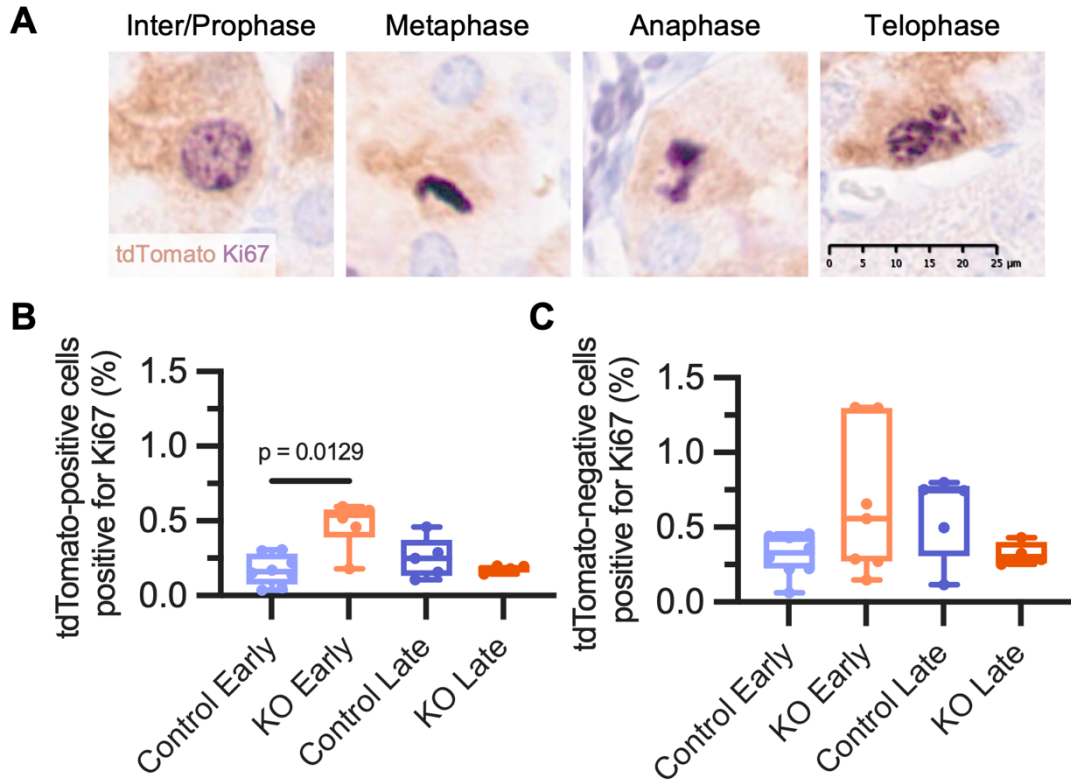


Figure 4.4: Proliferative status of *Vhl*-null cells in the RTE. (A) Representative tdTomato-Ki67 dual IHC depicting tdTomato-positive (brown), Ki67-positive (purple) cells in different stages of the cell cycle. Scale bar denotes 25 μm . (B, C) Proportion of tdTomato-positive (B), or tdTomato-negative (C), cells positive for Ki67 by dual IHC in kidneys of Control and KO mice given 5x 2 mg TMX and harvested at the early and late timepoints ($n = 8, 6, 5, 4$ for Control Early, KO Early, Control Late, and KO Late respectively). Median and inter-quartile range plotted. Pairwise comparisons by Kruskal-Wallis test with Dunn's correction. Only significant ($p < 0.05$) comparisons shown.

To test if this increase in proliferation was a systemic effect of TMX administration to KO mice, Ki67-positivity was also analysed in tdTomato-negative cells of the same kidneys. Ki67-positive tdTomato-negative cells were observed in the cortex and encompassed both tubular and interstitial cell types of the kidney (unlike tdTomato-positive

cells which were restricted by *Pax8-CreERT2* to the RTE). While the absolute proportion of cells positive for Ki67 was higher in tdTomato-negative compared to tdTomato-positive cells, no genotype- and timepoint-specific differences were observed for tdTomato-negative cells as they were for tdTomato-positive cells (**Figure 4.4C**). A greater amount of variability was observed in this dataset than for proliferation in tdTomato-positive cells, which may reflect the fact that the tdTomato-negative cell population in the kidney comprised both RTE and interstitial cell types. These observations indicate that the proliferation is induced specifically within cells that have undergone biallelic *Vhl* inactivation at the early timepoint.

A marked cell must generate two marked neighbouring daughter cells after cell division that share an equal probability of survival, at least in a short timeframe. Therefore, an increased proliferative capacity of marked cells must be associated with a greater chance of detecting recently proliferated marked cells as neighbours in the same tubule. I tested this hypothesis by analysing with dual IHC the incorporation of the deoxythymidine analogue 5-bromo-2'-deoxyuridine (BrdU)²¹⁰ over three weeks in tdTomato-positive or -negative RTE cells from Control and KO mice (n=3) following a single 1 mg TMX dose. A single TMX dose was used to avoid Cre activation in cells after BrdU-labelling.

Consistent with the infrequent detection of Ki67-positive cells in the papilla and inner medulla, few if any tdTomato-positive BrdU-labelled cells were detected in the papilla and inner medulla in kidneys of either genotype (**Figure 4.5A**). Few were seen in the Bowman's capsules too (data not shown). Cells in the outer medulla, however, were extensively BrdU-labelled in both KO and Control kidneys (**Figure 4.5A**). Most of the BrdU-labelled cells were tdTomato-negative, possibly as most cells in the outer medulla do not recombine *Vhl*^{*pjrf*} at this TMX dose (**Chapter 2**). This result suggests that cells in the outer medulla are inherently proliferative in kidneys in both genotypes and makes it difficult to detect *Vhl*-dependent phenotypes against a high level of background BrdU incorporation in this experiment.

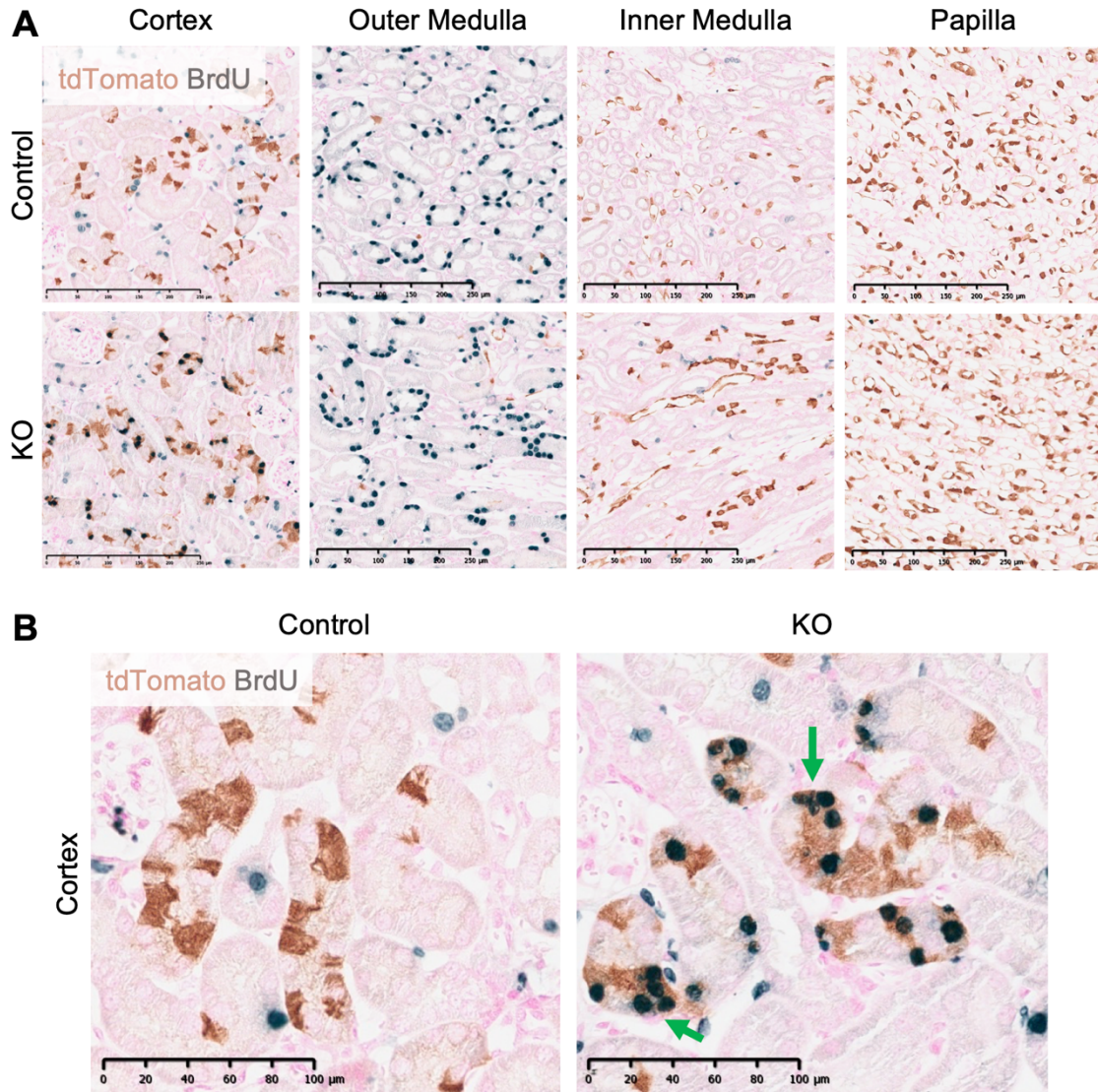


Figure 4.5: Cortical RTE cells proliferate early after *Vhl* loss. (A) Representative tdTomato-BrdU dual IHC depicting tdTomato-positive (brown), BrdU-positive (grey) cells in different renal regions of Control (top) and KO (bottom) mice given 1 mg TMX, continuously administered BrdU, and harvested three weeks after TMX dosing. (B) Higher power magnification view of renal cortex as above. Juxtaposed, recently-proliferated *Vhl*-null cells highlighted by green arrows, Scale bar denotes 250 µm (A) and 100 µm (B).

BrdU-labelled tdTomato-positive and -negative cells were observed in the renal cortex of both genotypes (Figures 4.5A and 4.5B). However, cortices of KO kidneys contained visibly more tdTomato-positive BrdU-positive cells compared to Control kidneys (Figures 4.5A and 4.5B). Moreover, many of these were detected as neighbours in the same

tubules, suggesting that they might be recently divided daughter cells of one *Vhl*-null cell; few if any such neighbouring dual-positive cells were detected in Control kidneys.

Collectively, these analyses indicate that the proliferative capacity of *Vhl*-null cells is impacted by their location in the kidney and changes with time. Biallelic *Vhl* inactivation induces proliferation in cortical cells at the early timepoint that leads to their propagation along the renal tubule, but this proliferative burst is not sustained over time.

4.2.4 Morphological alterations associated with *Vhl* inactivation in the RTE

Vhl inactivation alone has never been associated with tumorigenesis in the RTE, and evidence linking it to increased cyst formation and fibrosis lacks consensus. I thus tested if these phenotypes were reproduced with my experimental system, and to what extent *Vhl*-null cells contributed to their development. Kidneys from KO (n = 8) and Control (n = 11) mice given 5x 2 mg TMX and harvested at the late timepoint were analysed by a combination of H&E staining, Trichrome staining, and tdTomato IHC for the presence of tumours, dysplastic lesions, or cysts. An average of fourteen and nine 4 µm FFPE sections (2-3 sections sampled at 20 µm intervals through the tissue block) were analysed per KO and Control kidney respectively across all staining performed to increase likelihood for detecting rare lesions. No tumour or dysplastic lesion could be detected in any section of any kidney analysed in this experiment, confirming that *Vhl* inactivation is insufficient for tumorigenesis within 12 months of *Vhl* inactivation. Cystic dilatation of renal tubules was detected in 3 of the 8 KO kidneys but no Control kidneys. Interestingly, these were lined with tdTomato-negative cells indicating that these did not arise directly from *Vhl*-null cells and were likely associated with age (**Figure 4.6**).

Sections from 6 of 8 KO kidneys exhibited fibrotic lesions marked by increased collagen deposition and altered inter-tubular space as assayed by Trichrome staining (collagen, cytoplasm and nuclei stained blue, pink, and purple respectively) (**Figure 4.7**). This was seen in sections from only 2 of 11 Control kidneys, suggesting that KO kidneys are more likely to develop these lesions. Notably, kidneys carrying these lesions did not necessarily exhibit globally altered collagen deposition, indicating that the phenotype was focal and rare. KO kidneys harvested at 8 or 10 months after dosing exhibited more lesions than those harvested at 4 months after dosing.

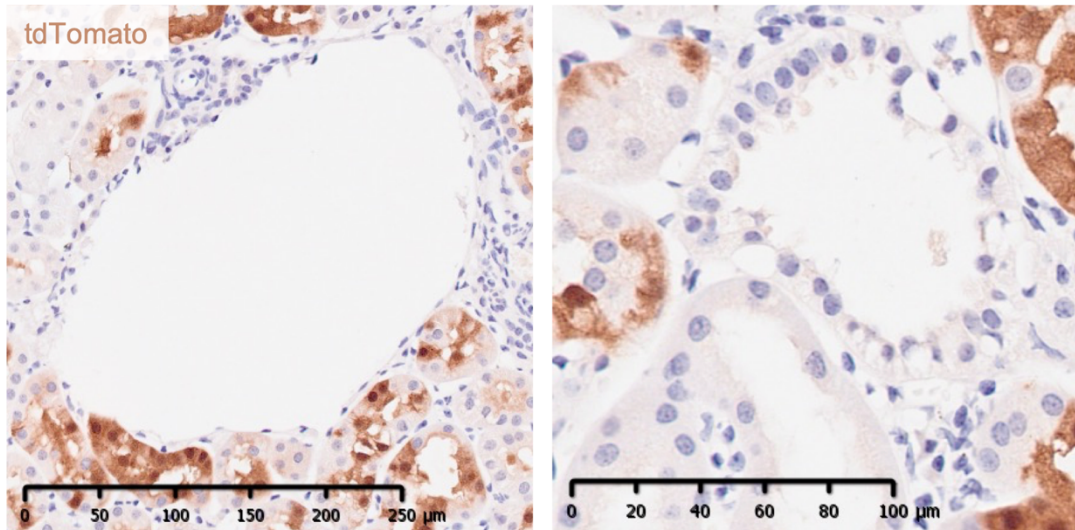


Figure 4.6: Cystic dilatation of renal tubules. Representative tubular cyst (left) and dilated renal tubule (right) observed by tdTomato IHC in kidneys of KO mice given 5x 2 mg TMX and harvested at the late timepoint. Note that cells lining these lesions are tdTomato-negative. Scale bar denotes 250 μm (left) and 100 μm (right).

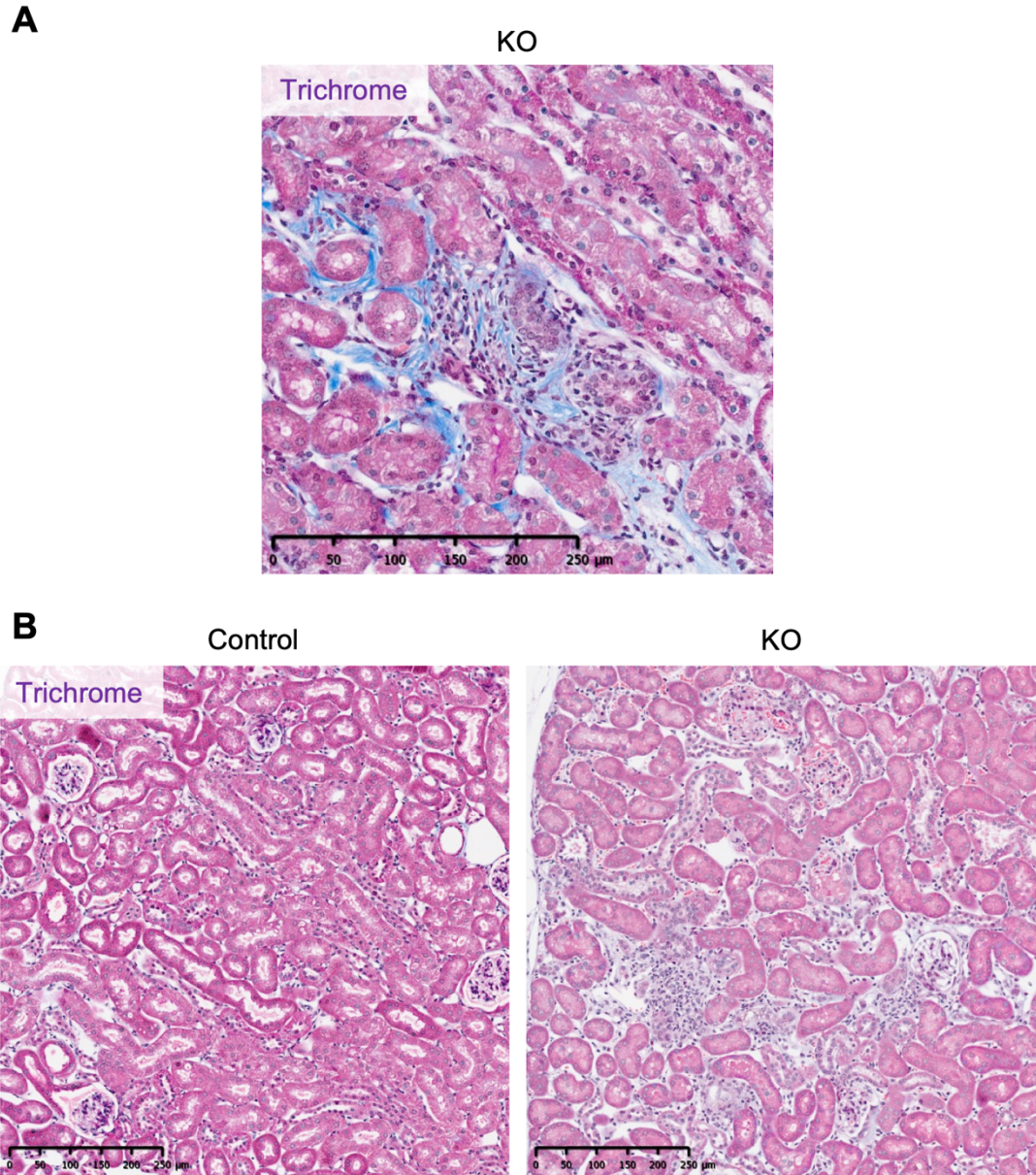


Figure 4.7: Fibrotic lesions in kidneys. (A) Representative fibrotic lesion in kidneys of KO mice given 5x 2 mg TMX and harvested at the late timepoint exhibiting increased collagen deposition (blue) as observed by Trichrome staining. (B) Representative Trichrome staining in kidneys of Control and KO mice given 5x 2 mg TMX and harvested at the late timepoint exhibiting altered inter-tubular space in KO samples. Scale bar denotes 250 μ m.

To test if these phenotypes in KO (*Vhl*^{*flac*.KO/*ptcr*.fl}; *Pax8-CreERT2*) kidneys depended on the inheritance of constitutively inactivated *Vhl* allele and not necessarily on the action of *Vhl*-null cells, kidneys from age-matched mice (10-15 months old) *Pax8-CreERT2*-negative

Vhl^{fl^{ae}.KO/wt} and *Vhl^{fl^{ae}.KO/pjr.fl}* mice, and *Vhl^{fl^{ae}.KO/pjr.fl}*; *Pax8-CreERT2* mice not treated with TMX (n = 13 combined) were analysed. Dilatated renal tubules and fibrotic lesions were detected in 2 and 4 of these kidneys respectively, indicating that a contribution of the inherited *Vhl*-inactivated allele cannot be excluded in interpreting these phenotypes in KO kidneys. Together, these results suggest that with my experimental system and a timeframe of 12 months following *Vhl* recombination, few effects on the morphology of the kidney can be directly linked to the actions of biallelic *Vhl* inactivation.

4.2.5 Immune infiltration in the kidney following *Vhl* inactivation

Previous work has associated loss of *Vhl* in the RTE with increased immune infiltration in the kidney^{128,129,226}; ccRCC is also known to be highly responsive to immunotherapy²²⁷. I tested if *Vhl* inactivation in my experimental system caused an immune phenotype using IHC for the pan-leukocyte marker CD45²⁰⁹.

Immune cells were detected in the inter-tubular space in cortex, outer medulla, and inner medulla, but not the papilla in kidneys of Control (n = 5) and KO (n = 6) mice given 5x 2 mg TMX and harvested at the late timepoint (**Figure 4.8A**). Most were present in groups of 1-3 cells and exhibited an elongated, thin morphology. “Inflammatory foci”, that were identified as clusters of >5 CD45-positive cells with enlarged morphology surrounding renal tubules were also detected in cortex and outer medulla (**Figure 4.8A**).

Quantification of the total number of CD45-positive cells by HALO in the cortex and outer medulla showed an upward trend that was not significant ($p = 0.20$) in the immune cell population in kidneys of KO compared to Control mice (n = 4) (**Figure 4.8B**). However, when only the numbers of ‘inflammatory foci’ were quantified, a significant increase ($p = 0.04$) was revealed in kidneys of KO mice (n = 6) compared to Control mice (n = 5) (**Figure 4.8C**). Kidneys of age-matched mice (10-15 months old) *Pax8-CreERT2*-negative *Vhl^{fl^{ae}.KO/wt}* (termed “Het-KO”) mice (n = 3) exhibited no difference in the numbers of inflammatory foci

compared to Control ($p > 0.99$) (**Figure 4.8C**), indicating that the phenotype in kidneys of KO mice is independent of the inheritance of one inactive *Vhl* allele.

The majority of CD45-positive cells in the kidney is composed of macrophages, most of which are ‘kidney resident macrophages’ that seed into the kidney during development; they are self-maintaining innate immune cells characterised by high expression of F4/80²⁰⁷ and perform functions in normal tissue development, homeostasis and repair of damaged tissue^{228,229}. I thus tested if the inflammatory foci were derived from these macrophages. F4/80 IHC in KO ($n = 5$) and Control ($n = 6$) kidneys at the late timepoint showed clusters or foci of macrophages like that seen for CD45-positive immune cells (**Figure 4.8D**), suggesting that inflammatory foci are composed of at least some renal macrophages.

Chronic inflammation in the kidney is known to cause renal fibrosis. Kidney macrophages have known roles in this process, wherein they undergo a ‘macrophage-to-myofibroblast’ transition and contribute to the deposition of extracellular matrix components such as collagen²³⁰. Such cells can be identified by the co-expression of the immune marker CD45 and myofibroblast marker α -SMA²³⁰. Analysis of CD45- and α -SMA-positivity in serial sections of inflammatory foci described above showed no co-expression of these markers in any focus across KO kidneys given 5x 2 mg TMX harvested at the late timepoint ($n = 6$) (**Figure 4.9**), suggesting that these did not comprise myofibroblasts.

Collectively, these results indicate that long-term retention of *Vhl*-null cells associates with increased inflammation in the renal cortex and outer medulla.

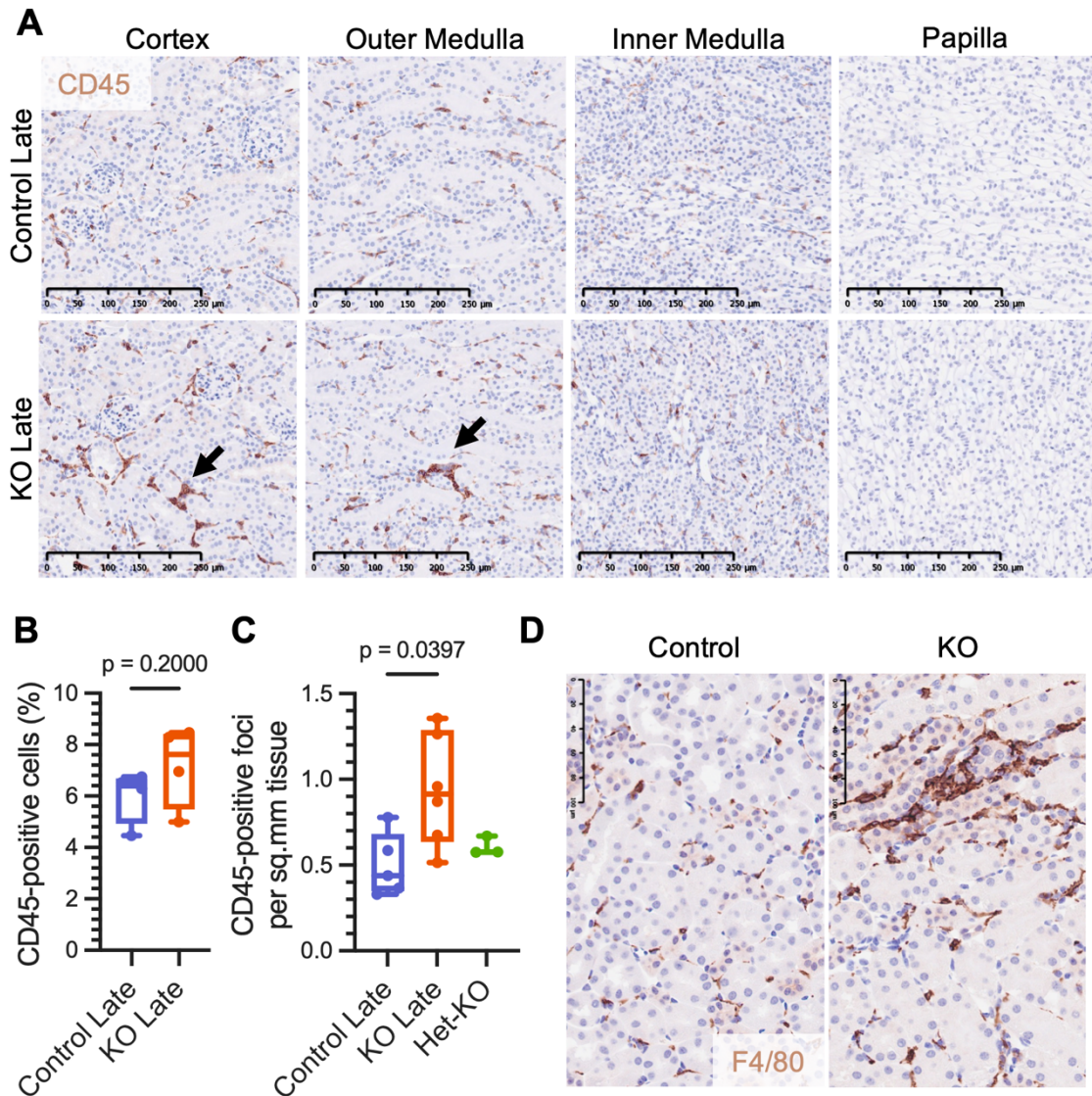


Figure 4.8: *Vhl* inactivation in the RTE is associated with inflammatory foci. (A) Representative CD45 IHC in different renal regions of Control and KO mice given 5x 2 mg TMX and harvested at the late timepoint. Inflammatory foci denoted by black arrows. Scale bar denotes 250 μ m. (B, C) Proportion of cortical and outer medullary cells that are CD45 positive as assayed by CD45 IHC in Control and KO mice (n = 4) given 5x 2 mg TMX and harvested at late timepoint (B). Number of inflammatory foci per mm² of cortical and outer medullary tissue as detected by CD45 IHC in Control (n = 5) and KO (n = 6) mice given 5x 2 mg TMX and harvested at late timepoint and age-matched het-KO mice (n = 3) (C). Median and inter-quartile range plotted. Pairwise comparisons by Mann-Whitney test (B) and Kruskal-Wallis test with Dunn's correction (C). (D) Representative F4/80 IHC in kidneys of Control (n = 6) and KO (n = 5) mice given 5x 2 mg TMX and harvested at the late timepoint depicting focus of macrophages. Scale bar denotes 100 μ m.

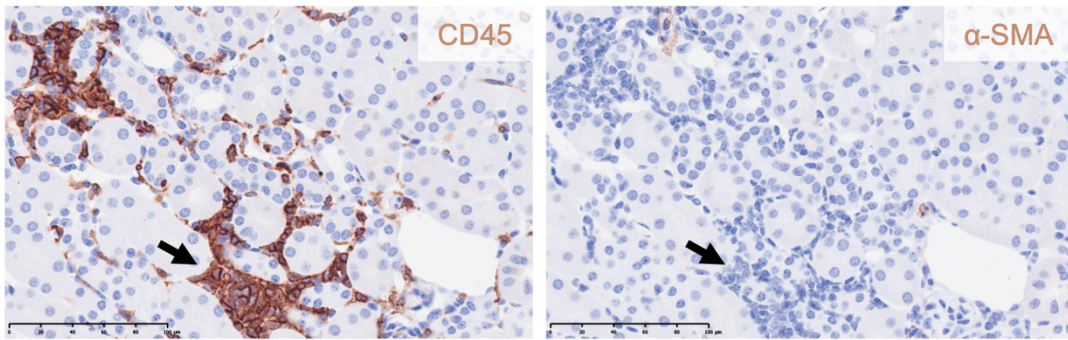


Figure 4.9: Inflammatory foci do not associate with markers for myofibroblasts. Representative inflammatory focus in serial cortical sections from KO mouse given 5x 2 mg TMX harvested at late timepoint stained for CD45 (left) and α -SMA (right) by IHC. Scale bar denotes 100 μ m. Inflammatory foci observed in serial sections denoted by black arrows.

4.3 Discussion

Here, I have employed the model system described in **Chapter 2**, and have considered the following questions:

1. Does *Vhl* inactivation in the RTE impact on the long-term survival and proliferation of cells?
2. If so, are these impacts specific to nephron segments and how does that relate to observed tissue-specificity in ccRCC?
3. How is the renal interstitium altered in association with presence of *Vhl*-null cells in the RTE?

I provide evidence that the survival of *Vhl*-null cells in the RTE for a year following *Vhl* inactivation is region-specific - *Vhl* inactivation confers a survival advantage in the cortex and outer medulla (and possibly the Bowman's capsule) but a disadvantage in the inner medulla and papilla (**Figures 4.1-4.3**). Associated with this advantage, entry into cell cycle was identified *in vivo* as a tissue-specific early consequence of *Vhl* inactivation; this effect was short-lived, and the early proliferative advantage lost between 3 weeks and 4 months following *Vhl* inactivation (**Figures 4.4 and 4.5**). Analysis of morphological changes in renal

tubules showed little that could be linked specifically to biallelic *Vhl* inactivation in RTE cells (**Figures 4.6** and **4.7**). Finally, long-term retention of *Vhl*-null cells in RTE was found to associate with a change in the organisation of renal immune cells, especially macrophages, resulting in the formation of discrete inflammatory foci (**Figures 4.8** and **4.9**); this response too was region-specific. These findings provide the first description of tissue-dependent cell fates entrained by *Vhl* inactivation *in vivo* in the RTE and reveal new insights on tissue-specific tumour suppression by *Vhl* for further investigation.

The experimental approach used differs from previous work on *VHL* inactivation in the kidney in two ways. First, phenotypes within *Vhl*-null cells are assessed with cellular resolution and distinguished from those in their *Vhl*-competent neighbours using the marking capabilities of the model system. Second, differences in initial recombination and cellular turnover in different regions of the kidneys are considered by comparing phenotypes in marked *Vhl*-null cells to marked *Vhl*-competent controls. These features of the model have allowed elucidation of phenotypes that might have otherwise been missed in unmarked mouse models of *Vhl* inactivation.

The distinct survival and proliferative phenotypes exhibited by *Vhl*-null cells in the cortex and outer medulla compared to the inner medulla and papilla provide empirical *in vivo* evidence for tissue-specific effects of *VHL* inactivation in the RTE. Positive effects observed in the cortex and outer medulla here are supported by previous work with primary RPTEC culture (derived from cortical and outer medullary proximal tubules) which showed that *Vhl*-null RPTECs can survive at least 25 days of culture under physiological oxygen tension^{120,122}, and that *VHL* inactivation confers proliferative advantage over *VHL*-competent cells at confluency¹²³. Negative effects observed here in the inner medulla and papilla are supported by and explain previous work with *Vhl*^{*fl/fl*}; *Pax8-CreERT2* animals which showed a small decrease in the total amount of recombined *Vhl* allele in kidneys over time¹⁰⁹. These findings also corroborate data from ccRCC patients which noted the presence of CA9-positive cells

in the cortical but not medullary tubules in normal tumour adjacent tissues of renal biopsies¹¹⁴. Taken together, the findings highlight the cortical and outer medullary RTE as ‘permissive’ and the inner medullary and papillary RTE as ‘non-permissive’ to *VHL* inactivation. Long-term survival in ‘permissive’ but not ‘non-permissive’ tissues might provide opportunities for cells to incur further mutations or experience other stimuli or insults, thereby supporting nephron-segment specific oncogenesis.

A striking discovery made here is of the transiency of proliferation entrained by *Vhl* inactivation in the cortical RTE – Ki67-positivity within *Vhl*-null cells increased within a 1-3 week time window following recombination but subsided to baseline levels within 4 months following *Vhl* inactivation. This alteration in the behaviour of *Vhl*-null cells is suggestive of an onco-protective adaptation in cells over time. These observations are partially supported by the absence of proliferative phenotypes observed by others at 6 or 12 months after inducing *Vhl* inactivation in proximal tubules¹⁴¹, or when *Vhl* is constitutively inactivated in the distal nephron *in utero*^{47,106}. It is possible that these published studies assayed proliferation only after the initial burst had subsided.

The mechanisms of both tissue-specific survival and proliferation associated with *VHL* inactivation need further study. The mechanisms underlying loss of cells from the papilla and inner medulla have been technically difficult to assay *in situ* (as seen in **Chapter 3**). No signs of increased apoptosis could be detected in the kidney by TUNEL or c-Caspase-3 IHC (data not shown). However, as apoptotic processes are short lived, gradual loss of cells taking place over months might not be captured by such assays. No CD45-positive cell was detected in any papilla analysed in kidneys of both genotypes and timepoints; this is evidence against the possibility that *Vhl*-null cells were being removed by the action of resident renal immune cells. As discussed in **Chapter 3**, *Vhl*-null cells in the inner medulla or papilla might be eliminated from the RTE by extrusion into the urine, though capturing this elimination process that occurs gradually over months might also be difficult.

Inactivation of tumour suppressor genes has been linked generally to release of mitotic restraints and dysregulated cell cycle progression²³¹. Additionally, HIF2A, which is stabilised in RTE cells after *Vhl* inactivation (**Chapter 2** and other work¹⁴⁵) has been shown to co-operate with PAX8 to upregulate expression of cyclin D1 (*CCND1*), which regulates transition from the G1 to S phase of the cell cycle^{13,123,143}. This may explain why cells enter cell cycle early after *Vhl* inactivation, but does not justify the tissue-specificity or temporality of the phenomenon. Considering that in the cortex *Vhl*-null cells expanded to occupy the profile of tubules but did not form dysplastic lesions, it is possible that the constraints of tubular anatomy are responsible for limiting proliferation. Whether and how this occurs merits further investigation.

An interesting association with the long-term retention of *Vhl*-null cells in tissue is of the reorganisation or activation of the renal immune compartment that was not necessarily linked to fibrosis or cystic dilatation of tubules. Surprisingly, immune phenotypes were observed in regions where *Vhl*-null cells had survived and proliferated, but not in regions from which *Vhl*-null cells were lost. This suggests that communication between *Vhl*-null RTE cells and renal immune cells might contribute either to their long-term survival or alternatively an onco-protective restraint on their proliferation. The cellular profiles and signalling pathways contributing to these phenotypes need further study.

The data presented in this chapter informs on the effects of *Vhl* inactivation in different regions of the kidney, each of which contains anatomically distinct parts of the nephron²³². However, it does not distinguish between different cell types found within the same renal region (e.g., proximal tubule *versus* distal tubule within the cortex), mainly because of technical difficulties in detecting co-expression of tdTomato and cell-type specific markers *in situ* by IHC, IF, or fluorescence microscopy. However, single-cell sequencing approaches can be used to gain cell-type resolution in this analysis.

Altogether, data in this chapter has illuminated hitherto unseen tissue- and time-dependent cellular effects of *VHL* inactivation *in vivo* in the RTE. Based on the absence of dysplasia and tumorigenesis, it has been presumed that *VHL* inactivation has little bearing on oncogenesis on its own in the absence of further perturbations of ccRCC-associated genes such *PBRM1*, *BAP1*, or *TP53*^{104,105,110,112,113}. While tumours were not observed in this study, multiple effects of potential relevance to oncogenesis were observed including cell-type specific proliferation, cell-type specific elimination, and long-term changes in the renal epithelium and interstitium.

Chapter 5:

Cell-Type Specific Transcriptomic Effects of *Vhl* Inactivation in the RTE

5.1 Introduction

VHL is the recognition component of a ubiquitin E3 ligase complex that targets hypoxia inducible transcription factors (HIF1A and HIF2A) for conditional oxygen-dependent degradation³⁴, and has also been implicated in a range of other roles including cytoskeletal functions⁴⁵ and regulation of the extracellular matrix⁴⁰ and other growth and inflammatory signalling pathways^{216,217}. Stabilisation of both isoforms of HIF – HIF1A and HIF2A – has been demonstrated after *Vhl* inactivation in the RTE in mice¹⁴⁵. Genes regulated by physiological HIF stabilisation in hypoxia, pathological HIF stabilisation by perturbation of the VHL/HIF signalling pathway, and after *VHL* inactivation have been extensively characterised in cell culture by a combination of RT-qPCR, microarray analysis, and bulk RNA-seq¹⁴⁹⁻¹⁵⁴. These studies demonstrate that the transcriptional effects of *Vhl* inactivation or HIF stabilisation are often cell-type specific. Genome-wide changes in gene expression following *Vhl* inactivation have been studied in the kidney *in vivo* but only in the context of cancer¹¹¹⁻¹¹³, ischaemic injury¹⁵⁶, and cystic or fibrotic disease²²⁶. Changes in affected cells were not distinguished from secondary effects in *Vhl* competent cells. Additionally, neither cell-type specific responses to *Vhl* inactivation nor responses entrained in rare cell populations were assayed.

Data presented in this thesis has demonstrated that *Vhl* inactivation has time-dependent, region-specific effects on the survival and proliferation cells in tissue that also associate with changes entrained in the renal interstitium (**Chapter 4**). Whether and how

these changes relate to cell-type specific and time-dependent gene expression changes caused by *Vhl* inactivation in cells of the RTE remains to be investigated.

Recently single-cell sequencing technologies have been applied to the kidney, providing a wealth of data on gene expression patterns, on nephron heterogeneity and on cellular responses in non-neoplastic disease^{89,179-182}. In this chapter, I employ the model described in **Chapter 2** and single-cell RNA sequencing technology to:

1. Identify the cell types within the RTE cells targeted by *Pax8-CreERT2* in my experimental system.
2. Describe gene expression changes that occur after *Vhl* inactivation in recombined and unrecombined cells in different parts of the RTE *vivo* in mice.
3. Assess whether responses to *Vhl* inactivation differ in different parts of the RTE.
4. Trace the evolution of gene expression in *Vhl*-null RTE or *Vhl*-competent RTE and renal interstitial cells for a year following *Vhl* inactivation.
5. Assess whether gene expression entrained over time relates to tissue-specific survival, proliferation, and inflammation observed previously.
6. Understand how changes caused by *Vhl* inactivation relate to gene expression in human ccRCC and generate insights into the necessity but insufficiency of *Vhl* inactivation for tumorigenesis.

5.2 Results

5.2.1 Renal cell heterogeneity in scRNA-seq data from flow-sorted renal cells

Single-cell RNA sequencing (scRNA-seq) was performed on FAC-sorted tdTomato-positive and -negative cells from Control (*Vhl*^{wt/pjr.fl}; *Pax8-CreERT2*) and KO (*Vhl*^{lae.KO/pjr.fl}; *Pax8-CreERT2*) mice given 5x 2 mg TMX and harvested at the early timepoint (1-3 weeks after the first TMX dose). After filtering for cell quality (see **Chapter 8**), I obtained data from 232,101 high-quality cells across 15 samples from 8 mice of both sexes, with an average

of $15,473 \pm 5731$ (SD) cells per sample, and $3,265 \pm 1490$ (SD) transcripts per cell that covered $1,281 \pm 380$ (SD) genes detected per cell per sample.

Cell types present in tdTomato-positive and -negative samples from Control mice were analysed first. This was done with two aims – first, to assess the recovery of renal cell populations after sorting and sequencing and thereby identify contexts in which the effects of *Vhl* inactivation can be assayed, and second, to identify cell populations targeted by *Pax8-CreERT2* with the given TMX dose. Most published scRNA-seq studies have identified cell types by clustering cells based on their global transcriptomic profile using dimension reduction analysis, identifying the top enriched genes in each cluster, and then matching those with well-characterised ‘gold-standard’ cell-type specific marker genes¹⁸³. This approach has been used by several groups to define renal cell populations from wild-type mice of both sexes and has generated a largely validated list of cell-type specific marker genes for renal cell populations¹⁷⁹⁻¹⁸². As the mice used in my study are not ‘wild-type’ for *Vhl*, and to avoid the potential effects of monoallelic or biallelic *Vhl* inactivation on dimension reduction from confounding cell type assignment, I determined cell types in this data by analysing the expression of reported renal cell type-specific marker genes in individual cells without reference to dimension reduction (see **Chapter 8**).

A range of renal cell types were recovered in tdTomato-negative samples from Control mice, including epithelial parietal (PEC), proximal tubular (PT), distal tubular (DCT), and collecting duct cells; non-epithelial interstitial and vascular cells and multiple immune cells subsets including macrophages, monocytes, granulocytes, NK cells, B cells and T cells (**Figure 5.1**); Loop of Henle (LoH) cells were not recovered.

By comparison, tdTomato-positive samples from the same mice contained mostly proximal tubular and intercalated collecting duct (CDIC) cells, consistent with the reported tubular epithelial (RTE) restriction of *Pax8-CreERT2*¹³⁷. Although tdTomato-positive cells were seen by IHC in the inner medulla where cells of the Loop of Henle reside (**Chapter 2**

and 4), these were not recovered after scRNA-seq. It is possible that the LoH cells and other cell types in the non-proximal tubular nephron were lost during the tissue dissociation and sorting steps. Interestingly, while distal tubular cells were present in tdTomato-negative samples, few were present in tdTomato-positive samples, suggesting that *Pax8-CreERT2* does not target this cell type at the given TMX dose.

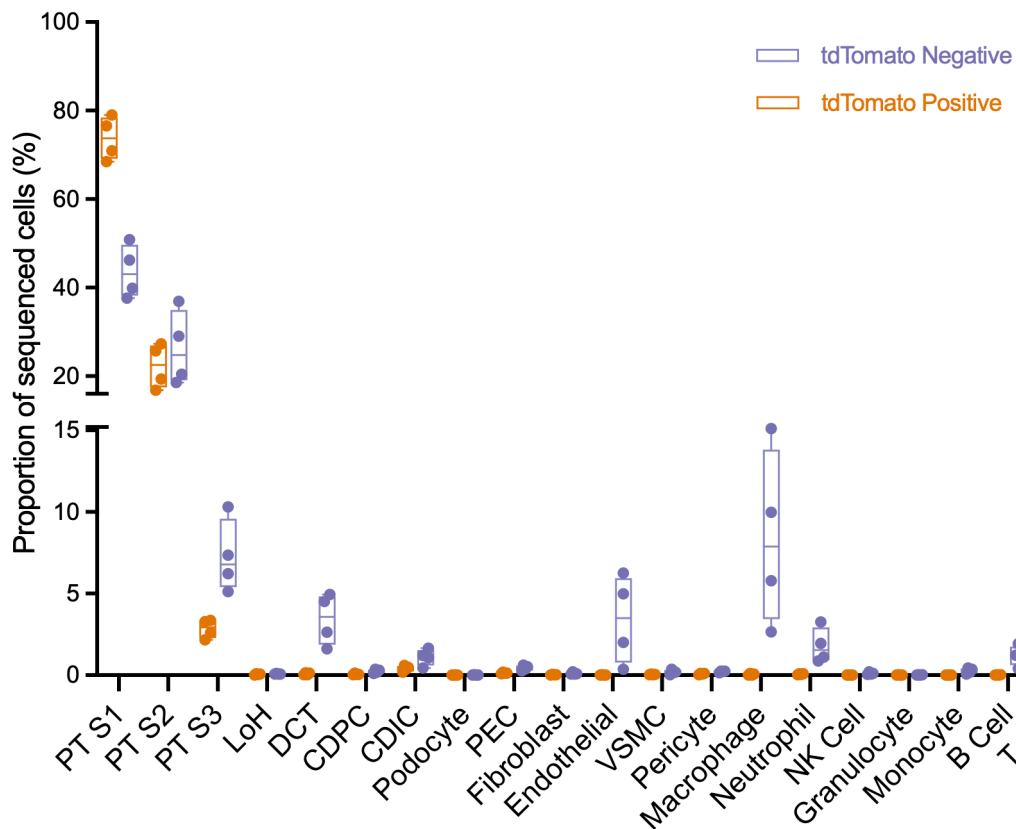


Figure 5.1: Cell types recovered after scRNA-seq. Proportion of total cells inferred to be of each cell type in tdTomato-positive or tdTomato-negative populations from kidneys of female ($n = 3$ (3F)) and male ($n = 1$ (1M)) Control mice harvested at the early timepoint. Median and inter-quartile range plotted. PT – Proximal Tubule; LoH – Loop of Henle; DCT – Distal Convolutd Tubule; CDPC – Collecting duct principal cell, CDIC – Collecting duct intercalated cell; PEC – Parietal epithelial cell; VSMC – Vascular Smooth Muscle Cell; NK – Natural Killer cell.

5.2.2 A novel dichotomy in patterns of proximal tubular gene expression

To validate cell type assignment, and to identify additional determinants of heterogeneity in the data, dimension reduction and clustering was performed using the 2,000 most variable genes in the dataset. Gene expression in RTE cells, and PT cells in particular, has been reported to be highly sex-specific^{182,233,234}. This was seen in my data too, as PT cells (but no other cell types) from male and female mice occupied distinct positions by uniform manifold approximation and projection (UMAP) (**Figure 5.2A**). Excluding sex-specific genes from the dimension reduction analysis (performed by batch correction; see **Chapter 8**) was sufficient for PT cells from the two sexes to occupy the same UMAP space (**Figure 5.2B**). This sex-corrected dimension reduction was used for further analysis as it highlighted better the heterogeneity present within individual samples. It should be noted that batch correction only affects the UMAP analysis but does not alter the raw data on gene expression; other downstream analyses such as differential expression testing are not affected.

Agreement between assigned cell types and global gene expression was then tested. In 36 of the 44 clusters resolved, >80% of cells within the cluster were from one or two closely related cell types as defined by reference to external published data¹⁷⁹⁻¹⁸² (**Figure 5.3**). This confirmed that cells expressing a set of cell type markers also shared global transcriptomic profiles.

Renal PT cells can be subdivided into S1 and S2 cells of the cortical convoluted tubule, and S3 cells of the outer medullary straight tubule²³⁵. Unexpectedly I found that each of these three cell types mapped to two distinct regions on the UMAP plot irrespective of tdTomato expression (**Figure 5.4**). This suggested that a determinant, independent of established cell type identity and sex, dichotomised gene expression in PT cells.

To identify this determinant, I analysed the 1,200 most variable genes within PT cells for mutually anti-correlated expression. Spearman correlations were calculated for every pair-wise combination of the variable genes and plotted onto a gene-by-gene matrix ordered

by hierarchical clustering (**Figure 5.5A**). This revealed two areas with a high degree of anti-correlation, resulting from genes in clusters A-F (termed PT Module A) and clusters G-H (termed PT Module B) (**Figures 5.5B** and **5.5C**). Genes within each module are listed in **Appendix 1**.

Scoring cells for expression of these modules effectively segregated S1, S2, and S3 cells, but not non-PT cells, into two populations (**Figure 5.6A**) that mapped to the two regions on the UMAP plot occupied by PT cells (**Figure 5.6B**). Genes in PT Module A included activator protein 1 (AP-1) transcription factors (*Jun* and *Fos*; **Appendix 1**) that are often upregulated in response to cellular stress. To test if PT Module A expression was a consequence of cellular stress imparted by tissue dissociation, sorting, or sequencing, I assayed for expression of candidate PT Module A and PT Module B genes in FFPE kidney sections from Control mice harvested at the early timepoint. Dual *in situ* RNA hybridisation for *Neat1* (for PT Module A) and *Fxyd2* (for PT Module B) demonstrated distinct spatial localisation of the two transcripts, thus confirming that this cellular segregation was not an artefact of the scRNA-seq protocol (**Figure 5.6C**). Cells with distinct *Neat1* or *Fxyd2* expression were often neighbours within the same tubule. scRNA-seq datasets gathered from renal cells from healthy adult wild-type mice not given TMX are available for public interrogation (see **Chapter 8**). Examination of these datasets also revealed anti-correlated expression of *Neat1* and *Fxyd2* in distinct populations of PT cells^{236,237} (**Figure 5.6D**), indicating that this dichotomy is not consequent on the administration of tamoxifen or on the transgenic nature of mice used in this study. I subsequently refer to cells with high PT Module A expression as “PT Class A” cells, and the others as “PT Class B” cells

Overall, this initial analysis demonstrated that renal cells could be successfully sorted and sequenced, that recombination was induced in the proximal nephron and the collecting duct but not distal convoluted tubules, and that the ‘class’ of PT cells can be considered in subsequent analysis in addition to the cell type.

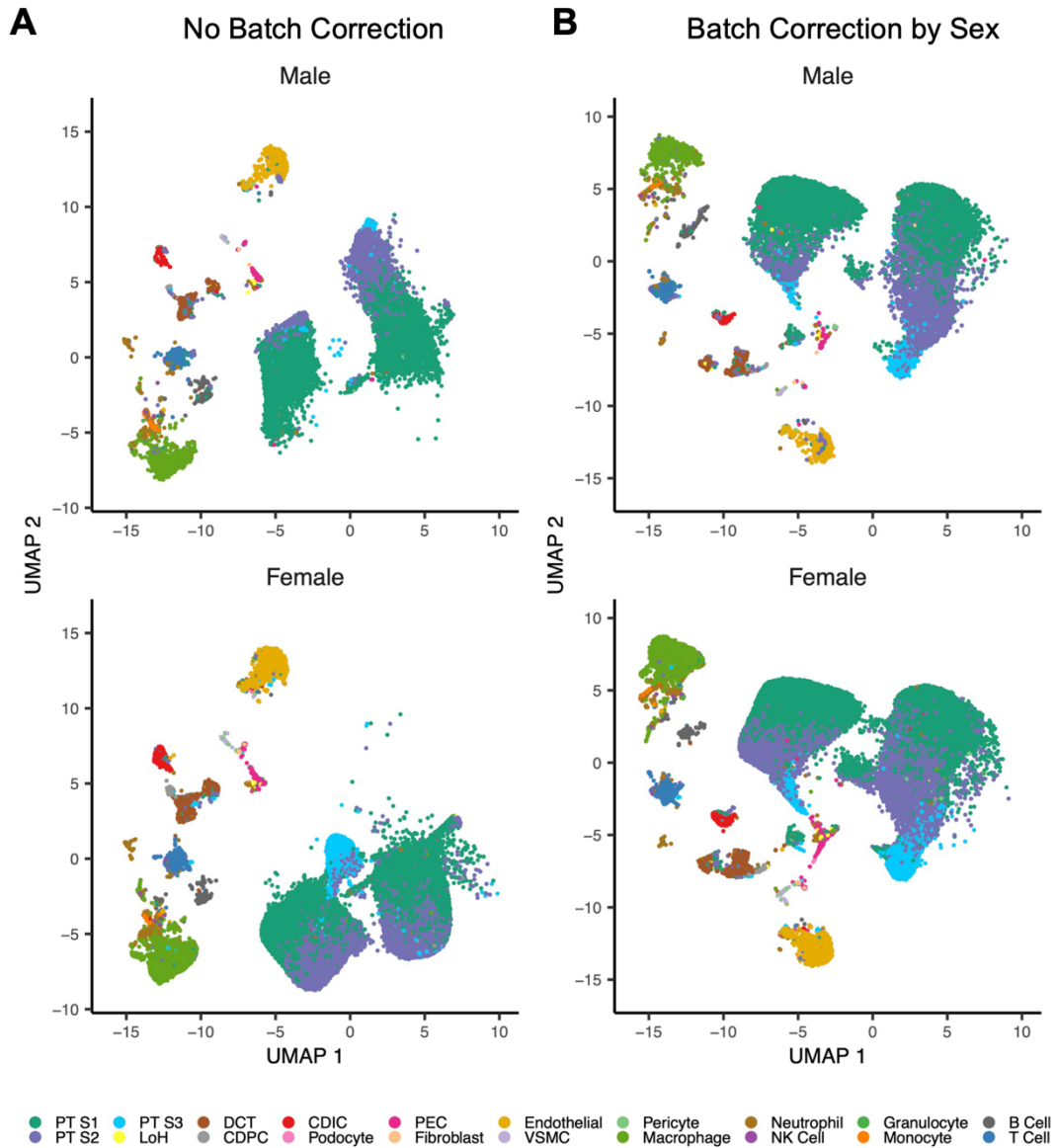


Figure 5.2: Sex-specific clustering of PT cells avoided by batch correction. (A, B) UMAP plot of renal cells from male (n = 1; top) or female (n = 3; bottom) Control mice given 5x 2 mg TMX and harvested at the early timepoint, without batch correction (**A**) or after batch correction for sex (**B**). Cells are coloured by inferred cell type. PT – Proximal Tubule; LoH – Loop of Henle; DCT – Distal Convolved Tubule; CDPC – Collecting duct principal cell, CDIC – Collecting duct intercalated cell; PEC – Parietal epithelial cell; VSMC – Vascular Smooth Muscle Cell; NK – Natural Killer cell. Batch correction performed by Dr Ran Li.

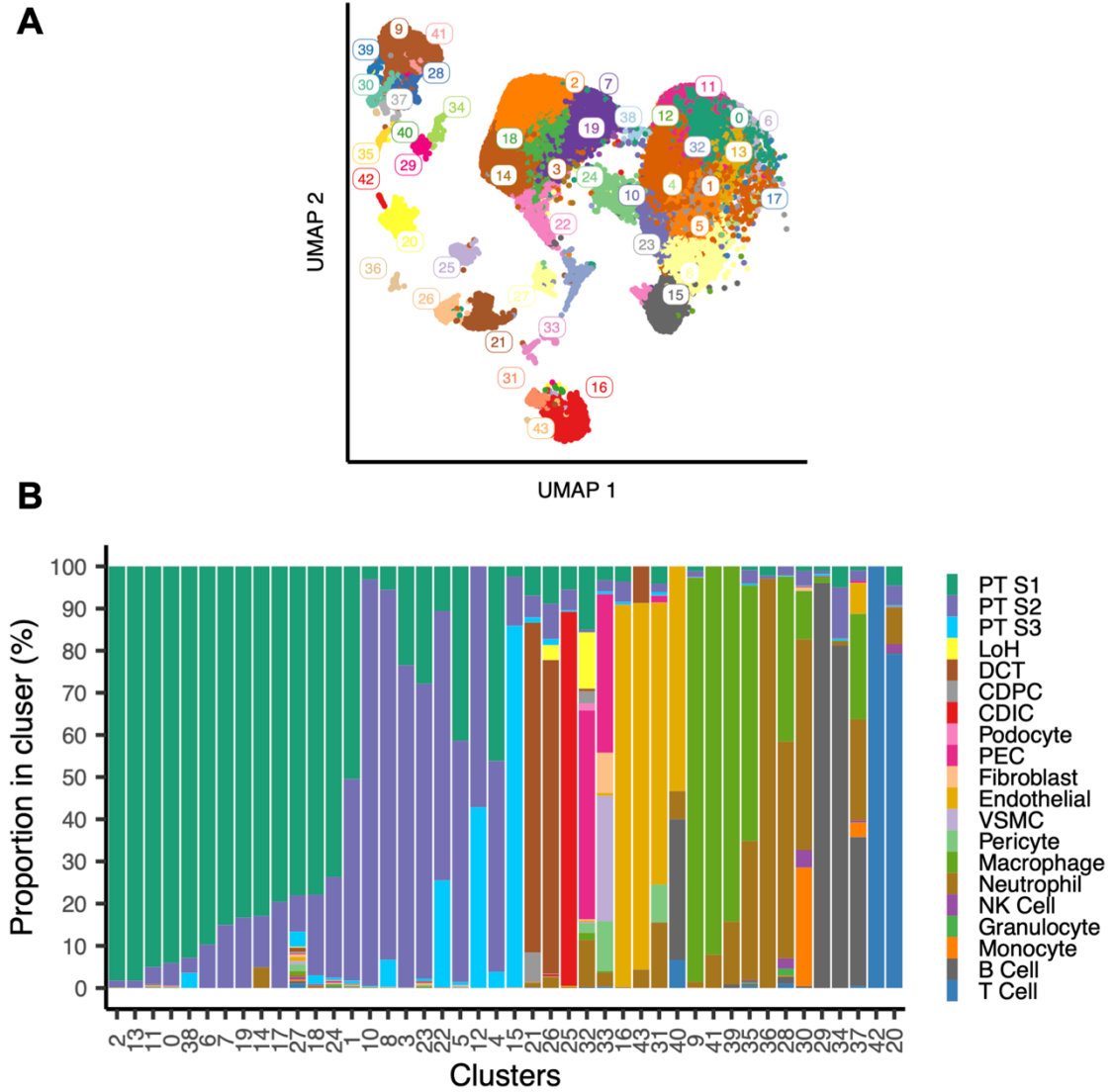


Figure 5.3: Cell type assignment agrees with global transcriptomic profile. (A) UMAP plot of renal cells from Control mice (n = 3F, 1M) given 5x 2 mg TMX and harvested at the early timepoint coloured by UMAP clusters. **(B)** Cell-type composition of each cluster. PT – Proximal Tubule; LoH – Loop of Henle; DCT – Distal Convolved Tubule; CDPC – Collecting duct principal cell, CDIC – Collecting duct intercalated cell; PEC – Parietal epithelial cell; VSMC – Vascular Smooth Muscle Cell; NK – Natural Killer cell.

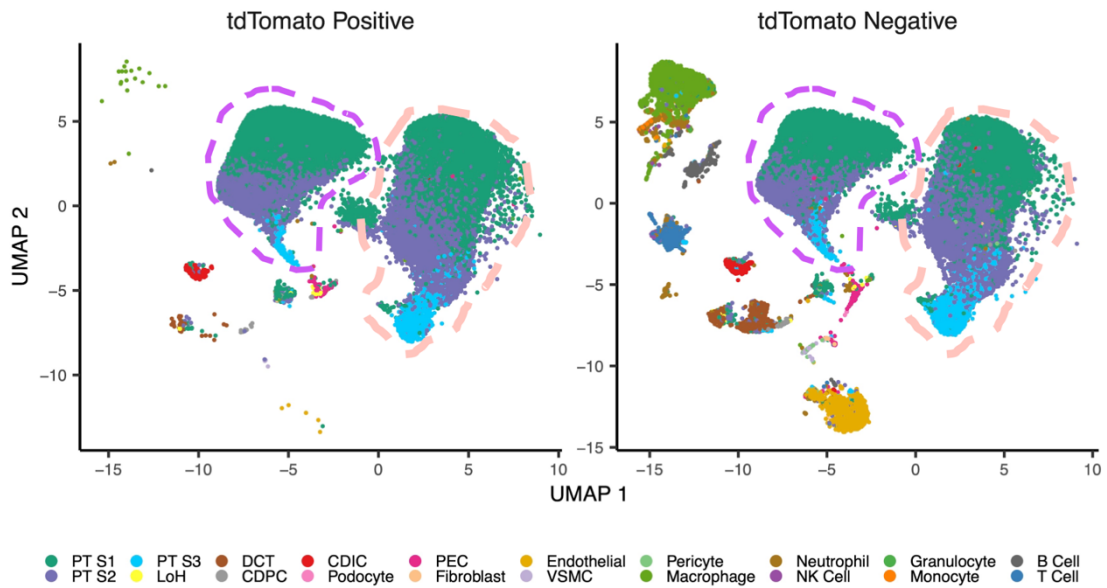


Figure 5.4: Two populations of proximal tubular cells. UMAP plot of tdTomato-positive (left) or tdTomato-negative (right) cells from Control mice ($n = 3F, 1M$) given 5×2 mg TMX and harvested at the early timepoint. Cells are coloured by inferred cell type. Two populations of PT cells denoted by pink and purple dashed lines. PT – Proximal Tubule; LoH – Loop of Henle; DCT – Distal Convolved Tubule; CDPC – Collecting duct principal cell, CDIC – Collecting duct intercalated cell; PEC – Parietal epithelial cell; VSMC – Vascular Smooth Muscle Cell; NK – Natural Killer cell.

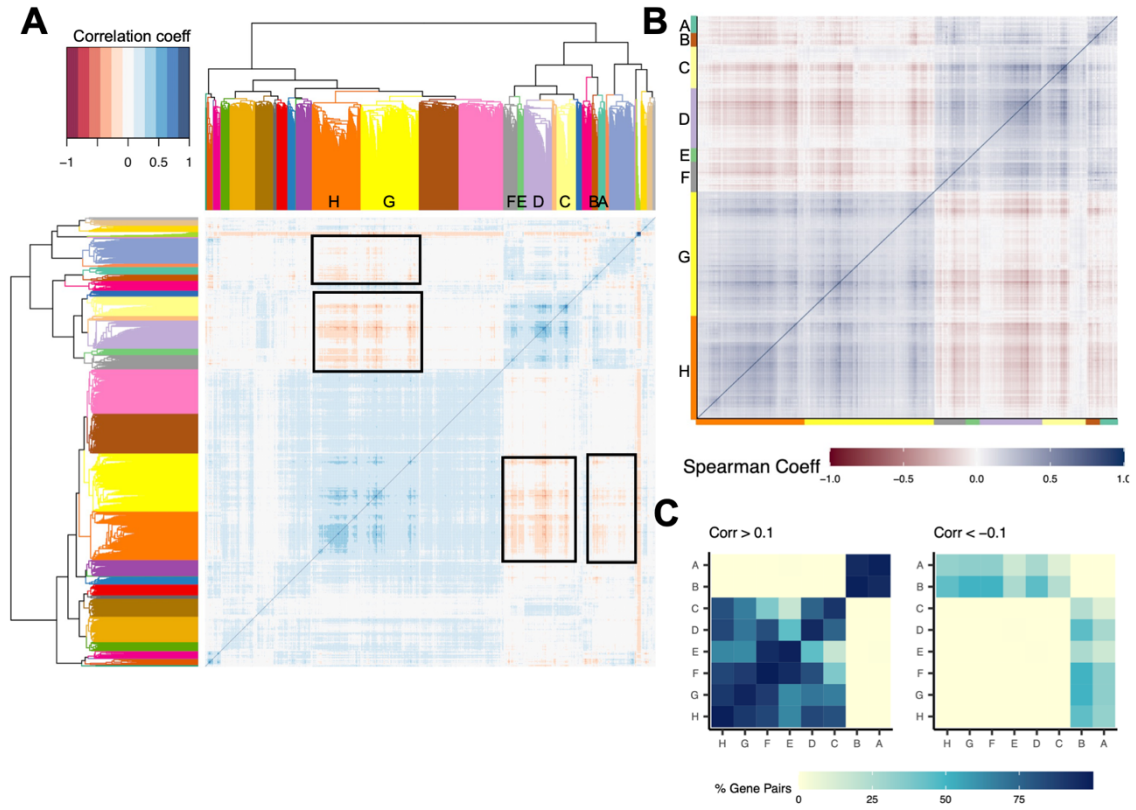


Figure 5.5: Anti-correlated expression of two gene modules in PT cells. (A) Correlation heatmap for top 1200 varying genes expressed in PT cells of Control ($n = 3F, 1M$) mice given 5x 2 mg TMX and harvested at the early timepoint after hierarchical clustering. Dendrogram coloured by hierarchical clusters. Two areas with high degree of anti-correlation marked by black rectangles. Hierarchical clusters contributing to the two areas are labelled A-H. (B) Heatmap depicting pairwise Spearman correlations between genes belonging to clusters A-F (PT Module A) and clusters G-H (PT Module B). (C) Proportion of gene-pairs between each cluster of genes with Spearman correlations >0.1 (left) or <-0.1 (right).

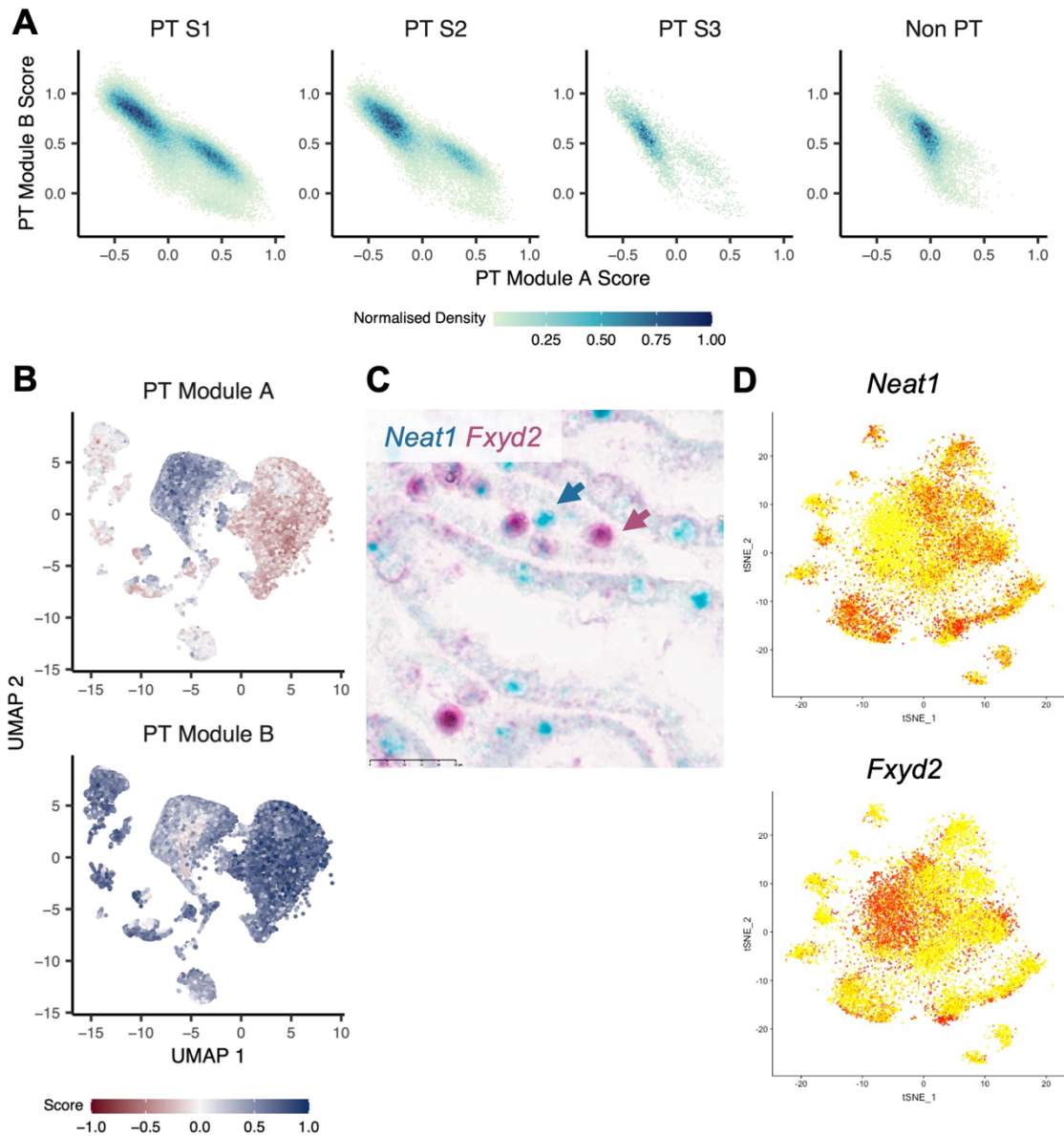


Figure 5.6: Anti-correlated expression of two gene modules in PT cells. (A) Density plot depicting expression scores for PT Module A and PT Module B in PT and non PT cells. Each plot is divided into 250 bins, and the number of cells in each bin is normalised relative to the densest bin. (B) UMAP plot depicting expression of PT Module A (top) and PT Module B (bottom) genes in PT cells. (C) *In situ* RNA hybridisation exhibiting spatially distinct expression of *Neat1* (blue) and *Fxyd2* (red) mRNA in kidneys from Control (n = 3) mice given 5x 2 mg TMX and harvested at the early timepoint. Scale bar denotes 25 μ m. *In situ* RNA hybridisation performed by Dr Joanna Lima. (D) t-SNE plots depicting expression of *Neat1* (top) and *Fxyd2* (bottom) in published snRNA-seq data. Red denotes high and yellow denotes low expression values. Data accessed at <http://humphreyslab.com/SingleCell> on 23 August 2023.

5.2.3 Biallelic *Vhl* inactivation entrains early cell-specific transcriptomic changes in renal tubular cells

5.2.3.1 Global, early transcriptomic changes following *Vhl* inactivation

The early effects of *Vhl* inactivation were elucidated by comparing cells from KO mice to those from Control mice at the early timepoint. To assess if biallelic *Vhl* inactivation caused a shift in global transcriptomic profiles of cells, I compared the distribution of tdTomato-positive and -negative cells from KO or Control mice by UMAP. tdTomato-negative cells from Control (n = 3F, 1M) and KO (n = 2F, 1M) mice, essentially *Vhl*^{wt/wt} and *Vhl*^{KO/wt} respectively, occupied the same positions and comprised the same clusters by UMAP (**Figures 5.7A** and **5.7B**), indicating that inheritance of one inactivated *Vhl* allele did not impact global gene expression in the kidney. tdTomato-positive cells from Control mice (n = 3F, 1M) (essentially *Vhl*^{KO/wt}) occupied the same clusters as tdTomato-negative PT and CDIC cells from Control and KO mice (**Figures 5.7A** and **5.7B**; see **Figure 5.3**), indicating that monoallelic *Vhl* inactivation in adult mice did not impact global gene expression. In contrast, tdTomato-positive cells from KO mice (n = 2F, 2M) (essentially *Vhl*^{KO/KO}) occupied distinct positions and formed new clusters by UMAP when compared to tdTomato-positive cells from Control mice (n = 3F, 1M), indicating transcriptomic changes specific to biallelic *Vhl* inactivation. Cells from KO mice (whether tdTomato-positive or -negative) comprised similar cell types to those from Control mice (**Figure 5.8**) at the early timepoint. This confirmed that the *Vhl*-dependent transcriptomic changes did not derive from a difference in the recovery or assignment of cell types for cells from the two genotypes.

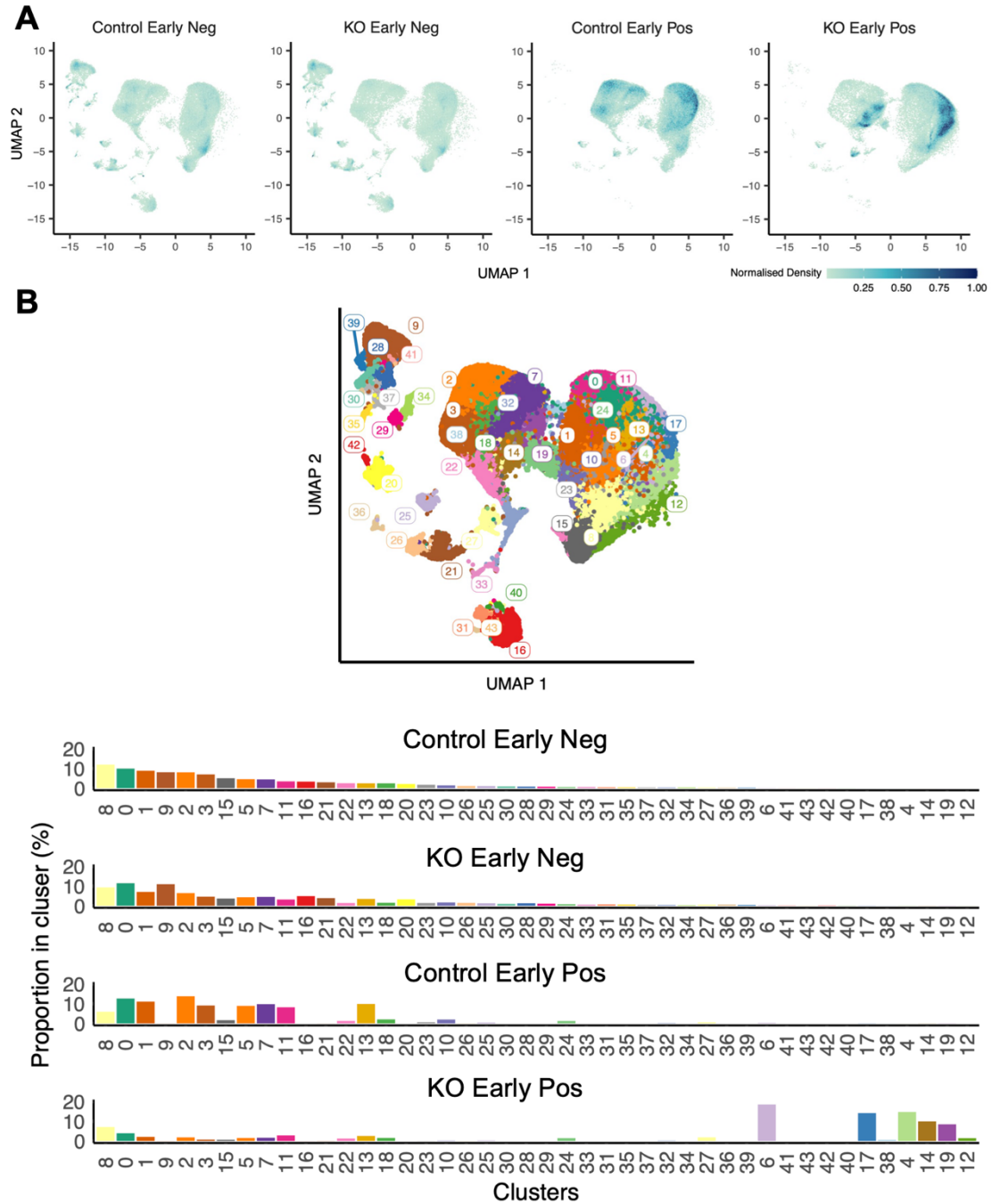


Figure 5.7: Biallelic *Vhl* inactivation causes shift in global transcriptomic profiles. (A)

Density plot depicting UMAP distribution of tdTomato-negative and positive cells from Control and KO mice harvested at the early timepoint ($n = 3F, 1M$ for Control negative; $n = 3F, 1M$ for Control positive; $n = 2F, 1M$ for KO negative; $n = 2F, 2M$ for KO positive). Each plot divided into 250 bins, and number of cells in each bin normalised relative to the densest bin. **(B)** UMAP plot depicting cells from Control and KO mice harvested at the early timepoint coloured by UMAP clusters (top). Proportion of cells from each condition belonging to any cluster (bottom).

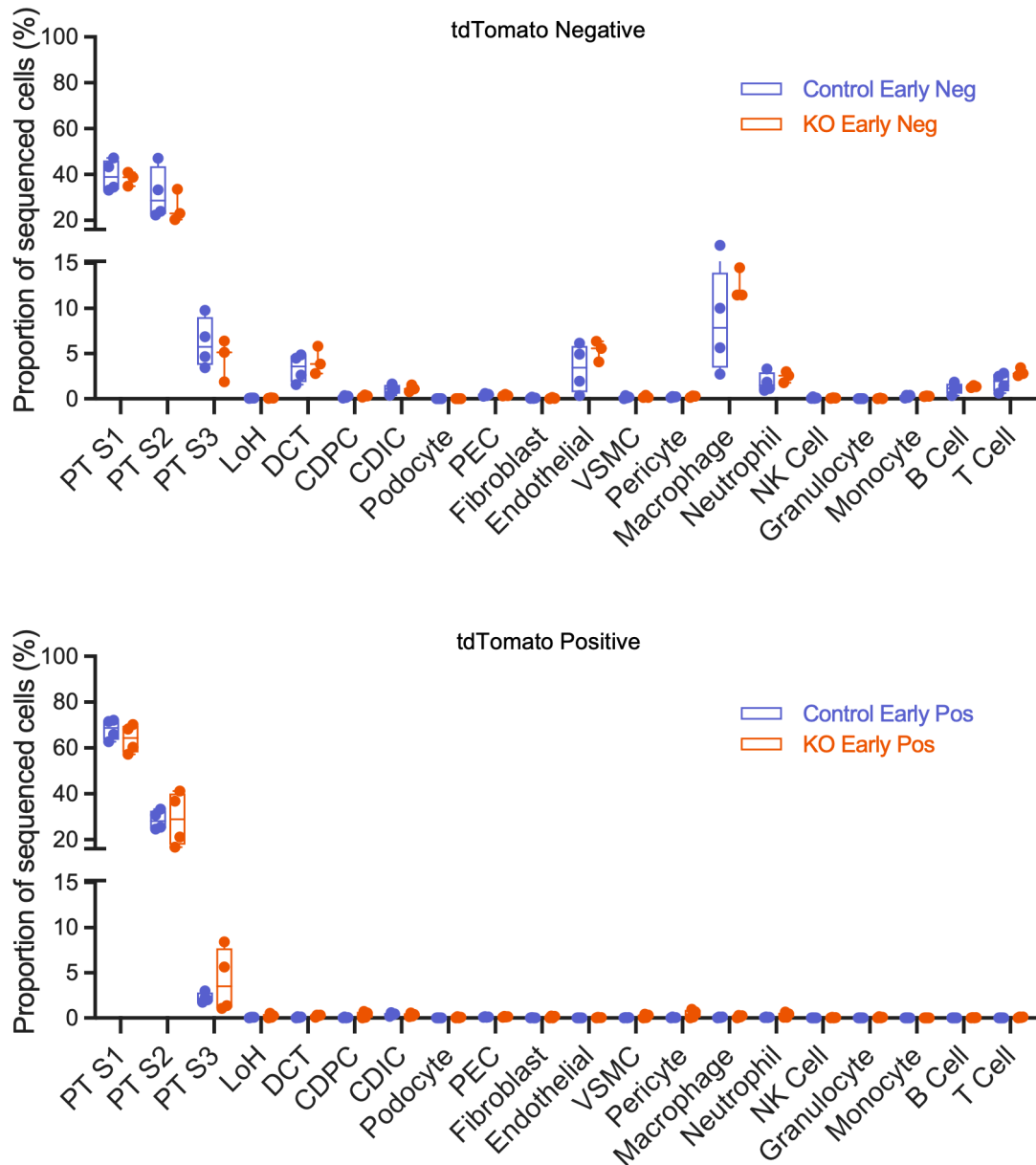


Figure 5.8: *Vhl* inactivation does not affect cell type assignment at the early timepoint.

Proportion of total cells inferred to be of each cell type in tdTomato-negative (top) or tdTomato-positive (bottom) cells from Control ($n = 3F, 1M$) and KO ($n = 2F, 1M$ for tdTomato-negative; $2F, 2M$ for tdTomato-positive) mice given 5×2 mg TMX and harvested at the early timepoint. Median and inter-quartile range plotted. Pairwise comparisons by 2-way ANOVA with Holm-Šidák correction. Only significant ($p < 0.05$) comparisons shown. PT – Proximal Tubule; LoH – Loop of Henle; DCT – Distal Convolved Tubule; CDPC – Collecting duct principal cell, CDIC – Collecting duct intercalated cell; PEC – Parietal epithelial cell; VSMC – Vascular Smooth Muscle Cell; NK – Natural Killer cell.

To identify the genes driving this transcriptomic change, differential expression was tested between cells from KO and Control mice, which were either tdTomato-positive or tdTomato-negative. Testing was performed on all cells together, or on individual cell types separately. When all cells were analysed together, 51 genes were upregulated in tdTomato-positive cells from KO compared to those from Control mice and 20 were downregulated ($|\log_2 \text{fold change}| > 0.25$, $p < 0.01$ after Bonferroni correction) (**Figure 5.9A** and **Appendix 2**). None of these genes were significantly altered in tdTomato-negative cells from KO compared to those from Control mice (**Appendix 2**). Genes differentially expressed after *Vhl* inactivation were significantly enriched ($p < 0.05$ after Bonferroni correction) for genes in the glycolytic pathway and MYC- or mTOR-regulated pathways (**Figure 5.9B**). However, many of the genes contributing to enrichment of MYC- and mTOR-regulated pathways overlapped with glycolysis genes (data not shown).

Cells from individual mice of the same genotype exhibited overlapping positions on UMAP (**Figure 5.10A**). Furthermore, alterations in the expression of 51 upregulated and 20 downregulated genes were highly correlated when individual KO mice were compared to Control mice (**Figure 5.10B** and **Appendix 3**; see **Chapter 8**). This confirmed that early *Vhl*-dependent changes are reproducible between mice. Notably, KO mice of the same sex showed slightly higher correlation with one another than with the opposite sex (**Figure 5.10B**). Accordingly, while most genes described above were regulated in both sexes, some showed sex-specific regulation (**Figure 5.10C**). It is worth noting here that the data contains only one male Control mouse at the early timepoint and thus, some of these apparent sex-specific differences might arise from sample variation.

Collectively, these results show that biallelic but not monoallelic *Vhl* inactivation has direct and reproducible effects on gene expression in the RTE at the early timepoint.

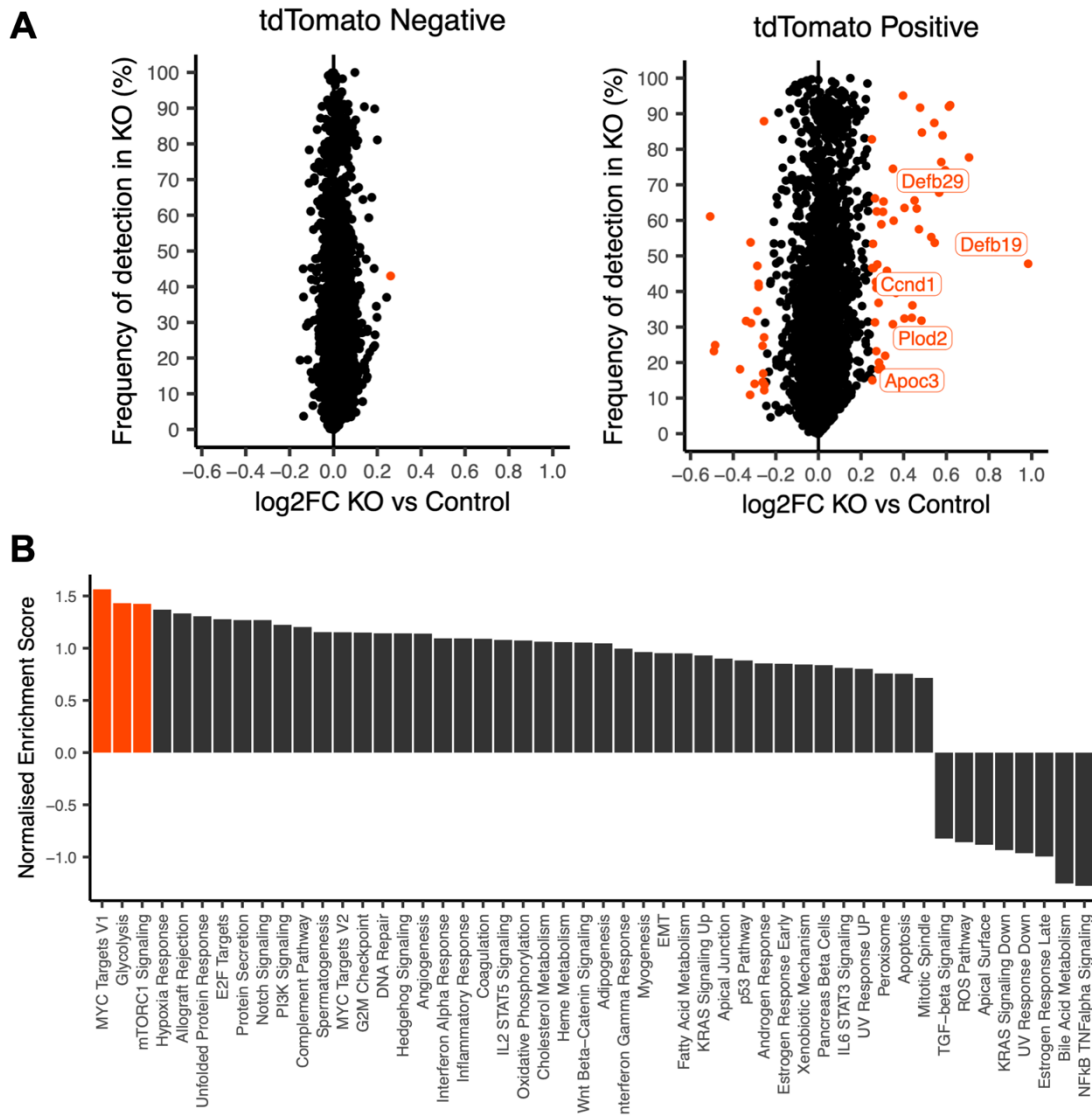


Figure 5.9: Global gene expression changes early after *Vhl* inactivation. (A) Scatter plot depicting frequency of expression in cells from KO mice against log₂-fold change between cells from KO *versus* Control mice for all genes in tdTomato-negative (left) and tdTomato-positive (right) cells harvested early after recombination. Significantly regulated genes (orange). Genes explicitly mentioned in the main text are labelled. **(B)** Enrichment of MSigDB Hallmark Gene Sets following *Vhl* inactivation. Significantly enriched pathways ($p < 0.05$ after Bonferroni correction) in orange.

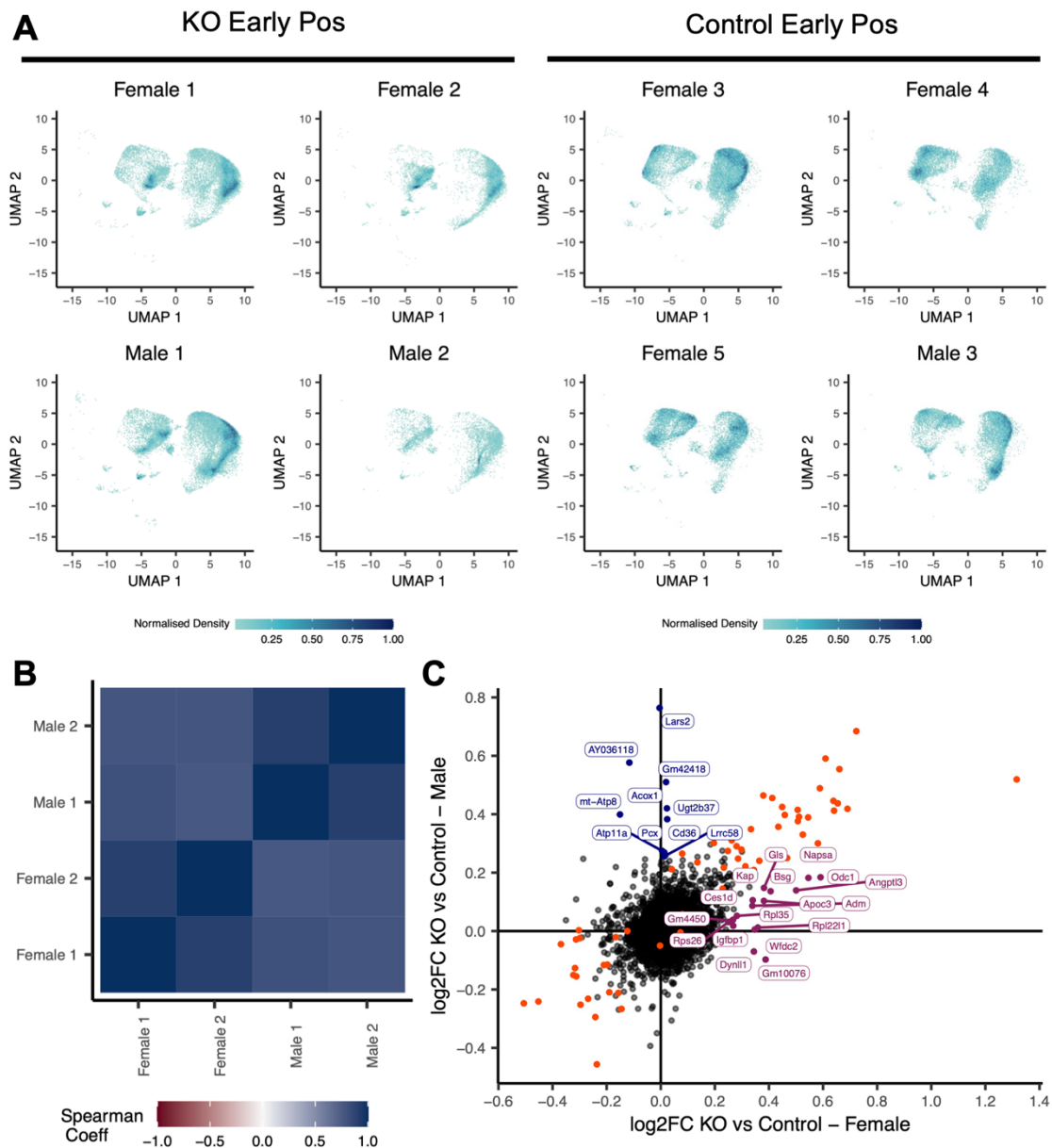


Figure 5.10: Changes entrained early after *Vhl* inactivation are reproducible. (A) Density plot depicting UMAP distribution of tdTomato-positive cells from individual KO or Control mice harvested at the early timepoint. Each plot is divided into 250 bins, and the number of cells in each bin is normalised relative to the densest bin. **(B)** Heatmap depicting Spearman correlation coefficients for changes in *Vhl*-regulated genes in tdTomato-positive cells derived from individual mice. **(C)** Scatter plot depicting log₂-fold changes in tdTomato-positive cells from male or female KO *versus* Control mice at the early timepoint (n = 2 KO, 2 Control for female; n = 2 KO, 1 Control for male). Genes regulated in both sexes (orange), and specifically regulated in males (blue) and females (maroon).

5.2.3.2 *Vhl* inactivation entrains cell-type specific gene expression changes

Next, I sought to define which elements of these transcriptional changes were common or specific to particular renal cell identities. Only those cell identities for which at least 100 cells were sequenced per condition were used for differential expression analysis – these included the three cell types and two classes of PT cells (PT S1, PT S2, PT S3, PT Class A, and PT Class B), and CDIC. 20 genes were upregulated after *Vhl* inactivation in every cell identity analysed (**Figure 5.11A** and **Appendix 4**), forming a “core” early response to *Vhl* inactivation in the RTE, while no genes were downregulated in all RTE cell types. 15 of these 20 genes had been previously characterised as HIF targets¹⁴⁹ (see **Chapter 8**), indicating that most of the core early response to *Vhl* inactivation is directly driven by HIF stabilisation.

Interestingly, Defensin-b 19 (*Defb19*), not a known HIF target gene, was the most upregulated gene after *Vhl* inactivation in all PT cells and showed the second highest enrichment after Lactate Dehydrogenase A (*Ldha*) in CDICs. *Defb19* transcript could be detected with RNA *in situ* hybridisation in kidney tubules of KO but not Control mice (**Figure 5.11B**), indicating that its upregulation is not an *ex-vivo* consequence of the preparation procedures associated with single cell sequencing. Another member of the β -Defensin family, *Defb29*, was upregulated in every cell identity except PT S3, in which it narrowly missed the regulation threshold ($\log_2$ -fold change = 0.23 instead of > 0.25) (**Appendix 4**). Together, this highlights defensin genes as novel targets of *Vhl* inactivation in the RTE.

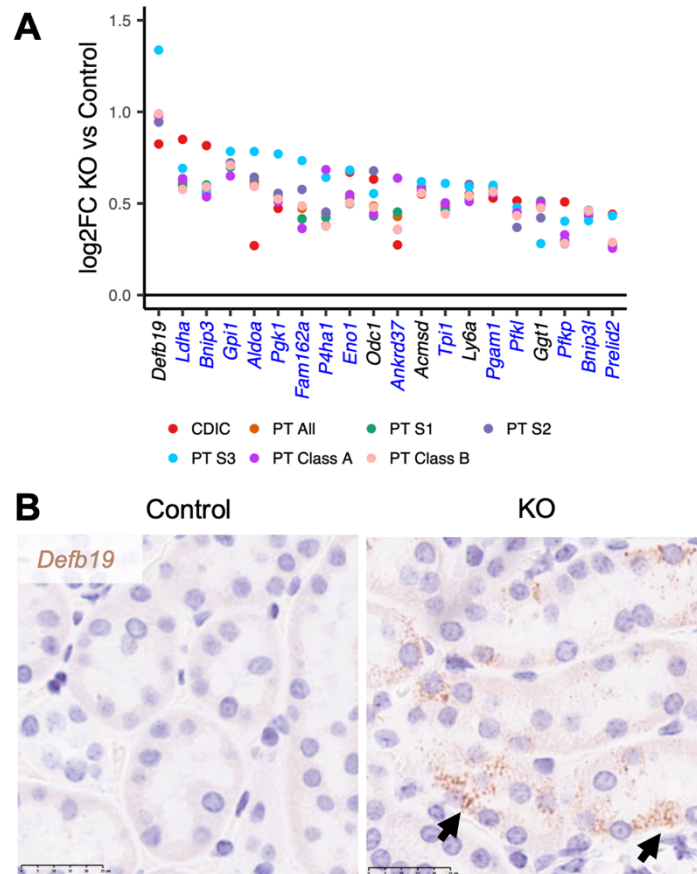


Figure 5.11: Core early responses to *Vhl* inactivation in the RTE. (A) Scatter plot depicting log₂-fold change between tdTomato-positive cells from KO *versus* Control mice for genes significantly regulated in every renal cell identity. Names of HIF target genes (blue). (B) Representative *in situ* RNA hybridisation for *Defb19* mRNA in Control or KO kidneys (n = 3 per genotype) harvested at the early timepoint. *Defb19* signal highlighted by black arrows. Scale bar denotes 25 μ m. *In situ* RNA hybridisation performed by Dr Joanna Lima.

I then investigated the genes, other than those in the ‘core’ response, that change following *Vhl* inactivation. 177 genes were upregulated and 105 were downregulated following *Vhl* loss in at least one cell identity (**Appendix 4**). Principal component analysis (PCA) segregated PT S3 and CDIC cells from other cell identities based on changes in expression of these genes (**Figure 5.12A**), indicating that these cell types exhibit distinct responses to *Vhl* inactivation compared to other PT cells. Only 2/14 and 1/29 genes that are specifically regulated in PT S3 and CDIC cells respectively (**Figures 5.12B and 5.12C**) were

previously characterised HIF targets¹⁴⁹. Notably, *Cdkn1a*, which codes for the cell cycle inhibitory protein p21, was specifically upregulated following *Vhl* inactivation in CDIC, but no PT cell (**Figure 5.12C**).

PT Class A and PT Class B cells respond to *Vhl* inactivation in a similar manner, as exhibited by their proximity in the PCA plot (**Figure 5.12A**). Consistent with this, all genes regulated by *Vhl* in PT Class B cells were also found to be regulated in PT Class A cells. However, 13 genes exhibited *Vhl*-dependent regulation in PT Class A but not PT Class B cells (**Figure 5.12D**). This indicates that PT Class A cells are more responsive to *Vhl* inactivation at the early timepoint than PT Class B cells. Interestingly, of the five genes specifically upregulated in PT Class A cells that have known human orthologues, four are prognostic markers for ccRCC (unfavourable - *Adm*, *Vegfa*, *Ddit4*; favourable – *Igsf3*) (**Figure 5.12E**).

Collectively, these results demonstrate that *Vhl* inactivation has early effects on gene expression that are remarkably tailored to renal cell identity, with only a fraction of total changes universally entrained in all renal cell types. They also highlight the regulation of defensin genes as an early *in vivo* response to *Vhl* inactivation in the RTE.

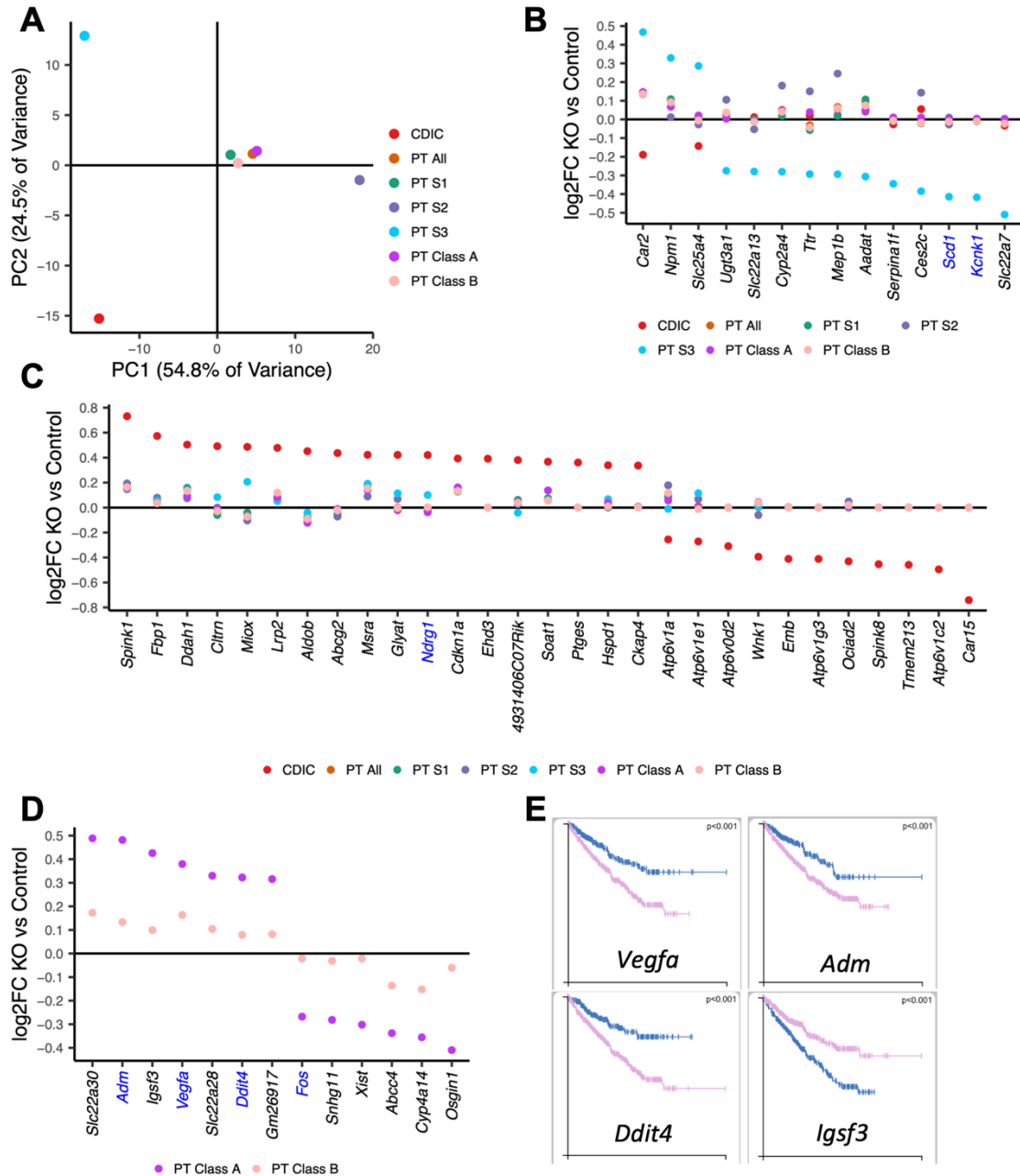


Figure 5.12: Cell identity-specific early responses to *Vhl* inactivation in the RTE. (A) Principal component analysis of gene expression changes early after *Vhl* loss in different cell identities. (B-D) Scatter plot depicting log₂-fold change between tdTomato-positive cells from KO *versus* Control mice in different renal cell identities for genes specifically regulated in (B) PT S3, (C) CDIC, or (D) PT Class A cells. Names of HIF target genes (blue). (E) Survival analysis of renal cancer patients stratified by expression of genes specifically regulated in PT Class A cells after *Vhl* loss. Cases with high (blue) and low (pink) expression. Data from The Human Protein Atlas, accessed on 23 July 2023.

5.2.4 *Vhl*-null cells specifically undergo time-dependent alterations in gene expression

I next sought to trace gene expression changes in *Vhl*-null cells over time, evaluate evidence for specific adaptation to *Vhl* inactivation in different parts of the RTE, as well as assess whether the long-term presence of *Vhl*-null cells in the RTE specifically alters gene expression in the renal interstitium. scRNA-seq was thus performed on flow-sorted tdTomato-positive and negative cells from KO and Control mice harvested at the late timepoint (4-12 months). This yielded data from an additional 242,561 high-quality cells across 16 samples from 9 mice, with an average of $15,160 \pm 5367$ (SD) cells per sample, and $3,232 \pm 665$ (SD) transcripts per cell that covered $1,254 \pm 191$ (SD) genes per cell per sample, which were integrated with data from cells from the early timepoint for subsequent analysis.

5.2.4.1 Time- and *Vhl*-dependent changes in cell type assignment or recovery

Cell types were assigned to the newly-sequenced cells based on the expression of marker genes defined by reference to published data. At the late timepoint tdTomato-negative cells from Control and KO mice, and tdTomato-positive cells from Control mice, contained significantly fewer PT S1 cells ($p = 0.01$, $p < 0.01$ and $p < 0.01$ respectively) than those at the early timepoint, with a difference of 6-10% between the two timepoints (**Figures 5.13A, 5.13B, and 5.14A**). tdTomato-negative cells from KO mice and tdTomato-positive cells from Control mice at the late timepoint also contained a significantly higher proportion of PT S2 cells ($p = 0.04$ and 0.02 respectively) than those at the early timepoint, with a 4 and 7% increase respectively over time (**Figures 5.13B and 5.14A**). This suggests that either the recovery of, or expression of cell type-specific marker genes in PT cells, changed with time. Notably, these effects were more pronounced in tdTomato-positive cells from KO mice, with samples at the late timepoint exhibiting a 24% drop ($p < 0.01$) in the proportion of PT S1 and 14.3% increase ($p < 0.01$) in the proportion of PT S2 cells compared to the early timepoint (**Figure 5.14C**). Together, this indicates a *Vhl*-dependent change either in the recovery of

cells or expression of cell type-specific marker genes that is more than can be accounted for by age at the late timepoint.

An unfortunate corollary of this is that cell type assignment on the basis of reference marker genes might not be accurate in tdTomato-positive cells from KO mice. As differences in marker gene expression cannot be distinguished from a differential recovery of cell types from kidneys of KO mice at the late timepoint, an assessment of cell type-specific gene expression changes in *Vhl*-null cells over time cannot be performed reliably. However, changes incurred in the population of recombined cells as a whole can still be reliably assayed based on categorisation of cells by tdTomato-positivity.

5.2.4.2 Global time-dependent gene expression changes in *Vhl*-null cells

Dimension reduction was performed on tdTomato-positive cells from KO and Control mice harvested at the early and late timepoints. This showed that cells from KO mice occupied different positions and clusters by UMAP at the late compared to the early timepoint; such differences were not seen for cells from Control mice (**Figures 5.15A and 5.15B**). Genes driving this change were identified by differential expression testing at late *versus* early timepoints in tdTomato-positive cells from KO mice; changes specific to *Vhl*-null cells (as opposed to those incurred with age) were identified by excluding genes regulated with time in tdTomato-positive cells from Control mice. This identified 24 upregulated and 16 downregulated genes (**Figure 5.16A and Appendix 5**). Interestingly, 32/40 of the genes regulated with time were distinct from those regulated early after *Vhl* loss and 36/40 were not known HIF targets (**Figure 5.16B**). *Defb19*, which was the top-enriched gene early after *Vhl* inactivation, was further upregulated over time.

Several genes involved with fatty acid and lipid metabolism – Apolipoprotein C3 (*Apoc3*), fatty acid transporter (*Cd36*), fatty acid binding protein 5 (*Fabp5*) – were also upregulated over time. I validated the upregulation of two time-regulated genes (*Apoc3* and *Defb19*) by *in situ* RNA hybridisation (**Figure 5.16C**).

The reproducibility of time-dependent changes was verified by analysing the UMAP positions of tdTomato-positive cells from individual KO mice at the late or early timepoints (n = 2F, 2M per timepoint). All four KO mice at the late timepoint exhibited shifts in UMAP positions compared to those at the early timepoint (**Figure 5.17A**). There was considerable overlap in the positions of the four late KO mice, though sex-specific clusters were seen in male samples at the late timepoint (**Figure 5.17A**). This suggested that time-dependent changes in *Vhl*-null cells might be partially sex-specific. Accordingly, while alterations in expression of time-regulated genes showed positive correlation across all samples, those between mice of opposite sex were lower than those between mice of the same sex (**Figure 5.17B** and **Appendix 6**; see **Chapter 8**). Sex-specific regulation of genes within *Vhl*-null cells is depicted in **Figure 5.17C**. Unfortunately, neither gProfiler analysis (for GO term enrichment) nor LISA analysis (for identifying regulatory transcription factors) on genes regulated specifically in either sex yielded any significant results (data not shown), keeping the drivers or biological consequences of these sex-specific differences unclear.

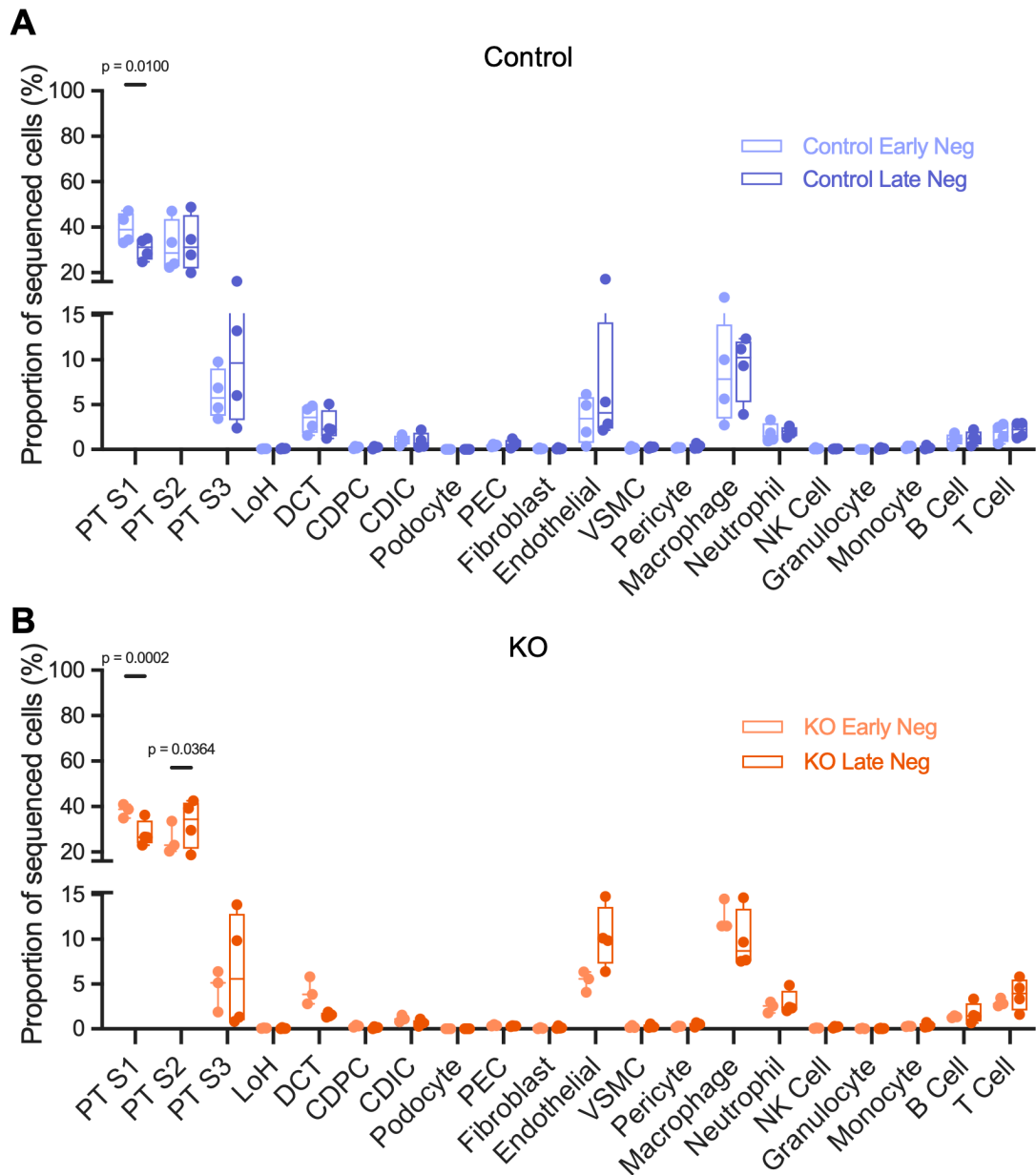


Figure 5.13: Effect of time after recombination on cell type recovery or assignment in tdTomato-negative cells. (A, B) Proportion of total cells inferred to be of each cell type in tdTomato-negative cells from kidneys of Control (n = 3F, 1M for Early; n = 2F, 2M for Late) (A), and KO (n = 2F, 1M for Early; n = 2F, 2M for Late) (B) mice given 5x 2 mg TMX and harvested at the early or late timepoint. Median and inter-quartile range plotted. Pairwise comparisons by 2-way ANOVA with Holm-Šídák correction. Only significant ($p < 0.05$) comparisons shown. PT – Proximal Tubule; LoH – Loop of Henle; DCT – Distal Convoluted Tubule; CDPC – Collecting duct principal cell, CDIC – Collecting duct intercalated cell; PEC – Parietal epithelial cell; VSMC – Vascular Smooth Muscle Cell; NK – Natural Killer cell.

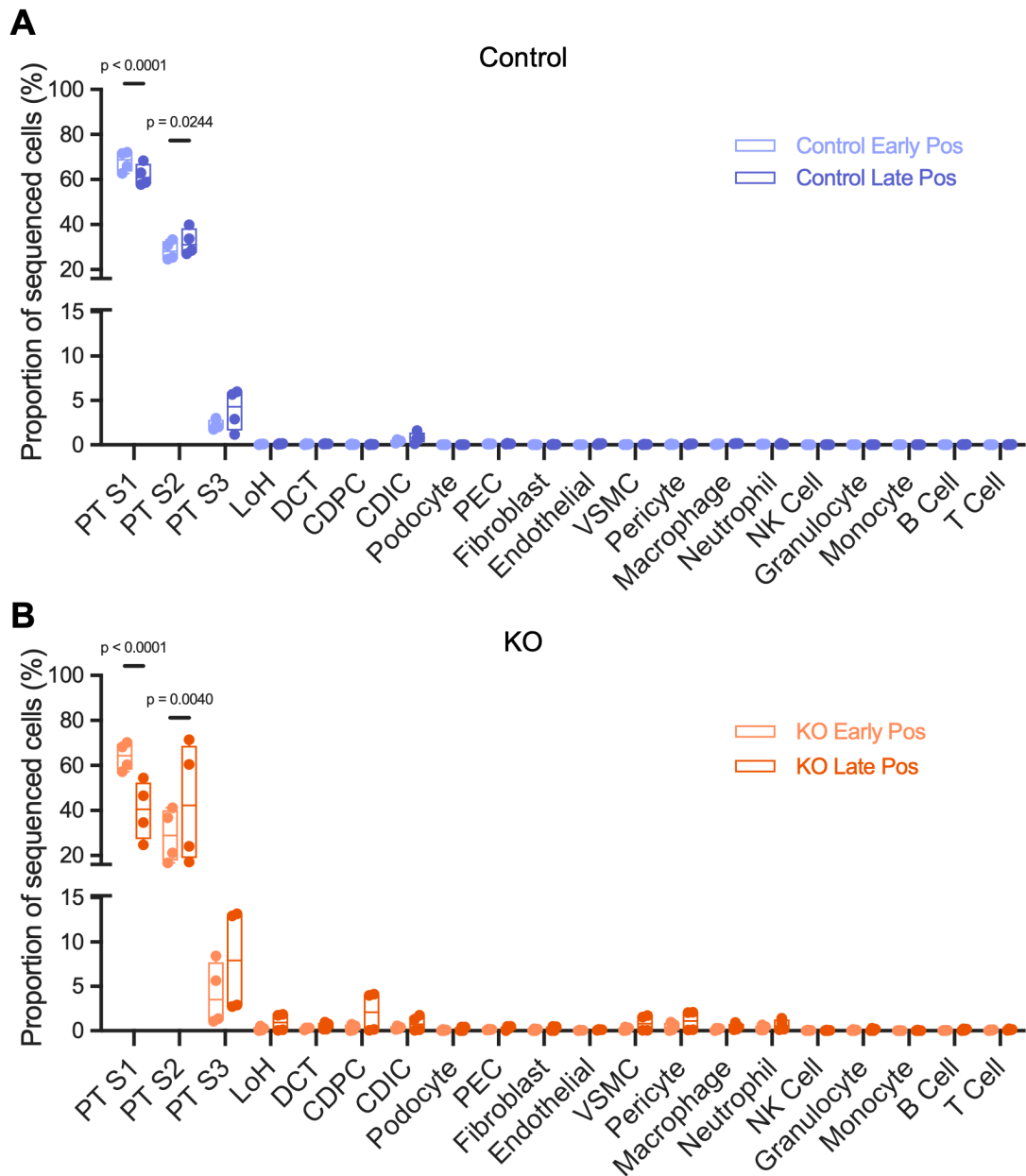


Figure 5.14: Effect of time after recombination on cell type recovery or assignment in tdTomato-positive cells. (A, B) Proportion of total cells inferred to be of each cell type in tdTomato-positive cells from kidneys of Control (n = 3F, 1M for Early; n = 2F, 2M for Late) (A), and KO (n = 2F, 2M for Early and Late) (B) mice given 5x 2 mg TMX and harvested at the early or late timepoint. Median and inter-quartile range plotted. Pairwise comparisons by 2-way ANOVA with Holm-Šídák correction. Only significant ($p < 0.05$) comparisons shown. PT – Proximal Tubule; LoH – Loop of Henle; DCT – Distal Convolved Tubule; CDPC – Collecting duct principal cell, CDIC – Collecting duct intercalated cell; PEC – Parietal epithelial cell; VSMC – Vascular Smooth Muscle Cell; NK – Natural Killer cell.

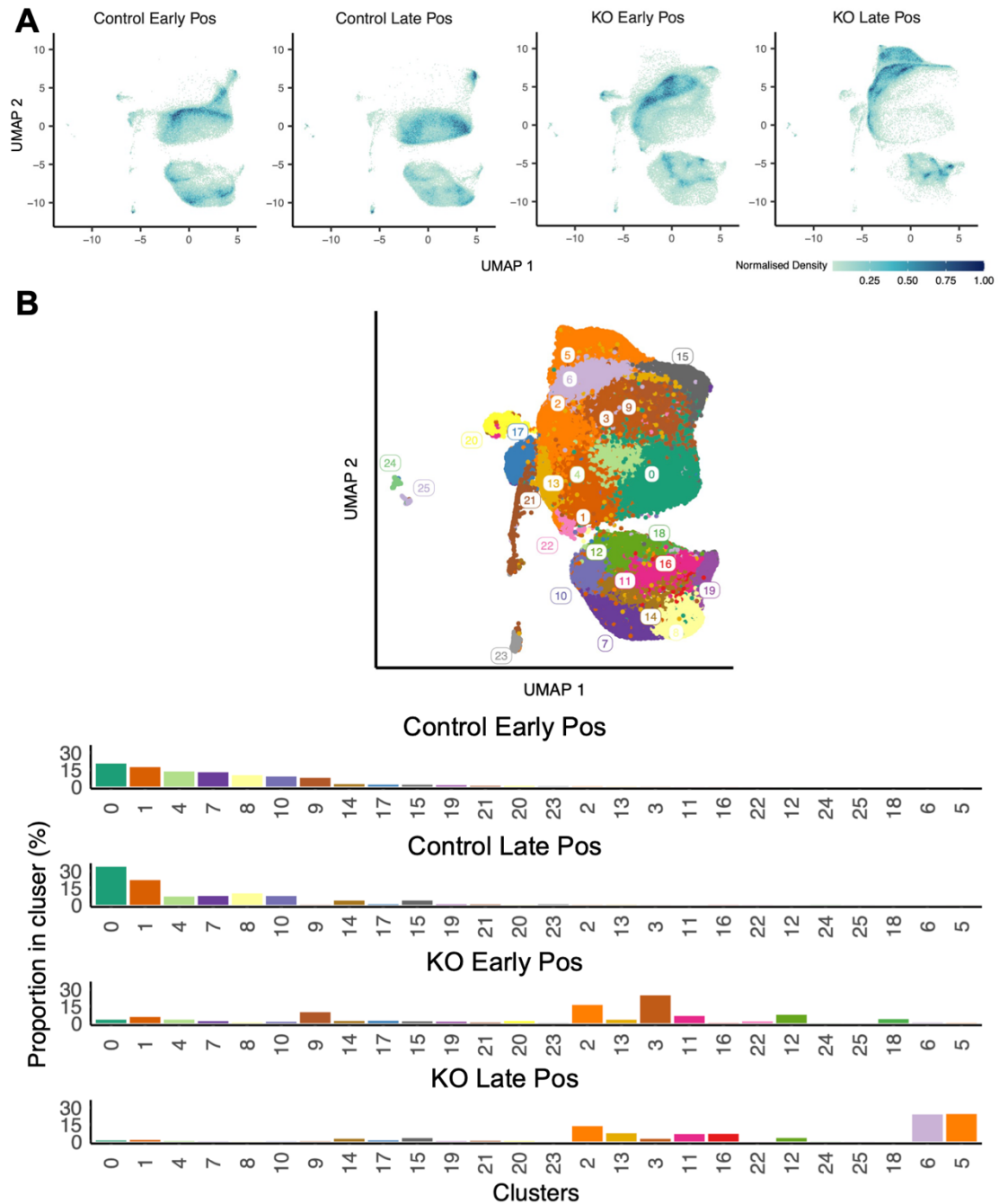


Figure 5.15: *Vhl*-null cells specifically alter gene expression over time. (A) Density plot depicting UMAP distribution of tdTomato-positive cells from Control (n = 3F, 1M for Early; n = 2F, 2M for Late) and KO (n = 2F, 2M for Early and Late) mice given 5x 2 mg TMX and harvested at the early and late timepoints. Each plot is divided into 250 bins, and the number of cells in each bin is normalised relative to the densest bin. (B) UMAP plot depicting tdTomato-positive cells from Control and KO mice harvested at the early and late timepoints coloured by UMAP clusters (left). Proportion of cells from each condition belonging to any cluster (right).

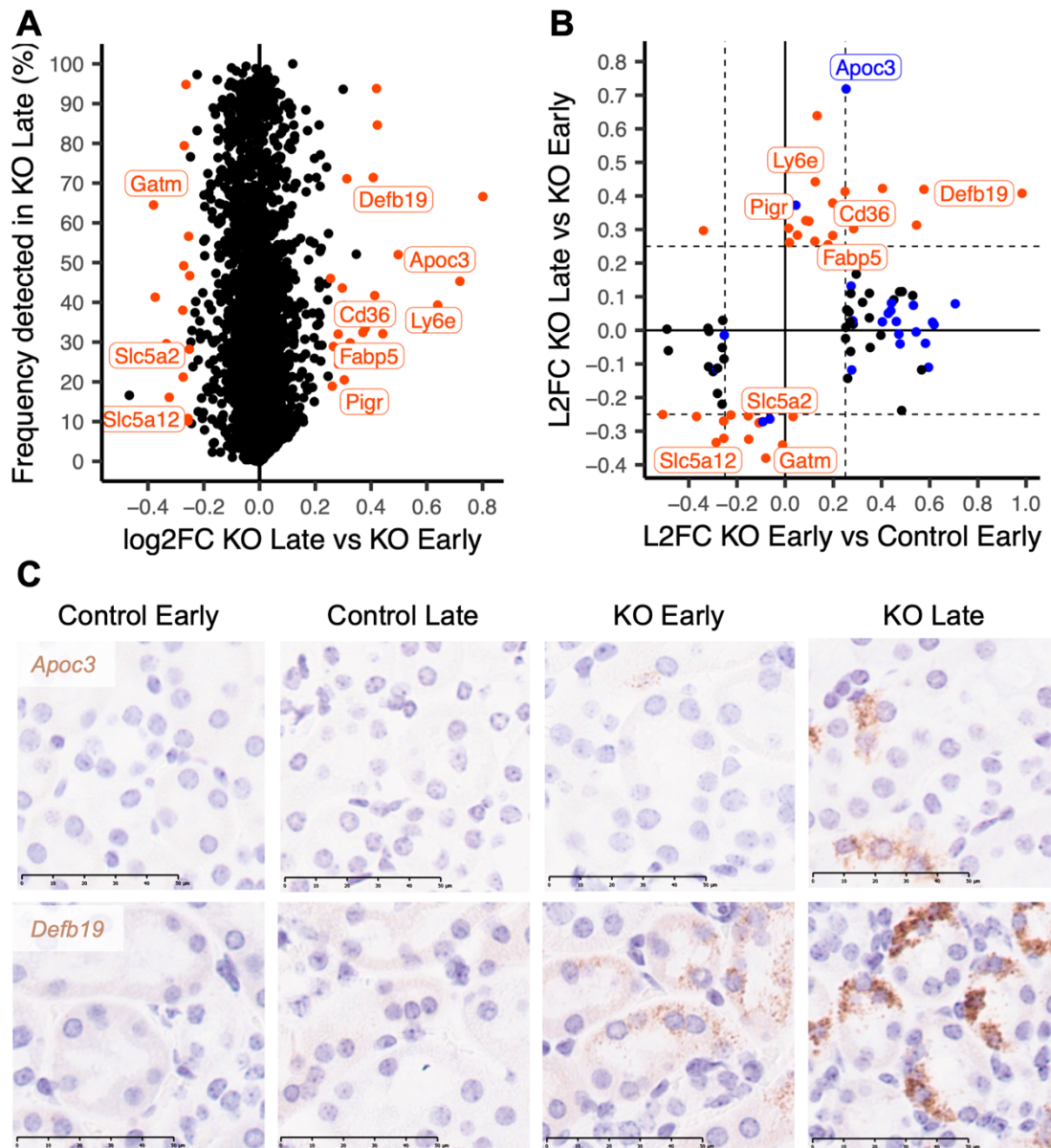


Figure 5.16: Genes regulated in *Vhl*-null cells over time. (A) Scatter plot depicting frequency of expression in cells from KO mice at the late timepoint against log₂-fold change between tdTomato-positive cells from KO mice at the late *versus* early timepoints for all genes. Genes regulated over time (orange). Genes explicitly mentioned in the main text are labelled. (B) Scatter plot depicting log₂-fold change early after *Vhl* inactivation against over time. Only significantly regulated genes plotted. Genes regulated over time (orange). HIF target genes (blue). Genes explicitly mentioned in the main text are labelled. (C) Representative *in situ* RNA hybridisation for *Apoc3* (top) and *Defb19* (bottom) mRNA in Control and KO (n = 3 per genotype and timepoint) kidneys harvested at the early or late timepoints. Scale bar denotes 50 μ m. *In situ* RNA hybridisation was performed by Dr Joanna Lima.

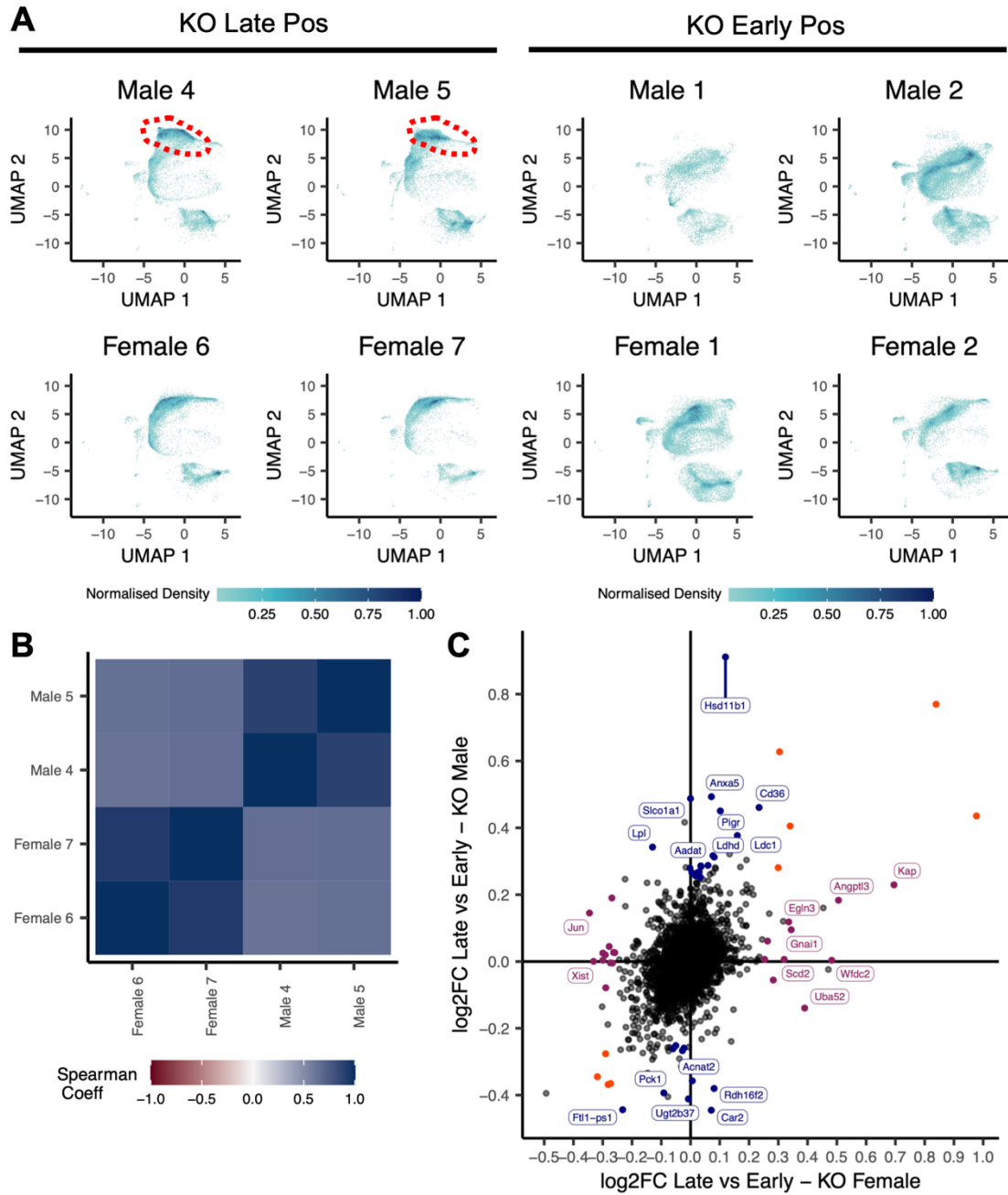


Figure 5.17: Changes entrained over time in *Vhl*-null cells are reproducible. (A) Density plot depicting UMAP distribution of tdTomato-positive cells from individual KO mice harvested at the early or late timepoints. Male-specific clustering of cells denoted by dashed lines. Each plot is divided into 250 bins, and the number of cells in each bin is normalised relative to the densest bin. **(B)** Heatmap depicting Spearman coefficients for changes in time-regulated genes over time in individual mice at the late timepoint. **(C)** Scatter plot depicting log₂-fold changes in tdTomato-positive KO cells at the late *versus* early timepoints from male plotted against female mice (n = 2F, 2M for both timepoints). Genes regulated in KO over time in both sexes (orange), males (blue) and females (maroon).

5.2.4.3 *Vhl*-null PT cells dedifferentiate over time

Several genes considered to be markers of proximal tubule differentiation (*Slc5a2*, *Slc5a12*, *Gatm*) were downregulated in *Vhl*-null cells over time (**Figure 5.16A**), suggesting a loss of proximal tubular identity. To test if this downregulation arose from a change in expression of PT-specific markers, as opposed to differential recovery of PT cells in tdTomato-positive KO mice at the late timepoint, I examined the data for cells that clustered with PT cells but were not assigned as a PT cell type. The data contained 7,673 such cells that were called “PT like” (**Figure 5.18A**; see **Chapter 8**).

tdTomato-positive cells from KO mice from the late timepoint contained a significantly higher ($p = 0.03$) proportion of PT like cells compared to other genotypes and timepoints (**Figure 5.18B**); no increase was seen for any condition among tdTomato-negative cells (**Figure 5.18C**). Compared to PT cells, PT like cells showed significant ($p = 4.6 \times 10^{-8}$) downregulation of PT marker genes (**Figure 5.18D** and **Appendix 7**), but no upregulation of marker genes of any non-PT cell type (data not shown). Notably, the PT developmental transcription factor²³⁸ *Maf* was downregulated ($p < 1 \times 10^{-16}$) in these cells but the pan-RTE transcription factor *Pax8* remained unchanged ($p > 0.01$) (**Figure 5.18E**). PT-like cells had a significantly higher proportion of cycling cells than other PT identities, as measured by the number of cells expressing the proliferation marker²³⁹ *Mki67* (**Figure 5.18F**).

Taken together, these results provide evidence supporting dedifferentiation of *Vhl*-null PT cells over time. This dedifferentiation process explains why, when cell types were identified based on the expression of PT differentiation marker genes, *Vhl*-null cells were found to comprise fewer PT S1 cells at the late timepoint.

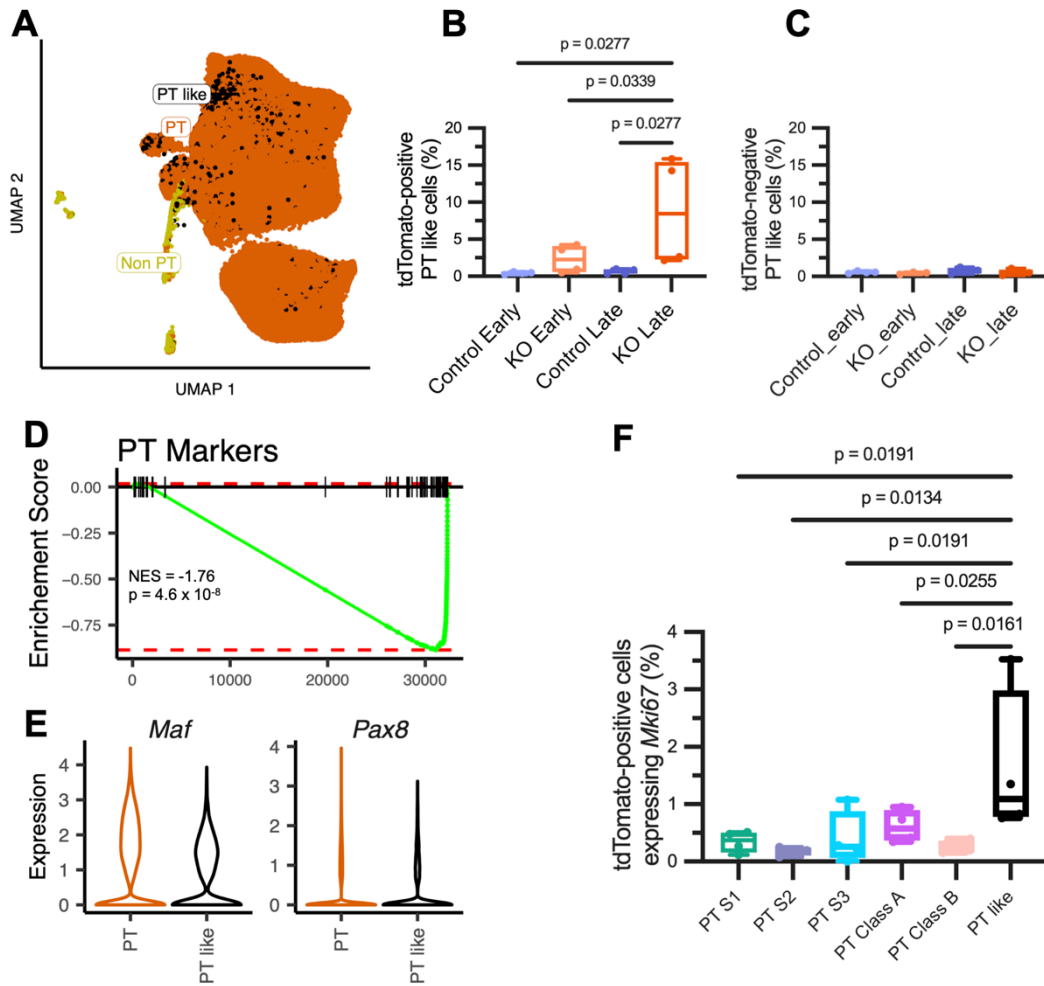


Figure 5.18: De-differentiation of *Vhl*-null PT cells over time. (A) UMAP plot depicting “PT like” cells among tdTomato-positive cells from Control and KO mice harvested at the early and late timepoints plotted together. “PT like” cells that did not express PT markers but clustered with PT cells coloured in black. (B, C) Proportion of cells inferred to be “PT like” within tdTomato-positive (B), or tdTomato-negative (C), cells across conditions. Median and inter-quartile range plotted. Pairwise comparisons tested by one-way ANOVA with Holm-Šidák correction. Only significant ($p < 0.05$) comparisons shown. (D) Gene set enrichment plot depicting downregulation of PT marker genes in PT like cells compared to PT cells at the late timepoint. NES = Normalised Enrichment Score. (E) Violin plots depicting expression of *Maf* and *Pax8* in tdTomato-positive PT or PT like cells from KO mice at late timepoint. (F) Proportion of tdTomato-positive cells of any PT identity in cell cycle assayed by scRNA-seq in cells from KO mice ($n = 4$) harvested at the late timepoint. Median and inter-quartile range plotted. Pairwise comparisons by one-way ANOVA with Holm-Šidák correction. Only significant ($p < 0.05$) comparisons shown.

5.2.4.4 Time-dependent gene expression changes in renal immune cells

Genes upregulated over time in *Vhl*-null cells included lymphocyte antigen 6E (*Ly6e*), poly-immunoglobulin G receptor (*Pigr*), and particularly *Defb19*, which all code for surface or secreted proteins predicted to communicate with immune cells²⁴⁰⁻²⁴². This was particularly interesting in the context of the inflammatory foci observed in KO kidneys at the late timepoint in **Chapter 4**. Consequently, gene expression changes in renal immune cells were analysed for signs of immune cell activation.

Dimension reduction was first performed on renal immune cells – cells assigned as macrophages, neutrophils, NK cells, monocytes, granulocytes, B cells, and T cells on the basis of marker gene expression – to assess for global gene expression changes associated with *Vhl* inactivation and/or time. ‘PT like’ cells that were assigned an immune cell type were excluded from this analysis. Intriguingly, no differences in UMAP positions were observed between conditions, suggesting that gene expression changes, if any, were minor (**Figure 5.19A**). Differential expression was then tested between late and early timepoints for each immune cell type separately and genes specifically regulated over time in cells from KO, but not Control mice, were analysed for GO term enrichment using gProfiler. Of the renal cell types analysed, only macrophages exhibited time-dependent upregulation of genes involved with leukocyte activation ($p = 0.0068$) (**Figures 5.19B and 5.19C**). This suggests that kidney macrophages are activated in association with time-dependent changes in *Vhl*-null RTE cells.

Collectively, these results demonstrate that biallelic inactivation of *Vhl* leads to changes in gene expression in affected cells which evolve after the initial HIF-dependent response, and which are sufficient to drive dedifferentiation in the epithelium and immune and inflammatory changes in the renal interstitium.

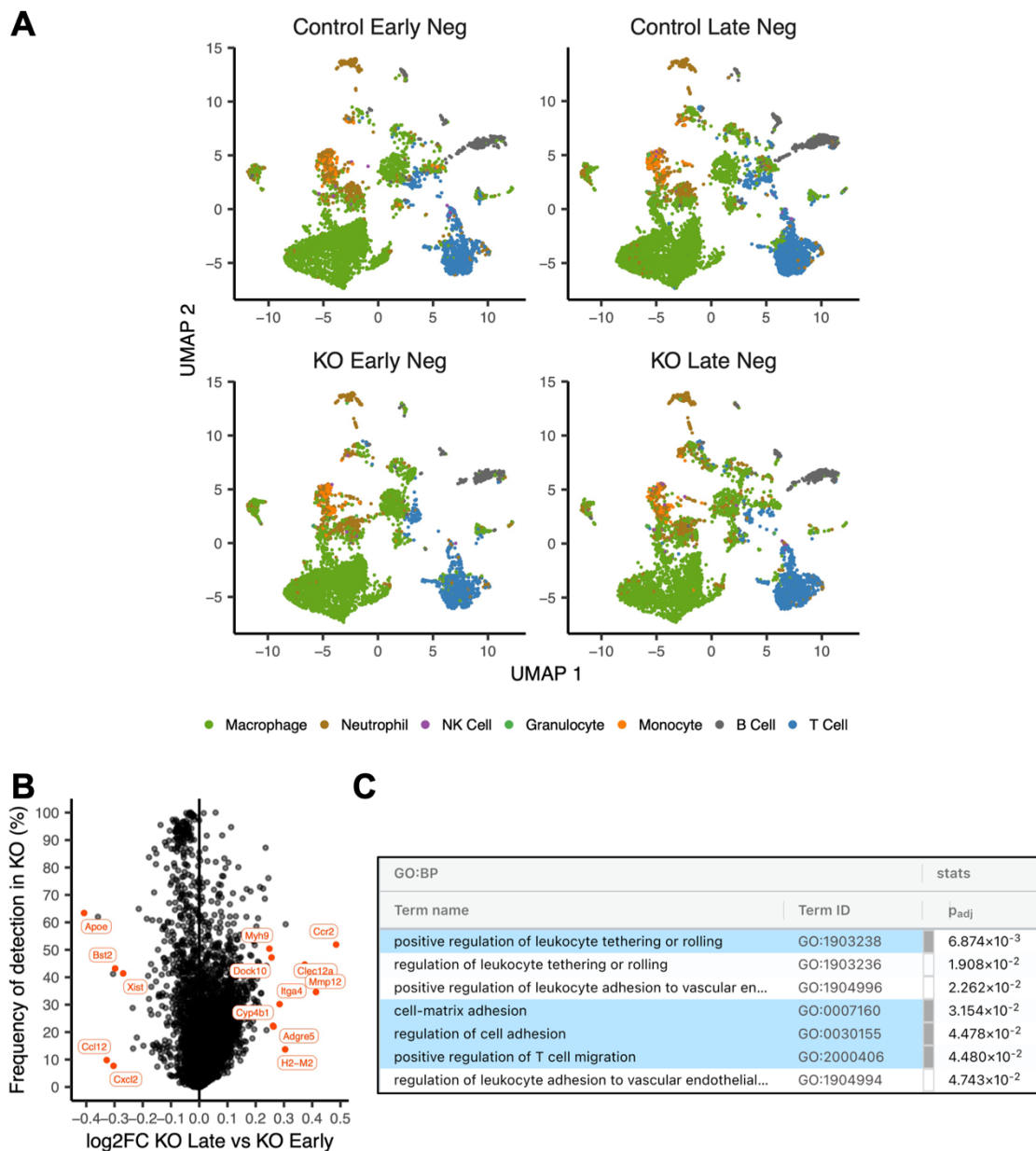


Figure 5.19: Renal macrophages are activated in association with long-term retention of *Vhl*-null cells in the RTE. (A) UMAP plot of tdTomato-negative renal immune cells from Control and KO mice harvested at the early and late timepoints (n = 3F, 1M for Control Early; n = 2F, 1M for KO Early; n = 2F, 2M for Control Late and KO Late). Cells are coloured by inferred cell type. NK – Natural Killer cell. (B) Scatter plot depicting frequency of expression in macrophages from KO mice at the late timepoint against log₂-fold change between tdTomato-negative macrophages from KO mice at the late *versus* early timepoints for all genes. Genes regulated in macrophages over time (orange). (C) Significantly enriched Gene Ontology terms for genes upregulated over time in tdTomato-negative macrophages in KO mice. Results from gProfiler.

5.2.5 *Vhl*-null cells exhibit time-restricted proliferation

Data reported in **Chapter 4** indicated that *Vhl* inactivation confers a selective advantage to cortical tubular cells, in part by facilitating an early entry into cell cycle. It was also observed that this increase in cycling behaviour is not sustained over time, suggesting a non-oncogenic adaptation of *Vhl*-null cells. Therefore, the proliferation status of *Vhl*-null cells was determined from scRNA-seq data.

Cells in cell cycle were defined as those that express at least one copy of the proliferation marker²³⁹ Ki67 (*Mki67*); this definition was supported by observing that cells expressing *Mki67* also expressed other cell cycle-associated genes such as topoisomerase²³⁹ 2A (*Top2a*) (**Figure 5.20A**). A greater ($p < 0.0001$) proportion of tdTomato-positive cells from KO mice were in cell cycle compared to those from Control mice at the early timepoint (**Figure 5.20B**), confirming an early proliferative burst entrained by *Vhl* inactivation. Such an increase was not seen in tdTomato-negative RTE cells (**Figure 5.20B**), supporting proliferation as a direct, cell autonomous effect of *Vhl* inactivation in the RTE. Notably, the proportion of tdTomato-positive cells in KO mice in cell cycle subsided to baseline levels at the late timepoint, confirming the transiency of induced proliferation (**Figure 5.20B**). To test if the proliferation induced was specific to any PT identity, the proportion of tdTomato-positive PT cells in cell cycle was calculated separately. This showed that cells of all PT cell identities exhibited an upward trend in proliferation early after *Vhl* inactivation. This was statistically significant in both classes (A and B) of PT cells. However, only PT S1 exhibited a statistically significant difference in proliferation among the three types of PT cells (**Figure 5.20C**). Interestingly, while PT S1 cells of both classes significantly increased proliferation early after *Vhl* inactivation, the increase was more significant ($p < 0.0001$ for PT Class A; $p = 0.03$ for PT Class B) among PT Class A cells (**Figure 5.20D**).

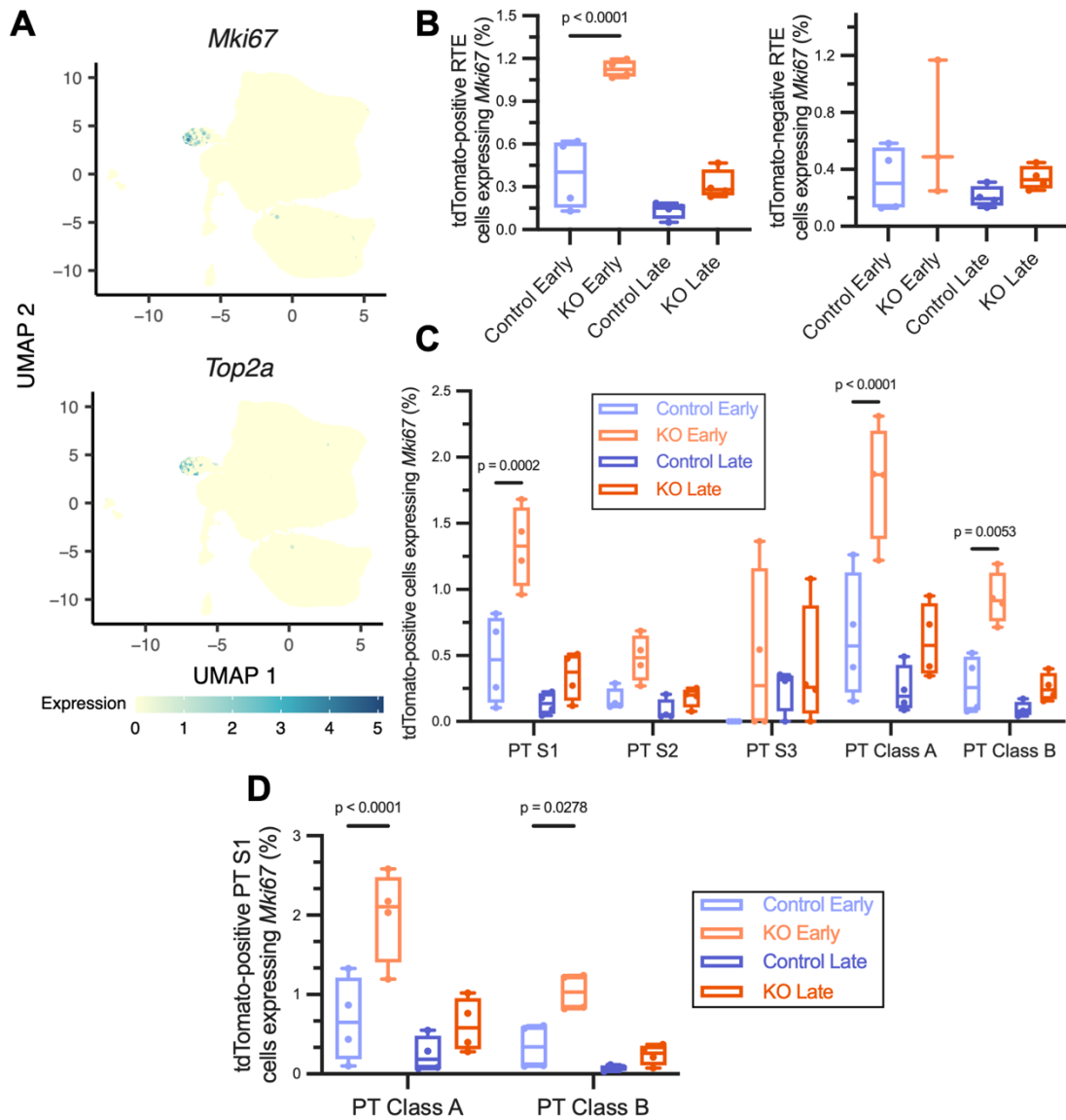


Figure 5.20: Transient proliferation of *Vhl*-null cells. (A) UMAP plot of tdTomato-positive cells from Control and KO mice at the early and late timepoints depicting expression of *Mki67* and *Top2a*. (B) Proportion of tdTomato-positive (right) or tdTomato-negative (left) RTE cells in cell cycle in Control and KO mice at the early and late timepoints ($n = 2F, 1M$ for KO early negative, $n = 3F, 1M$ for Control early; $n = 2F, 2M$ for all other conditions). (C) Proportion of tdTomato-positive cells of different PT identities in cell cycle in Control and KO mice at the early and late timepoints. (D) Proportion of tdTomato-positive PT S1 cells of different PT Classes in cell cycle in Control and KO mice at the early and late timepoints. (B, C, D) Median and inter-quartile range plotted. Pairwise comparisons tested by one-way (B), or two-way ANOVA (C, D) with Holm-Šidák correction. Only significant ($p < 0.05$) comparisons shown.

5.2.6 Associations with ccRCC-like transcriptomic profiles

Finally, to determine the relevance to human ccRCC, I examined if there were overlaps between the changes in *Vhl*-null cells and a “ccRCC-like transcriptomic profile”. The latter was inferred using a list of genes specifically upregulated in tumour cells compared to normal proximal tubular cells that was generated by reference to published scRNA-seq studies on ccRCC samples¹⁷⁰ (see **Chapter 8**). Genes were categorised based on their known regulation by HIF¹⁴⁹, and their expression was scored in tdTomato-positive KO and Control cells at early and late timepoints.

Expression scores were analysed for all HIF target genes together and then separately for the complement of HIF target genes that were, or were not, upregulated in ccRCC. All groupings of HIF target genes exhibited an increase ($p < 1 \times 10^{-16}$ in KO cells compared to Control at the early timepoint (**Figure 5.21A**). However, expression of HIF target genes also upregulated in ccRCC exhibited a further increase in KO cells at the late *versus* early timepoints (**Figure 5.21A**), suggesting an amplification of ccRCC-associated HIF-driven gene expression in *Vhl*-null cells over time. PT Class A cells exhibited the highest response among all renal cells (**Figure 5.21B**).

Expression scores for all ccRCC-upregulated genes mirrored the pattern seen with ccRCC-upregulated HIF target genes (**Figure 5.21A**). However, expression of ccRCC-upregulated genes not known to be HIF targets remained unchanged early after *Vhl* inactivation and decreased significantly and specifically within *Vhl*-null cells over time ($p < 1 \times 10^{-16}$) (**Figures 5.21A and 5.21B**). Interestingly, ‘PT like’ cells showed the least such decrease among all PT cell identities. These results suggest that the specific time-dependent alterations within *Vhl*-null cells in genes not known to be direct HIF targets do not associate with those seen in ccRCC.

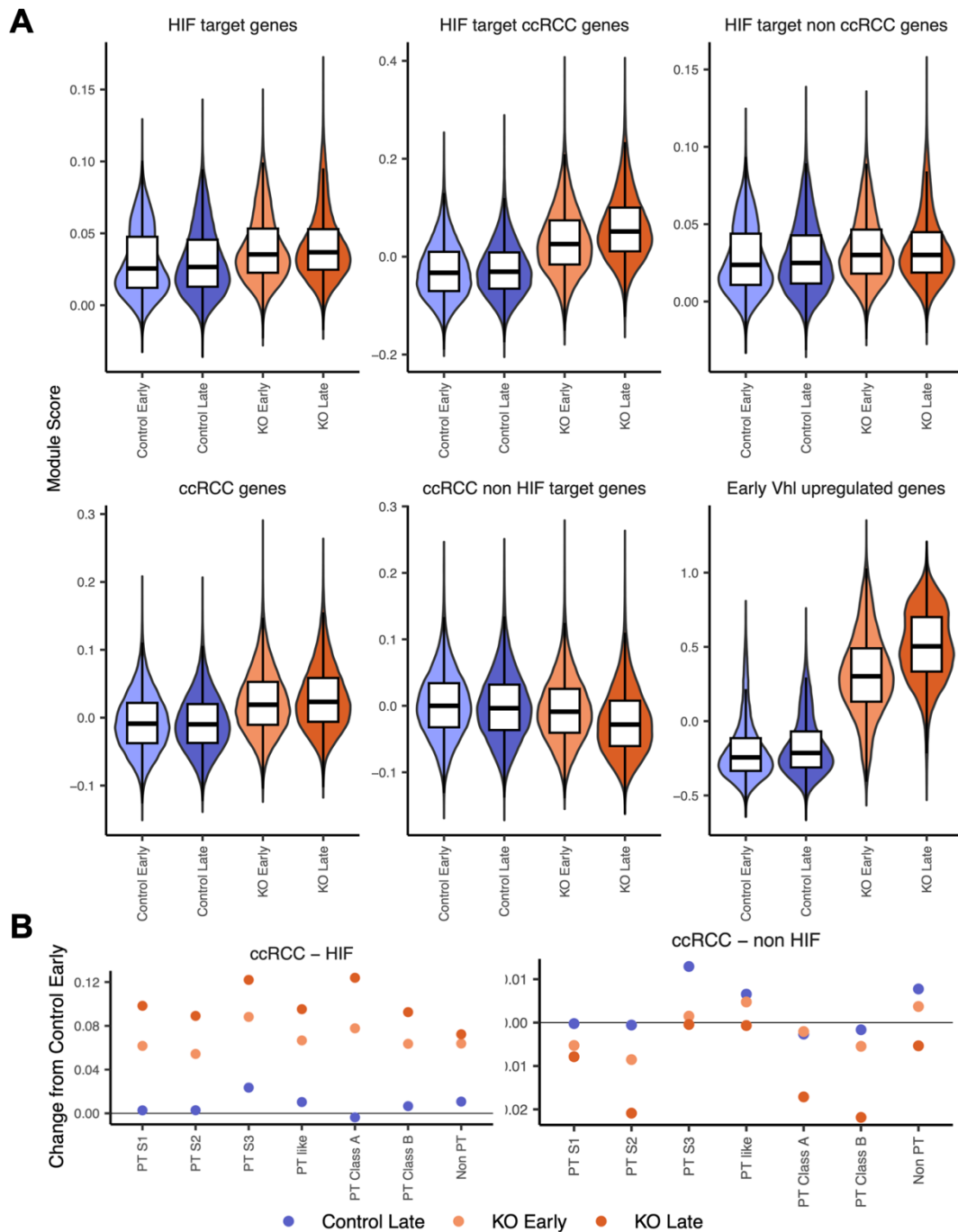


Figure 5.21: Association between gene expression in *Vhl*-null cells with that seen in ccRCC. (A) Violin plots overlaid with boxplots depicting expression score for genes upregulated early after *Vhl* inactivation, and different intersections of HIF target genes and genes upregulated in ccRCC in tdTomato-positive cells from Control and KO kidneys harvested at early or late timepoints. **(B)** Scatter plot depicting changes in mean expression scores for HIF-target (top) and non-HIF-target (bottom) genes specifically upregulated in ccRCC, in cells of different PT identities from different conditions when compared to Control at the early timepoint.

5.3 Discussion

5.3.1 Summary of results

In this chapter, I have employed the model described in **Chapter 2** and taken advantage of developments in the application of single-cell RNA sequencing methodologies to the murine kidney, to identify gene expression changes entrained *in vivo* early after *Vhl* inactivation and those incurred over time. Changes within *Vhl*-null RTE cells and those coincident in *Vhl*-competent epithelial and interstitial cells are described. I have benchmarked data generated in this study to those published in the literature and in the process identified cell types targeted by *Pax8-CreERT2* in my experimental system which, contrary to reports of pan-RTE activity of this Cre allele¹³⁷, did not include distal tubular cells (**Figures 5.1 - 5.3**). A novel dichotomy in proximal tubular gene expression was discovered and validated *in situ* in ‘normal’ kidneys (**Figures 5.4 - 5.6**).

The consequences of *Vhl* inactivation were then assessed in relation to the described renal cell heterogeneity. Remarkable cell type-specificity was observed among genes regulated by *Vhl* at the early timepoint and expression of β -Defensin genes was identified as a conserved *in vivo* response to *Vhl* inactivation in the RTE (**Figures 5.7 - 5.12**). Despite no further interventions performed on *Vhl*-null cells, gene expression was found to be significantly altered in *Vhl*-null cells over a 4–12-month period after recombination to a greater degree than was seen with age in *Vhl*-competent cells of the RTE. These alterations impacted classes of genes not affected by *Vhl* inactivation at the early timepoint and involved fatty acid, immune, and dedifferentiation pathways (**Figures 5.13 - 5.19**). Transient proliferation induced by *Vhl* inactivation was demonstrated (**Figure 5.20**) and finally evidence furnished for a paradoxical diminution in the expression of non-HIF target ccRCC-associated genes specifically within *Vhl*-null cells over time (**Figure 5.21**).

These findings provide the first description of cell-type specific time-resolved effects of *Vhl* inactivation in the RTE *in vivo* and highlight its tumour suppressive functions for further investigation.

5.3.1.2 Caveats and limitations of the experimental system

A general caveat to interpreting this work is the low read-depth in scRNA-seq. Due to the practicalities of reverse transcription in single cell droplets and limits on the total cDNA that can be amplified and sequenced without introducing amplification errors, read depths with single-cell RNA sequencing are considerably smaller than those considered standard for bulk RNA-seq. scRNA-seq studies using the same technology as utilised here generally report ~5000 cells per sample, with an average of ~10,000 useful ‘reads’ generated per cell^{179,181,183,228}. In contrast, data generated here contains ~15,000 cells per sample at a read depth to ~3,500 reads per cell. Therefore, it is likely that my findings under-represent the totality of gene expression changes incurred. Though the same protocols for single-cell droplet generation and sequencing were followed here as reported by others, FAC-sorting of samples may have increased cellular yield compared to the other studies which generated single-cell droplets directly from dissociated tissue suspensions.

The loss of medullary cells (Loop of Henle cells and principal cells of the collecting duct), and the overall low representation of non-proximal tubular cell types might also be a consequence of the tissue dissociation and sorting procedures used that were optimised for maximal recovery of tdTomato-positive (largely PT) cells. It is also possible that not all types of, and states of renal immune cells were recovered by the protocols used here. Nonetheless, FAC-sorting was essential to enriching the dataset for recombined cells that otherwise are a small minority of the total renal population. Furthermore, considering the low read-depth in scRNA-seq, identification of tdTomato-positive cells by FAC-sorting was deemed more reliable than by detecting tdTomato transcript in sequenced cells.

5.3.3 Heterogeneity in the response to *Vhl* inactivation in the RTE

5.3.3.1 Cell-type specific responses associate with region-specific survival and proliferative phenotypes

A major finding of my analyses is that the *in vivo* effects of *Vhl* inactivation are substantially cell type-specific and encompass changes in genes that are not known to be direct HIF targets. Of the 277 genes regulated by *Vhl* in any cell type in the RTE, only 20 exhibited ubiquitous regulation, and only 43 were known HIF targets. There are three implications of these observations. First, that HIF has cell-type specific targets in renal cells that were not identified in studies of other cell types; this is especially possible if HIF binding is impacted by cell-type specific organisation and accessibility of genomic DNA as reported by others²⁴³. Second, that HIF activation has cell type-specific indirect effects, possibly induced by the cooperation of HIF target genes and cell type-specific transcription factors. Third, that *Vhl* regulates transcription factors other than HIF in a cell-type specific manner.

Although causality is difficult to infer, the cell-type specific responses identified in these analyses closely associate with the region-specific proliferative and survival phenotypes observed in **Chapter 4**. Cyclin D1 (*Ccnd1*) was significantly upregulated in all PT cell types, but not in CDIC cells early after *Vhl* inactivation; conversely *Cdkn1a* that codes for the cell cycle inhibitory regulator p21^{Cip1} was significantly upregulated in CDICs, but not in any PT cell type. The absence of tdTomato-positive distal tubular cells in my data suggests that most, if not all, cortical and outer medullary phenotypes observed in **Chapter 4** arise from PT cells; likewise, the behaviour of CDICs may be linked to phenotypes in the papilla which is composed of collecting ducts. Collectively, these suggest that inverse regulation of Cyclin D1 and p21^{Cip1} in PT and CDICs might underlie survival advantages and disadvantages conferred by *Vhl* inactivation in these cells.

5.3.3.2 PT Class A cells are potential cells-of-origin for ccRCC

An unexpected dichotomy in gene expression was also revealed within renal proximal tubular cells which cut across sex-specific, and cell-type specific patterns of gene

expression identified in this and other single-cell studies¹⁷⁹⁻¹⁸². This allowed the analysis of *Vhl* inactivation within novel categorisations of PT cells and added to the heterogeneity associated with *Vhl*-dependent responses. This dichotomy arose from expression of genes in PT Module A that include long non-coding RNAs *Neat1* and *Malat1* and AP-1 transcription factors *Jun*, *Jund*, *Fos*, and *Fosd*, among others. Expression of AP-1 genes in a subset of sequenced cells has been reported by some studies as an artefact of tissue dissociation in scRNA-seq²⁴⁴. However, this assertion has been contested by others who demonstrated baseline expression of *Jun* and *Fos* in a subset of cells in normal tissues and cancers²⁴⁵. I have validated this dichotomy as being present in normal kidneys using *in situ* RNA hybridisation and by reference to published single-nuclear RNA sequencing datasets, that unlike scRNA-seq are reported to yield fewer dissociation artefacts²³⁶. Thus, while some contribution of tissue dissociation cannot be excluded, there is sufficient evidence to support the categorisation of PT cells based on PT Module A expression in mice *in vivo*. At present, whether the two classes represent distinct differentiation pathways or interchangeable cell states cannot be discerned.

PT Class A cells exhibited a greater magnitude of changes in *Vhl*-dependent genes early after inactivation, specific regulation of genes associated with adverse prognosis of ccRCC, and manifested the greatest increase in expression of ccRCC-associated genes that are known transcriptional targets of HIF. Furthermore, these cells were most likely to enter proliferation after *Vhl* inactivation was induced. Previous studies have associated ccRCC cells-of-origin with PT cells expressing the cell adhesion molecule *VCAM1*^{89,90}. While *Vcam1* was not identified in our analysis, we observed a considerable overlap in PT Module A genes and those whose expression is highly correlated with *VCAM1* in human PT cells (unpublished data from Dr Olivia Lombardi and Dr Ran Li). These observations support the relevance of PT Class A cells to cancer development. Altogether, these results reinforce the

scope for further *in vivo* investigations into the actions of *Vhl* with specific attention given to renal cell heterogeneity.

5.3.2 Non-oncogenic adaptation in *Vhl*-null cells over time

Another major conclusion drawn from this work is that despite no further interventions performed, and a HIF transcriptional cascade expected to be fully active within days of *Vhl* inactivation, *Vhl*-null cells undergo specific transcriptomic changes in tissue over time that are distinct from what is seen with age. This finding supports the assertion that *Vhl* inactivation is not a passive event in tissue-specific tumorigenesis.

5.3.2.1 A potential role of β -defensin genes as alarm signals generated by *Vhl*-null cells

One of the most marked and widely observed changes was that of transcripts encoding β -Defensins. *Vhl* inactivation caused an early upregulation of β -Defensin-19 (*Defb19*) and -29 (*Defb29*) and a further increase in *Defb19* expression was observed over time. Others have identified *Vhl*-dependent *Defb19* upregulation in kidneys but only after ischaemic injury was induced¹⁵⁶. My data suggest that this upregulation occurs immediately after *Vhl* inactivation and is independent of acute injury. *DEFB119*, the human orthologue of *Defb19*, is not upregulated in hypoxia in cell culture¹⁴⁹ suggesting that its upregulation might be specifically an *in vivo* response to *Vhl* inactivation. β -Defensins are small proteins secreted by epithelial cells and are thought to have roles in innate immunity²⁴². This is of particular note in relation to the inflammatory foci of macrophages found in KO kidneys at the late timepoint (**Chapter 4**). An involvement of innate immunity was highlighted in the current analysis by observations of gene expression changes in renal macrophages that resembled leukocyte activation. These findings raise the possibility that defensin upregulation is a potential ‘alarm’ signal generated by cells of the RTE in response to pathological *Vhl* inactivation. Its role in the survival and transient proliferation of *Vhl*-null RTE cells and in interactions with the renal interstitium merit further investigation.

5.3.2.2 Gene expression in *Vhl*-null cells does not associate with ccRCC-like profiles over time

It has been hypothesised that indiscriminate gene expression changes entrained by the pathological stabilisation of HIF following *VHL* inactivation are ‘fine-tuned’ over time until a net pro- and anti-oncogenic transcriptional profile is achieved, in a phenomenon called ‘pathway adaptation’¹⁴⁴. My analysis partially supports this hypothesis. Among all HIF target genes, expression of those upregulated in ccRCC increased in *Vhl*-null cells over time to a greater extent than those that are not upregulated in ccRCC. This is indicative of a selective amplification of HIF responses associated with ccRCC in *Vhl*-null cells over time. However, the converse was not observed – expression of HIF target genes not upregulated in ccRCC did not subside over time, nor did any gene regulated at the early timepoint return to baseline expression at the late timepoint. Unlike early upregulation and further amplification of the ccRCC-associated HIF response, expression of ccRCC-upregulated non-HIF target genes was not altered at the early timepoint, and in fact decreased specifically in *Vhl*-null cells over time. This suggests the presence of an active adaptive process incurred in *Vhl*-null cells that might be independent of direct HIF activity.

Associated with this decrease, I also observed that markers of proliferation that had initially increased in *Vhl*-null cells at the early timepoint had subsided to baseline levels at the later timepoint, a result that reproduced similar findings in **Chapter 4**. Taken together, these results suggest that an oncogenic drive entrained early in *Vhl*-null cells is restrained over time, possibly by elements of *Vhl*-dependent biology that are not known to be directly affected by HIF.

5.3.3 Implications for tumorigenesis

Overall, this work adds to our understanding of *VHL*-associated tumorigenesis in two ways. It first provides empirical data on the heterogeneous effects of *Vhl* loss in different populations of the RTE and highlights PT Class A as particularly entraining oncogenic consequences early after *Vhl* inactivation. Second, it demonstrates (along with data in

Chapter 4) that *Vhl*-null cells change with time; while some of these changes associate positively with oncogenesis (dedifferentiation, lipid metabolism, and amplification of cancer-upregulated HIF response), others negatively impact on their proliferative capacity and expression of other genes involved in oncogenesis. It further highlights the role of genes not known to be direct HIF targets in adaptations incurred in *Vhl*-null cells over time. These changes might occur because:

1. *Vhl*-null cells incur mutations that affect gene expression over time.
2. Early effects of *Vhl* inactivation gradually impact on the epigenome of *Vhl*-null cells and thus alter gene expression.
3. Long-term interactions between *Vhl*-null cells and renal immune cells alter gene expression in *Vhl*-null cells.

The first hypothesis is supported by previous work connecting *Vhl* inactivation to defects in the mitotic spindle and increased chromosomal aneuploidies⁴⁷ but is difficult to test *in vivo* with current methodology unless specific genomic events are selected over time. The second hypothesis is supported by the observation that the top 3 genes inactivated in ccRCC (barring *VHL*) – *PBRM1*, *BAP1*, and *SETD2* – are all known to be epigenetic modulators; it is possible that some of the time-dependent non-oncogenic adaptations entrained in *Vhl*-null cells are dependent on the actions of these genes. The third hypothesis has emerged from observations in this work of inflammatory foci and defensin upregulation.

Further investigation into the effects of *Vhl* loss that accumulate over time, their mechanistic basis, and the impact of secondary mutations on these effects will be key to unravelling the enigma of tissue-specific tumour suppression by *VHL*.

Chapter 6:

Effects of Concomitant *Pbrm1* Inactivation on *Vhl*-null Cells in the RTE

6.1 Introduction

While biallelic inactivation of *VHL* is the truncal genomic event in the evolution of ccRCC, it is insufficient for tumorigenesis⁷⁰. Tumorigenesis requires the additional inactivation of genes such as *PBRM1*, *SETD2*, or *BAP1*, that lie on the same chromosome as *VHL*^{51,55}. Of these, biallelic inactivation in *PBRM1* (one *PBRM1* allele generally inactivated in a Chr 3p loss event and the other allele inactivated by a nonsense or missense mutation) is most common⁹⁵. *PBRM1* is a poly-bromodomain containing protein and is best characterised as the accessory subunit of the PBAF SWI/SNF chromatin remodelling complex⁹⁹. It also interacts with methylated microtubules and may thus have additional roles in cytoskeletal organisation¹⁶⁶. Unlike mice in which *Vhl* alone has been inactivated, mice in which both alleles of *Vhl* and *Pbrm1* have been concomitantly inactivated in cells of the renal tubular epithelium (RTE) develop renal cancers^{104,109,111}; such cancers have not been observed in mice modelling biallelic *Pbrm1* inactivation on its own, and biallelic *Vhl* inactivation coupled to monoallelic *Pbrm1* inactivation^{104,109,111}. This has implicated *PBRM1* as a tumour suppressor in ccRCC, the biallelic inactivation of which is sufficient to drive oncogenesis in *Vhl*-null cells of the RTE^{104,109,111}. However, the mechanism by which additional *Pbrm1* inactivation drives oncogenesis in *Vhl*-null cells remains unknown.

Data presented so far in this thesis on the cellular and transcriptomic effects of *Vhl* inactivation in the RTE has shown that *Vhl*-null cells exhibit tissue- and cell type-specific survival phenotypes and gene expression patterns. Also, that while some *Vhl*-null cells proliferate early after *Vhl* inactivation in the renal cortex, this proliferation is restricted at later timepoints, and that the continued retention of these cells in proximal tubules is associated with adaptive changes in their gene expression. I hypothesise that *Pbrm1* inactivation drives oncogenesis in *Vhl*-null cells by altering their time-dependent adaptation and serves to relieve the restraints on their proliferation at the late (>4 months after recombination is induced) timepoint.

In this chapter, I aim to explore this hypothesis and study cell fates and gene expression changes entrained after concomitant *Vhl* and *Pbrm1* inactivation in cells of the RTE and compare these to phenotypes observed previously in *Vhl*-null cells. Specifically, I set out to:

1. Couple the novel reporter model described in **Chapter 2** to a conditional *Pbrm1* allele and establish the utility of this experimental system to accurately identify and assay *Vhl/Pbrm1*-null cells *in situ* and *ex vivo*.
2. Reproduce dysplasia and renal cancer with concomitant *Vhl* and *Pbrm1* inactivation as reported by others and then trace them back to earlier stages of oncogenesis using the reporter mouse model.
3. Identify gene expression changes caused by *Vhl* and *Pbrm1* inactivation in cells of the RTE in comparison to those caused by *Vhl* alone and assess their relation to the development of cancer.
4. Test if *Pbrm1* inactivation affects the region-specific survival and elimination phenotypes associated with *Vhl* inactivation on its own.

5. Test if *Pbrm1* inactivation relieves proliferative restraints on *Vhl*-null cells at the late (> 4 months after induction) timepoints.

This exploration leads to an evaluation of my starting hypotheses and suggests new approaches that might inform on understanding how *Vhl* and *Pbrm1* inactivation cooperate in the development of ccRCC.

6.2 Results

6.2.1 *Vhl/Pbrm1*-null cells form dysplastic lesions in the renal cortex at 17 months after recombination

6.2.1.1 Lesions in a mouse modelling concomitant inactivation of *Vhl* and *Pbrm1* at 17 months after recombination

I first tested the hypothesis that concomitant *Vhl* and *Pbrm1* inactivation causes dysplasia and oncogenesis in the kidney using mice in which *Vhl*^{*flae.KO/pjr.fl*} was coupled to homozygous conditional alleles for the *Pbrm1* gene (*Pbrm1*^{*fl/fl*}). In these *Pbrm1* alleles, a pair of *loxP* sites flank *Pbrm1* exon 4 to enable its Cre-mediated excision. Excision of exon 4 introduces a frameshift in the 24 *Pbrm1* exons downstream to exon 4 which results in the translation of a dysfunctional PBRM1 protein¹⁰⁹. Others have reported incidence of renal cancer in *Vhl*^{*fl/fl*}; *Pbrm1*^{*fl/fl*}; *Pax8-CreERT2* mice given 5x 2 mg TMX and harvested >20 months after recombination¹⁰⁹. While I could not maintain mice for this long after TMX dosing due to the constraints of the mouse procedural licence, one *Vhl*^{*flae.KO/pjr.fl*}; *Pbrm1*^{*fl/fl*}; *Pax8-CreERT2* (“*Vhl Pbrm1* KO”) mouse given 5x 2 mg TMX could be harvested at 17 months after TMX dosing. Histological (tdTomato IHC and Trichrome staining) analyses on a total of twenty-six 4 µm sections (4 sections cut at 50 µm intervals through the tissue block) revealed extensive lesions present in the cortex but not the outer medulla, inner medulla, and papilla of the kidney from this mouse. The lesions could be classified into three categories – dysplasia (**Figure 6.1A**), cystic dilatation (**Figure 6.1B**), and disorganised tubules (**Figure 6.1C**).

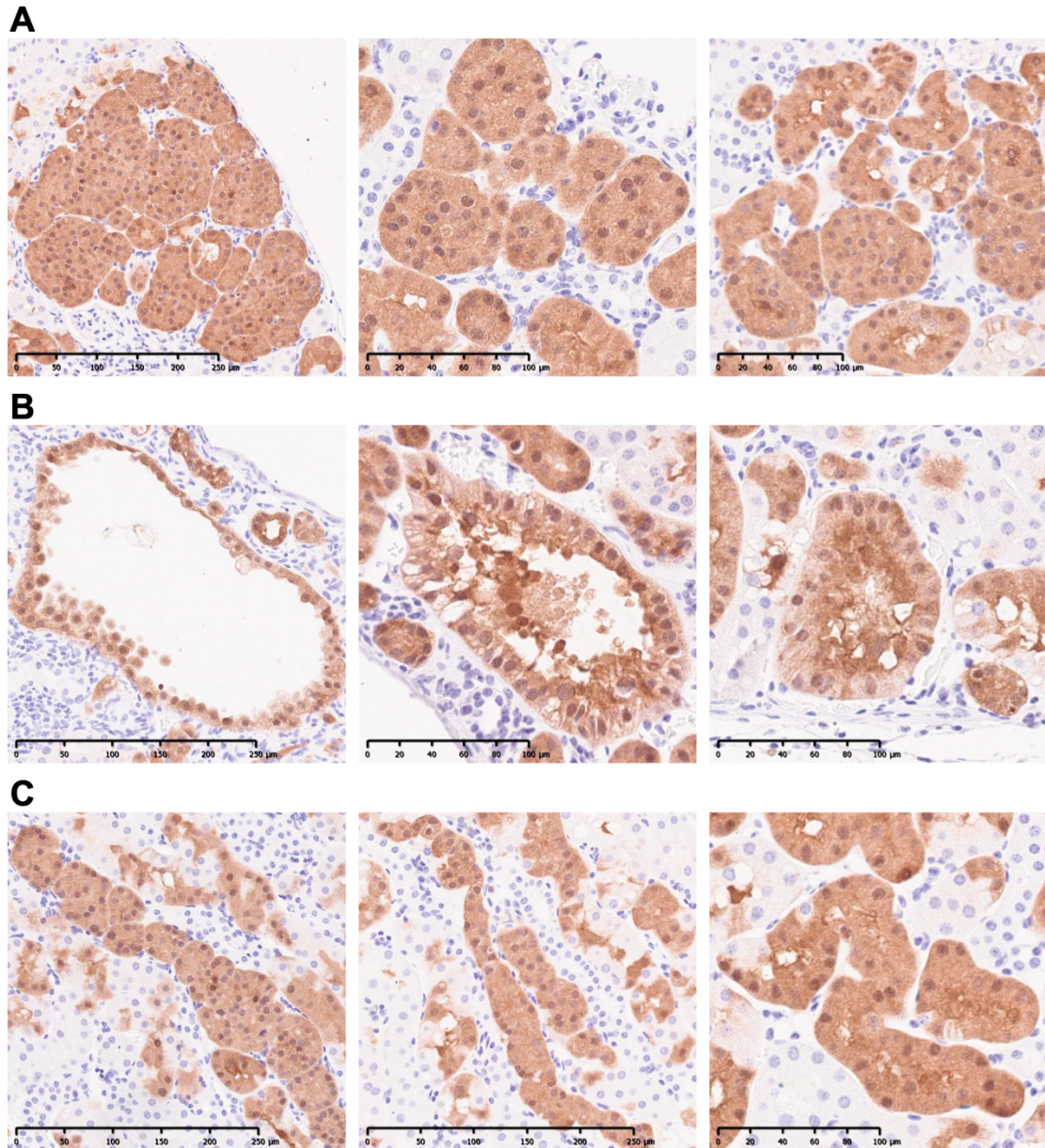


Figure 6.1: An atlas of lesions associated with concomitant *Vhl* and *Pbrm1* inactivation in the RTE. Representative tdTomato IHC on kidneys of one *Vhl^{flae.KO/pjr.fl}; Pbrm1^{fl/fl}; Pax8-CreERT2* mouse given 5 x 2 mg TMX and harvested at 17 months after TMX dosing. Images represent lesions classified as (A) dysplasia, (B) cystic dilatation, and (C) disorganised tubules. Scale bar denotes 250 µm for left panels in (A), (B), and left and middle panels for (C); 100 µm for middle and right panels in (A), (B), and right panel in (C). tdTomato IHC performed in collaboration with Dr Joanna Lima.

Lesions classified as ‘dysplasia’ appeared as aggregations of renal tubules composed entirely of tdTomato-positive cells; the tubules lacked defined boundaries and contained multi-layered epithelia that did not conform to the normal circumferential arrangement around a lumen. The lesions were interspersed with tdTomato-negative interstitial cells (**Figure 6.1A**). Cycling cells were also detected within these lesions, as identified by dual tdTomato/Ki67 IHC (**Figure 6.2**). The largest lesions (exemplified by the left-most panel in **Figure 6.1A**) were present on the outer edge of the renal cortex and resembled the dysplastic and cancerous lesions reported by others¹⁰⁹.

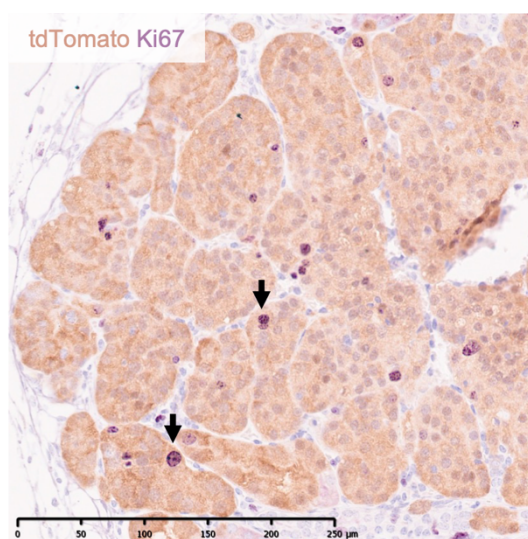


Figure 6.2: Dysplastic lesions in *Vhl Pbrm1* KO mice contain proliferating cells. Representative tdTomato (brown) and Ki67 (purple) dual IHC performed on kidney sections from a *Vhl Pbrm1* KO mouse given 5 x 2 mg TMX and harvested 17 months after TMX dosing. Black arrows point to tdTomato-positive Ki67-positive cells. Scale bar denotes 250 μm .

Lesions classified as ‘cystic dilatations’ comprised renal tubules with mono-layered epithelia composed entirely of tdTomato-positive cells that exhibited a columnar morphology and elongated nuclei. These tubules had enlarged lumens that contained tdTomato-positive cells or nuclei which appeared as if extruded into the lumen (**Figure 6.1B**). Disorganised

tubules were identified as renal tubules whose profiles almost entirely comprised tdTomato-positive cells, and which exhibited a jagged tubular boundary; the width of the tubule was non-uniform along its length and the nuclei were not linearly organised along its circumference (**Figure 6.1C**). Multi-layered epithelia were also observed in these tubules.

The tubular cells in these lesions were overwhelmingly tdTomato-positive, indicating that the constituent cells were *Vhl*-null. To test if they were also *Pbrm1*-null, PBRM1 IHC was performed on kidney sections from this *Vhl Pbrm1* KO mouse. A *Vhl^{flacKO/pjrf1}; Pax8-CreERT2* (“*Vhl* KO”) mouse given 5x 2 mg TMX and harvested 6 months after dosing was used as a positive control for PBRM1 staining as it carried two wild-type alleles of *Pbrm1*. PBRM1 signal could be detected in all RTE cells of the *Vhl* KO mouse (**Figure 6.3A**). In contrast, none of the tubular cells in the dysplastic lesion were positive for PBRM1 (**Figure 6.3A**). Interestingly, some, but not all cells in morphologically normal renal tubules of the *Vhl Pbrm1* KO mouse also lacked PBRM1 signal (**Figure 6.3A**). These results indicate that the lesions in the *Vhl Pbrm1* KO kidney have arisen from cells with concomitant *Vhl* and *Pbrm1* inactivation but also that many *Pbrm1*-null cells in the same kidney are morphologically normal.

6.2.1.2 tdTomato-positive cells in *Vhl Pbrm1* KO mice are *Vhl/Pbrm1*-null

To corroborate the latter observation and to identify the extent to which tdTomato-positivity (and thus *Vhl* inactivation) related to *Pbrm1* inactivation, I evaluated the recombination status of *Pbrm1* by genomic PCR on FAC-sorted tdTomato-positive and -negative cells from the contralateral kidneys of the *Vhl Pbrm1* KO and *Vhl* KO mice used for the IHC analysis described above. Amplicons corresponding to the wild-type *Pbrm1* allele were detected in tdTomato-positive and -negative cells from the *Vhl* KO mouse (**Figure 6.3B**). While the amplicon corresponding to the unrecombined *Pbrm1^{fl}* allele was detected in tdTomato-negative cells from the *Vhl Pbrm1* KO mouse, it was not detected in tdTomato-positive cells from the *Vhl Pbrm1* KO mouse (**Figure 6.3B**). This indicates that tdTomato-positive cells in kidneys from *Vhl Pbrm1* KO mice do not carry the unrecombined *Pbrm1*

allele. Furthermore, PBRM1 protein (expected MW 200 kDa) was detected by immunoblotting in tdTomato-positive and -negative cells from *Vhl* KO mice and tdTomato-negative cells from *Vhl Pbrm1* KO mice but was not detected in tdTomato-positive cells from *Vhl Pbrm1* KO mice (**Figure 6.3C**). Thus, tdTomato-positive cells in kidneys from *Vhl Pbrm1* KO mice have undergone recombination of both *Vhl* and *Pbrm1* and are essentially *Vhl/Pbrm1*-null. The presence of *Vhl*-competent (tdTomato-negative) but *Pbrm1*-null cells in kidneys of *Vhl Pbrm1* KO mice cannot be excluded by these analyses. Furthermore, the order and time at which *Vhl* and *Pbrm1* are recombined in the same cells cannot be discerned.

Taken together, findings from this one mouse provide proof-of-principle that concomitant *Vhl* and *Pbrm1* inactivation directly drives substantial alterations in the organisation of the RTE and the formation of dysplastic lesions when observed at 17 months after recombination. This supports the proposition that *Pbrm1* inactivation makes *Vhl*-null cells oncogenic. It should be noted here that while this supports the sufficiency of *Vhl* and *Pbrm1* inactivation in modelling dysplasia, the necessity of *Pbrm1* inactivation cannot be inferred as *Vhl* KO mice were not analysed in this study at the 17-month timepoint.

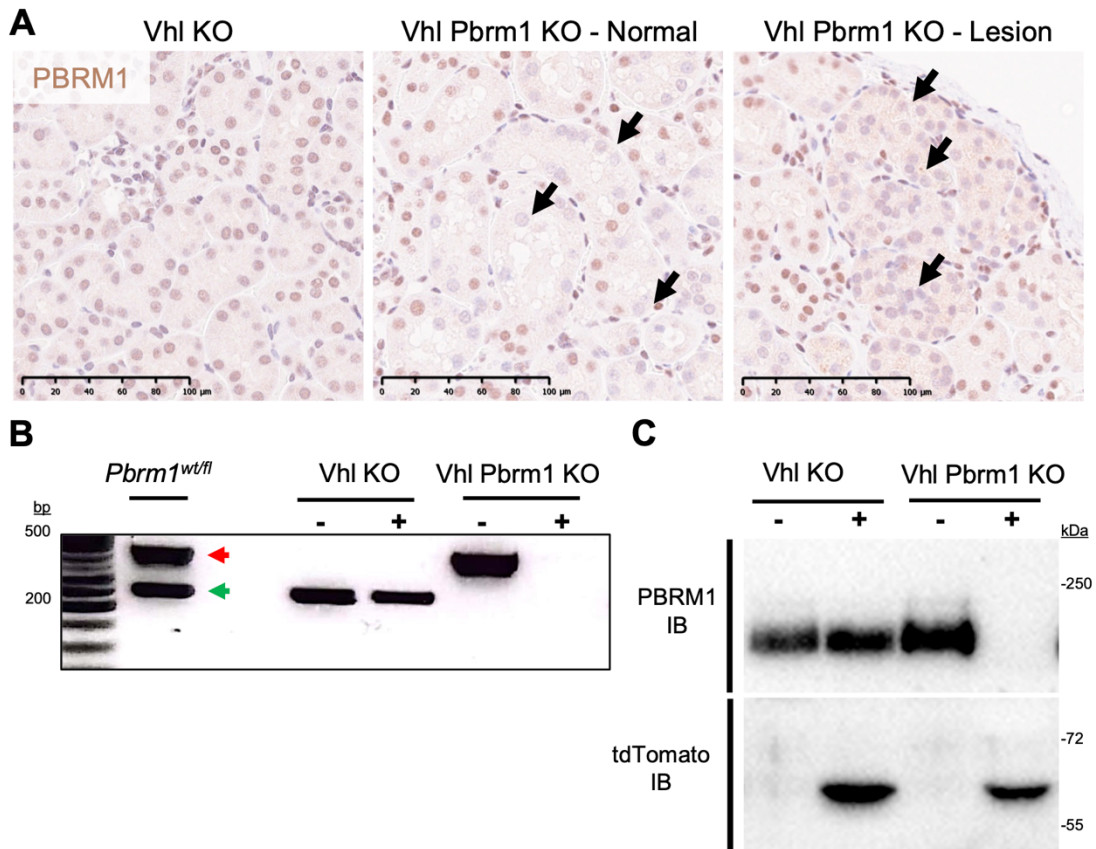


Figure 6.3: tdTomato-positive cells in *Vhl Pbrm1* KO mice have undergone *Pbrm1* inactivation. (A) Representative PBRM1 IHC on kidneys from *Vhl* KO or *Vhl Pbrm1* KO mice given 5x 2 mg TMX and harvested at 8 and 17 months after dosing respectively. Black arrows point to cells in which PBRM1 has not been detected. Scale bar denotes 100 μ m. PBRM1 IHC performed by Dr Joanna Lima. (B) Gel electrophoresis of genomic PCR for wild-type (*Pbrm1*^{wt}) or 'floxed' (*Pbrm1*^{fl}) *Pbrm1* alleles on FAC-sorted tdTomato-positive (+) and tdTomato-negative (-) cells from kidneys of *Vhl* KO or *Vhl Pbrm1* KO mice given 5x 2 mg TMX and harvested at 8 and 17 months after dosing respectively. Green and red arrows point to expected amplicons for *Pbrm1*^{wt} or *Pbrm1*^{fl} alleles. Genomic DNA from mouse heterozygous for wild-type and floxed *Pbrm1* (*Pbrm1*^{wt/fl}) used as positive control for PCR. (C) Representative immunoblots for PBRM1 or tdTomato protein in FAC-sorted tdTomato-positive (+) or tdTomato-negative (-) cells from dissociated kidneys of *Vhl* KO or *Vhl Pbrm1* KO mice given 5x 2 mg TMX and harvested 8 and 17 months after dosing respectively. Immunoblotting performed by Dr Norma Masson.

6.2.2 Pronounced morphological alterations in cortical cells following concomitant *Vhl* and *Pbrm1* inactivation at 10 months after recombination

The timepoint at which the *Vhl Pbrm1* KO mouse was analysed above was much longer than the 4–12-month timepoint at which *Vhl* KO mice have been analysed in this thesis (**Chapters 4 and 5**). It had been left as long as possible as a ‘proof’ that lesions reported in mice with conditional, inducible *Vhl* and *Pbrm1* inactivation¹⁰⁹ can be reproduced with my experimental system. Within the 4–12-month timepoint, I had not observed dysplasia or tubular disorganisation driven by *Vhl*-null cells. To test if *Vhl/Pbrm1*-null cells specifically displayed any morphological alterations at an equivalent timepoint, tdTomato IHC and Trichrome staining were performed on one section each of kidneys from *Vhl^{flac.KO/pjr.fl}; Pbrm1^{fl/fl}; Pax8-CreERT2* (*Vhl Pbrm1* KO) (n = 10) mice given 5x 2 mg TMX and harvested at 10 months after dosing. Phenotypes in *Vhl Pbrm1* KO mice that are not observed in *Vhl* KO mice might arise either from cooperation of *Vhl* and *Pbrm1* inactivation, or from *Pbrm1* inactivation on its own. To discern the former effects from the latter, *Vhl^{wt/pjr.fl}; Pbrm1^{fl/fl}; Pax8-CreERT2* (Control *Pbrm1* KO) (n = 6) mice were also dosed and assayed at equivalent timepoints. Cre-mediated recombination in Control *Pbrm1* KO mice is expected to generate tdTomato-positive cells that are *Vhl^{wt/KO}* (and thus *Vhl*-competent) but *Pbrm1^{KO/KO}* (and thus *Pbrm1*-null), making them marked ‘comparators’ for the tdTomato-positive *Vhl^{KO/KO}* and *Pbrm1^{KO/KO}* (*Vhl/Pbrm1*-null) cells generated in the *Vhl Pbrm1* KO mice. Lesions were identified and classified into the three categories – dysplasia, cystic dilatation, and disorganised tubules – as exemplified in **Figure 6.1**, and the number of lesions observed per mm² of renal tissue was quantified.

No lesions were observed in the outer medulla, inner medulla, and papilla of any mouse analysed, suggesting that concomitant *Vhl* and *Pbrm1* loss does not affect the

morphology of cells in these tissues. In contrast, several lesions were observed in the renal cortex (Figure 6.4).

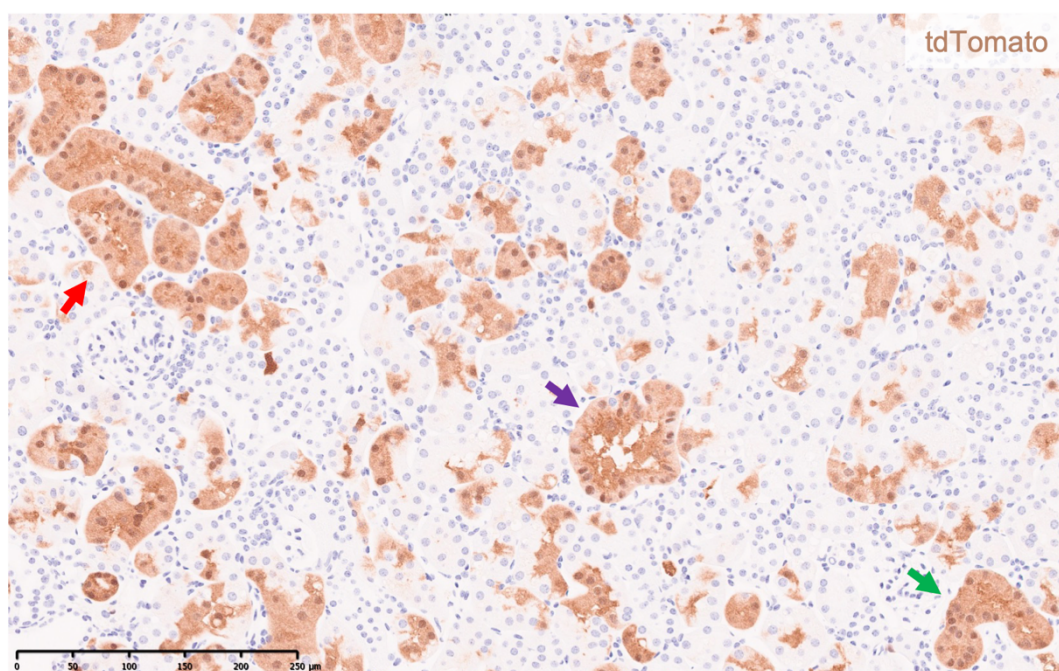


Figure 6.4: Lesions observed in *Vhl Pbrm1* KO mice at 10 months after dosing. Representative tdTomato IHC on kidneys of *Vhl Pbrm1* KO mice given 5x 2 mg TMX and harvested at 10 months after dosing. Red, green and purple arrows denote lesions classified as ‘precursor dysplasia’, ‘disorganised tubules’, and ‘cystic dilatation’ respectively. Scale bar denotes 250 μ m. tdTomato IHC performed in collaboration with Ayslan Barros.

6.2.2.1 *Vhl/Pbrm1*-null cells form lesions resembling precursors of dysplasia at 10 months after recombination

Lesions that resembled those classified as overt ‘dysplasia’ (Figure 6.1A) were not detected in the renal cortex of any mouse analysed at this timepoint. However, numerous lesions that appeared to be precursors of the large dysplastic lesions were observed and classified as ‘precursor dysplasia’. These were characterised by: 1) the aggregation of several renal tubules comprised almost entirely of tdTomato-positive cells, 2) multi-layered epithelia detected in the profiles of at least some of these tubules, and 3) interspersed clusters of tdTomato-negative cells between the aggregated renal tubules (Figure 6.5A). These differed from the ‘dysplastic’ lesions observed at the 17-month timepoint on account of their smaller size, and because they also contained tubules with defined boundaries or mono-layered

epithelia (**Figure 6.5A**). The presence of tdTomato-negative interstitial cell clusters in these lesions was particularly interesting, as clusters of renal macrophages were found in the renal cortex of *Vhl* KO mice at the late timepoint (**Chapter 4**). Interestingly, analysis of serial sections showed that while some of the tdTomato-negative cells in these lesions colocalised with F4/80 signal and were therefore likely to be macrophages, many of the clusters did not (**Figure 6.5A**), suggesting these lesions were formed by other cell types. The renal cortex of *Vhl Pbrm1* KO mice contained significantly higher ($p = 0.0005$) numbers of such lesions than Control *Pbrm1* KO mice (**Figure 6.5B**), suggesting that their incidence was incumbent on concomitant *Vhl* and *Pbrm1* inactivation, but not *Pbrm1* inactivation on its own. These results suggest that the presence and expansion of *Vhl/Pbrm1*-null cells in cortical renal tubules initiates the formation of dysplastic lesions at 10 months after recombination.

6.2.2.2 *Vhl/Pbrm1*-null cells cause disorganisation of the RTE at 10 months after recombination

Lesions that resembled those classified as “disorganised tubules” (**Figure 6.1C**) were frequently observed in the renal cortex of *Vhl Pbrm1* KO mice at the 10-month timepoint (**Figures 6.6A and 6.6B**). These exhibited rough tubular boundaries, nuclei misaligned in relation to the tubular circumference, and sometimes contained multi-layered epithelia (**Figure 6.6A**); the altered tubular boundary and the presence of multi-layered epithelia could be discerned by Trichrome staining (collagen, cytoplasm, and nuclei stained blue, pink and purple respectively) (**Figure 6.6A**). These lesions were infrequently observed in Control *Pbrm1* KO mice (**Figure 6.6B**), suggesting that *Pbrm1* inactivation on its own might contribute to the formation of these lesions. However, disorganised tdTomato-positive tubules were observed at a significantly higher frequency ($p = 0.0005$) in the renal cortex of *Vhl Pbrm1* KO mice compared to those of Control *Pbrm1* KO mice, indicating that concomitant *Vhl* and *Pbrm1* inactivation affected tubular organisation to a greater extent than *Pbrm1* inactivation on its own. These results suggest that the presence and expansion of

Vhl/Pbrm1-null cells in cortical renal tubules alters the organisation of these tubules at 10 months after recombination.

6.2.2.3 *Vhl/Pbrm1*-null cells did not significantly induce cystic dilatation of renal tubules at 10 months after recombination

Lesions resembling those classified as “cystic dilatations” (**Figure 6.1C**) were observed in the renal cortex of 6/10 *Vhl Pbrm1* KO mice and 2/6 Control *Pbrm1* KO mice at the 10-month timepoint (**Figure 6.7A**). Though the proportion of mice that exhibited these lesions and the median number of lesions observed per mm² cortical tissue in kidneys were higher for *Vhl Pbrm1* KO mice compared to Control *Pbrm1* KO mice, the difference was not statistically significant ($p = 0.28$) (**Figure 6.7B**). Cystic dilatation of renal tubules was previously seen in ageing mice irrespective of *Vhl* inactivation (**Chapter 4**). Therefore, it is not possible to conclude that concomitant *Vhl* and *Pbrm1* inactivation directly induced cystic dilatation in the renal tubules here.

6.2.2.4 Morphological effects of *Pbrm1* inactivation are likely to be specifically entrained in *Vhl*-null cells and occur at late timepoints

The necessity of *Pbrm1* inactivation for inducing these morphological effects in *Vhl*-null cells can be assayed by comparing *Vhl Pbrm1* KO mice to *Vhl* KO mice given the same TMX dose and harvested at the same timepoints. Cystic dilatation of tubules was observed in 3/8 *Vhl* KO mice given 5x 2 mg TMX and harvested 4-12 months after dosing, though the lesions in those mice were lined by tdTomato-negative cells (**Chapter 4**). However, lesions resembling “precursor dysplasia” and “disorganised tubules” were not observed in any of the *Vhl* KO mice harvested 4-12 months after dosing with 5x 2 mg TMX ($n = 8$) or 1 mg TMX ($n = 6$). This suggests that precursors of dysplastic lesions and tubular disorganisation does not occur with *Vhl* inactivation on its own as it does with concomitant *Vhl* and *Pbrm1* inactivation, thereby supporting additional *Pbrm1* inactivation as necessary for transformation of *Vhl*-null cells. A formal, systematic analysis of multiple sections from the *Vhl* KO mice is necessary to substantiate these preliminary observations.

Histological analysis of kidneys from *Vhl Pbrm1* KO mice harvested 1-3 weeks after TMX dosing (n = 6; one section per mouse) did not reveal any class of lesion in any renal region of any mouse, suggesting that they might not occur in this early time-window after concomitant *Vhl Pbrm1* inactivation.

Altogether, the results obtained so far demonstrate that *Vhl/Pbrm1*-null cells cause cortex-specific tubular disorganisation and have begun to form dysplastic lesions in the RTE at 10 months after recombination that might go on to form tumours at even later timepoints. They possibly suggest that these lesions do not occur early after *Vhl* and *Pbrm1* inactivation and thus involve some oncogenic process entrained in some *Vhl/Pbrm1*-null cells over time.

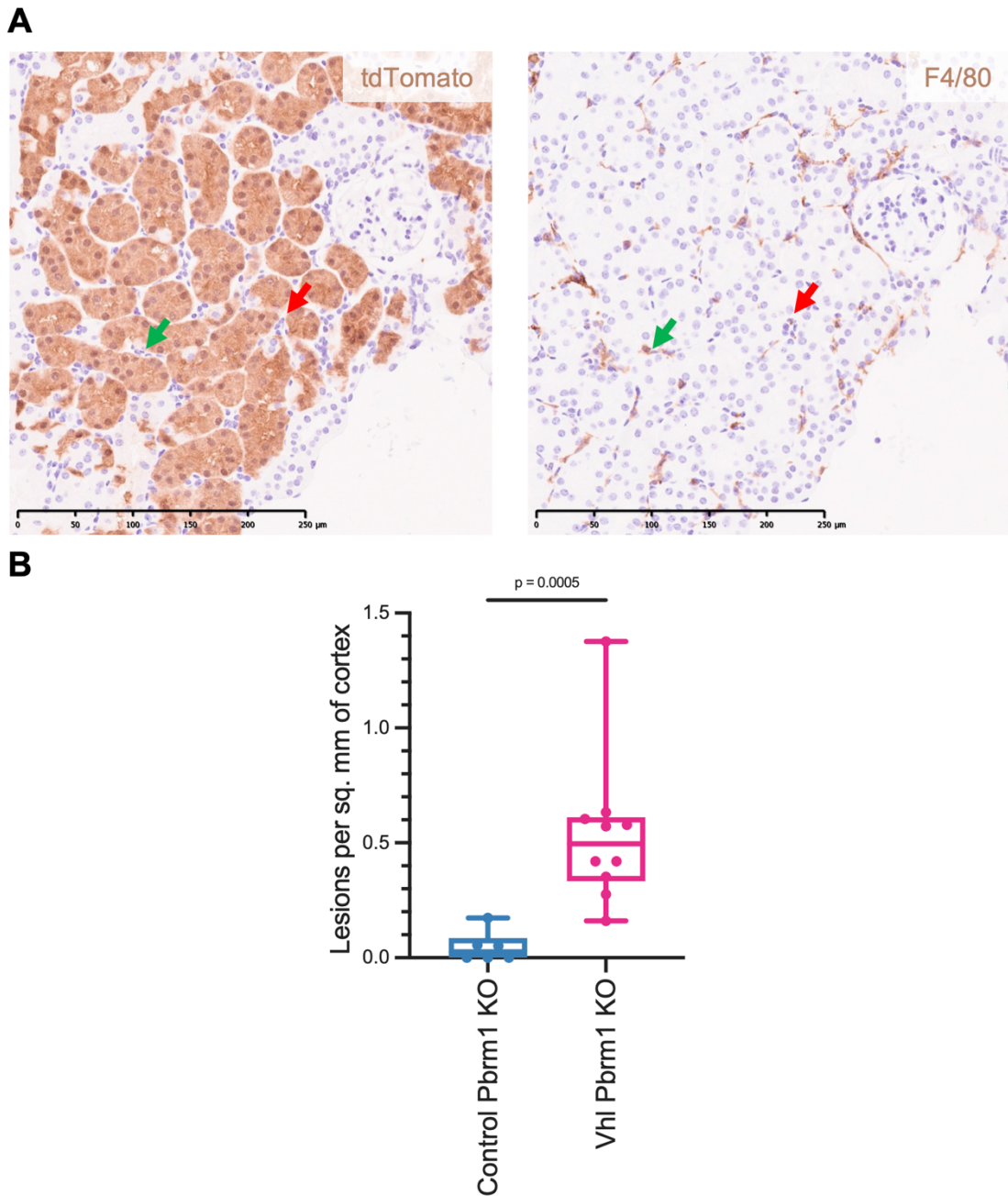


Figure 6.5: *Vhl Pbrm1* KO mice exhibit increased incidence of precursor dysplastic lesions at 10 months after dosing. (A) Representative tdTomato (left) and F4/80 (right) IHC performed on serial sections of kidneys from *Vhl Pbrm1* KO mice given 5x 2 mg TMX and harvested at 10 months after dosing depicting a lesion classified as ‘precursor dysplasia’. Green and red arrows point to interstitial cells that did or did not colocalise with F4/80 signal respectively. Scale bar denotes 250 μ m. (B) Number of ‘precursor dysplasia’ lesions per mm² cortical tissue detected in kidneys of Control *Pbrm1* KO (n = 6) and *Vhl Pbrm1* KO (n = 10) mice given 5x 2 mg TMX and harvested at 10 months after dosing. Median and inter-quartile range plotted. Pairwise comparison by Mann-Whitney test.

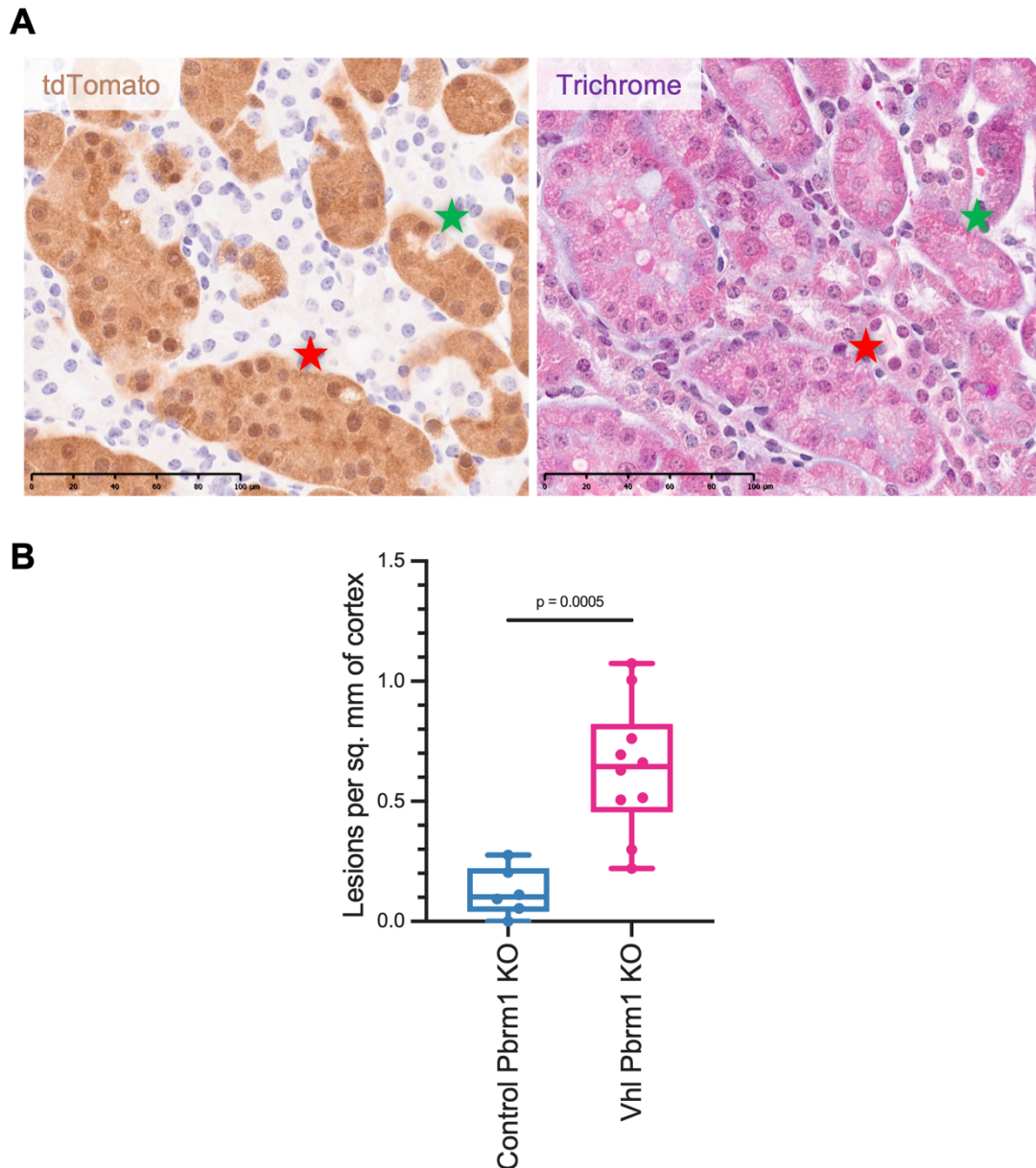


Figure 6.6: *Vhl Pbrm1* KO mice exhibit increased incidence of disorganised tubules at 10 months after dosing. (A) Representative tdTomato IHC (left) and Trichrome staining (right) performed on serial sections of kidneys of *Vhl Pbrm1* KO mice given 5x 2 mg TMX and harvested at 10 months after dosing depicting disorganised tubules. Green and red stars denote morphologically ‘normal’ and ‘disorganised’ tubules respectively. Scale bar denotes 100 μ m. Trichrome staining performed by Dr Joanna Lima. (B) Number of ‘disorganised tubules’ lesions per mm² cortical tissue detected in kidney of Control *Pbrm1* KO (n = 6) and *Vhl Pbrm1* KO (n = 10) mice given 5x 2 mg TMX and at harvested 10 months after dosing. Median and inter-quartile range plotted. Pairwise comparison by Mann-Whitney test.

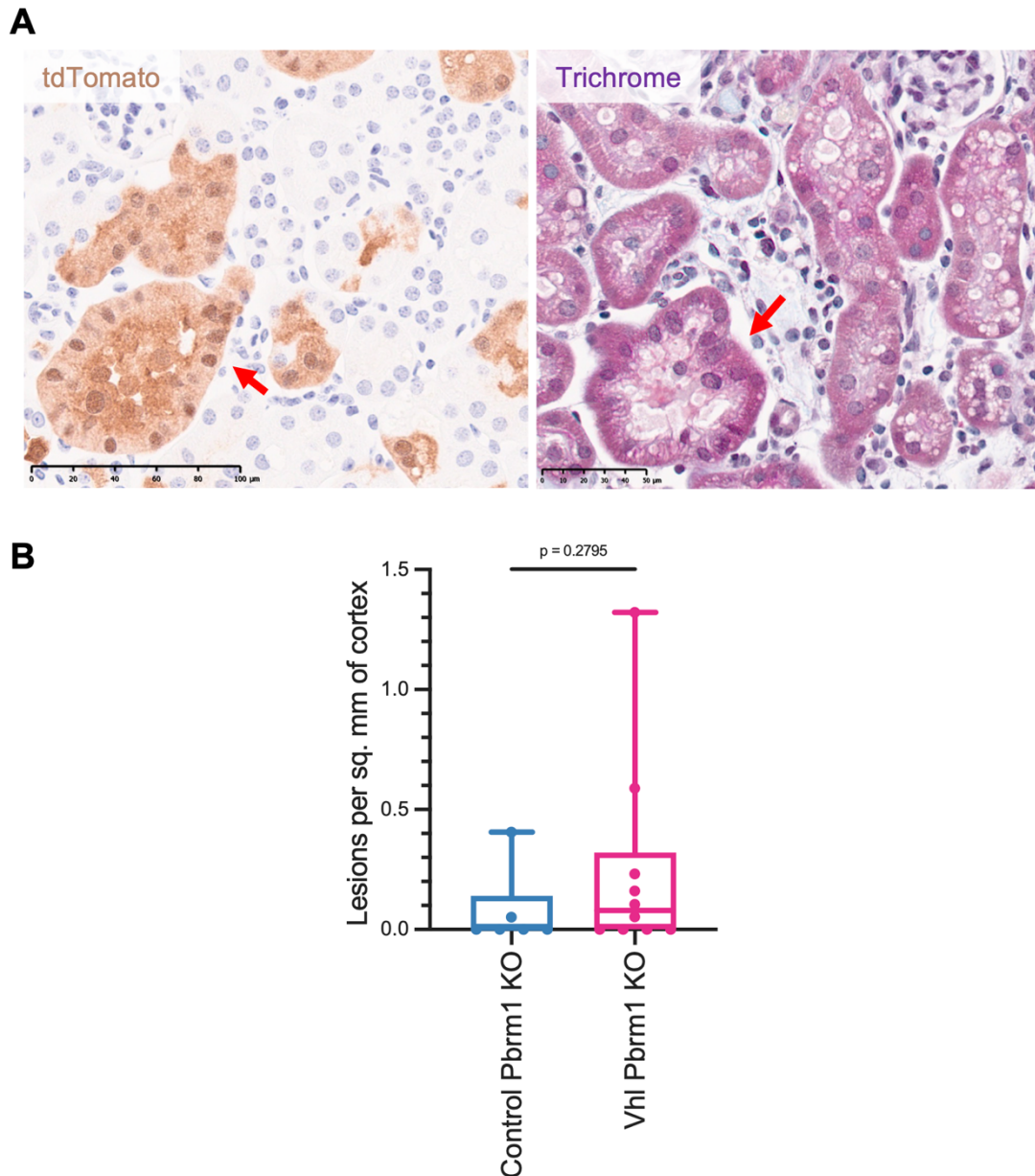


Figure 6.7: Cystic dilatation observed in *Vhl Pbrm1* KO mice at 10 months after dosing. (A) Representative tdTomato IHC (left) and Trichrome staining (right) performed on serial sections of kidneys from *Vhl Pbrm1* KO mice given 5x 2 mg TMX and harvested at 10 months after dosing depicting a cystic dilatation lesion (red arrows). Scale bar denotes 100 μ m (left) and 50 μ m (right). Trichrome staining performed by Dr Joanna Lima. (B) Number of 'cystic dilatation' lesions per mm² cortical tissue detected in kidney sections from Control *Pbrm1* KO (n = 6) and *Vhl Pbrm1* KO (n = 10) mice given 5x 2 mg TMX and harvested at 10 months after dosing. Median and inter-quartile range plotted. Pairwise comparison by Mann-Whitney test.

6.2.3 Region-specific expansion of *Vhl/Pbrm1*-null cell population in the renal cortex

Survival of *Vhl*-null RTE cells 4-12 months after recombination was shown to be supported in the cortex and outer medulla but not in the inner medulla and papilla (**Chapter 4**). To test if this phenotype is impacted by concomitant *Pbrm1* inactivation, I analysed the number and distribution of tdTomato-positive cells in the renal cortex, outer medulla, inner medulla, and papilla of kidneys from *Vhl Pbrm1* KO and Control *Pbrm1* KO mice at the early (1-3 weeks after TMX dosing) and late (4-12 months after TMX dosing) timepoints. As seen for *Vhl* recombination on its own (**Chapters 2 - 4**), no significant differences were observed in the distribution (**Figure 6.8A**) or proportion (**Figure 6.8B**) of tdTomato-positive cells between Control *Pbrm1* KO mice and *Vhl Pbrm1* KO mice at the early timepoint, and between Control *Pbrm1* KO mice at the late *versus* early timepoints, indicating that both recombination is induced equally in all genotypes and that *Pbrm1* inactivation with monoallelic *Vhl* inactivation does not affect the long-term survival of cells in the RTE. In contrast, region-specific genotype-dependent differences were observed in *Vhl Pbrm1* KO mice at the late timepoint. Neither the proportion ($p > 0.05$) nor the distribution of tdTomato-positive cells was different in the outer medulla of *Vhl Pbrm1* KO mice compared to Control *Pbrm1* KO mice (**Figures 6.8A and 6.8B**). The papilla and inner medulla of kidneys of *Vhl Pbrm1* KO mice contained significantly fewer ($p = 0.0002$ and $p = 0.0091$ respectively) tdTomato-positive cells than in the Control *Pbrm1* KO mice (**Figures 6.8A and 6.8B**). In contrast, the cortex of *Vhl Pbrm1* KO mice contained a significantly higher ($p > 0.002$) proportion of tdTomato-positive cells compared to that of Control *Pbrm1* KO mice (**Figures 6.8A and 6.8B**). Consistent with this increase, tdTomato-positive cells in the renal cortex of *Vhl Pbrm1* KO often encompassed the entire profile of renal tubules (**Figure 6.8A**).

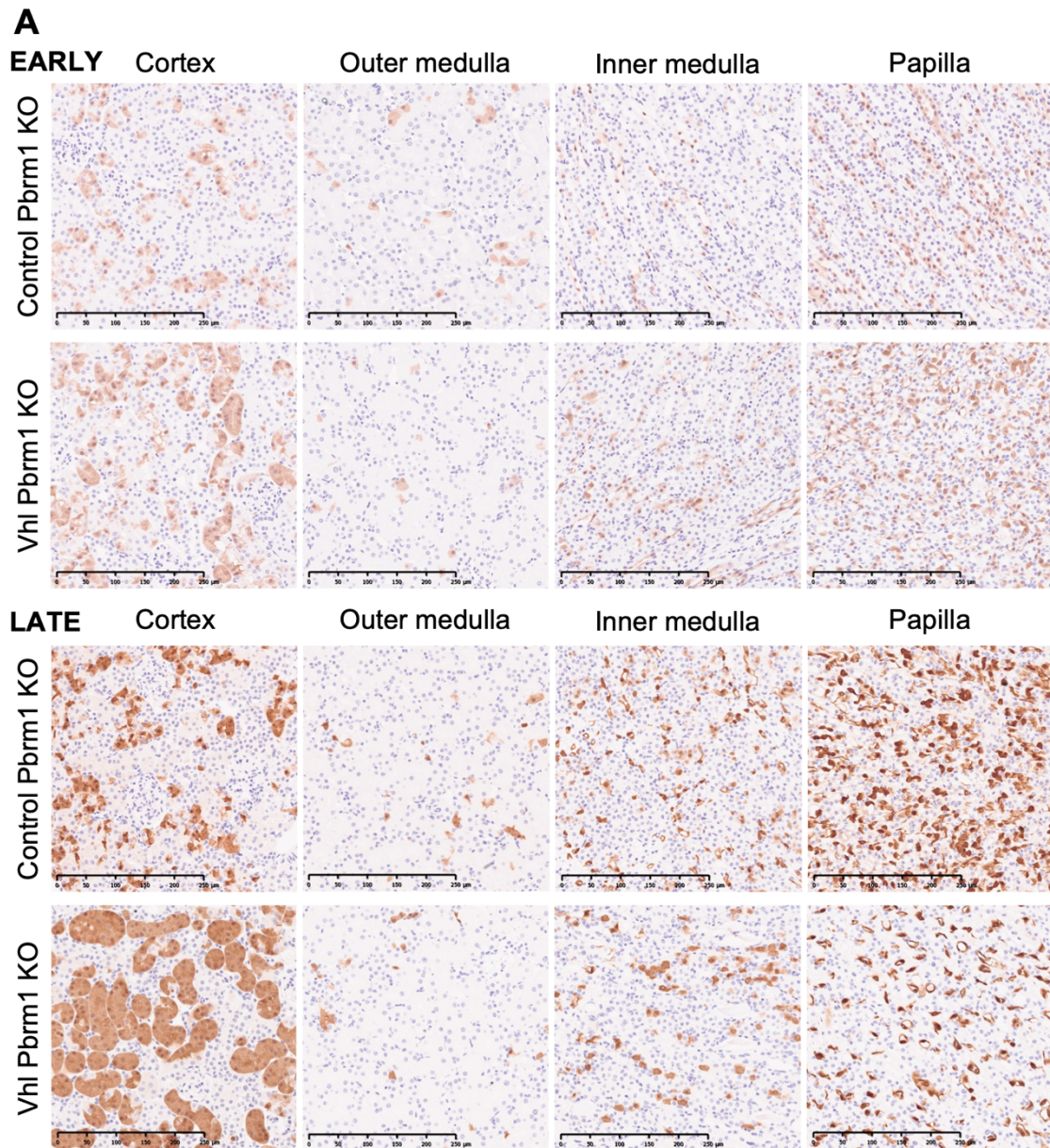


Figure 6.8: Region-specific retention of *Vhl/Pbrm1*-null cells in the kidney. (A) Representative tdTomato IHC in different renal anatomical regions of Control *Pbrm1* KO or *Vhl Pbrm1* KO mice given 5x 2 mg TMX and harvested at the early or late timepoints. Scale bar denotes 250 μ m. IHC performed in collaboration with Dr Joanna Lima and Ayslan Barros.

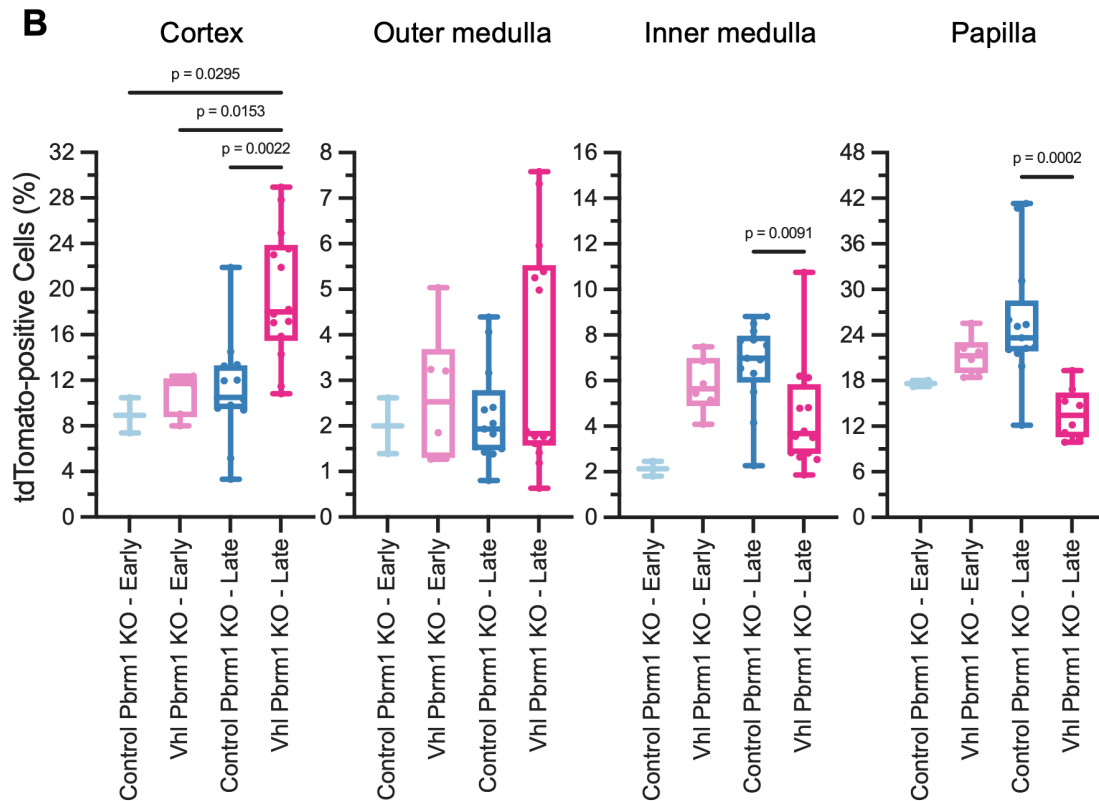


Figure 6.8 (contd.): Region-specific retention of *Vhl/Pbrm1*-null cells in the kidney. (B)

Proportion of total cells that are tdTomato-positive in different regions of the kidney of Control *Pbrm1* KO or *Vhl Pbrm1* KO mice given 5x 2 mg TMX and harvested at the early and late timepoints ($n = 2, 6, 13$ for all renal regions of Control *Pbrm1* KO at the early timepoint, *Vhl Pbrm1* KO at the early timepoint, and Control *Pbrm1* KO at the late timepoint respectively; $n = 14, 14, 14, 8$ for renal cortex, inner medulla, outer medulla, and papilla of *Vhl Pbrm1* KO at the late timepoint respectively). Median and inter-quartile range plotted. Pairwise comparisons using multiple Kruskal-Wallis test with Dunn's correction. Only significant ($p < 0.05$) comparisons shown.

I next compared how time-dependent survival of *Vhl/Pbrm1*-null cortical cells and loss of *Vhl/Pbrm1*-null papillary cells compared with that observed for *Vhl*-null cells. The proportion of cells positive for tdTomato in either the cortex or the papilla was not significantly different in *Vhl Pbrm1* KO versus *Vhl* KO mice given 5x 2 mg TMX and harvested at the early timepoint, suggesting that similar levels of recombination were induced by TMX dosing in mice of the two genotypes (**Figure 6.9**). While a time-dependent

difference in the proportion of cells was observed for mice of both genotypes (data from **Figure 6.8** and **Chapter 4**), no differences were observed between *Vhl Pbrm1* KO versus *Vhl* KO mice at the late timepoint. This indicates that the region-specific survival phenotypes entrained in *Vhl/Pbrm1*-null cells are not dissimilar to those entrained in *Vhl*-null cells over time.

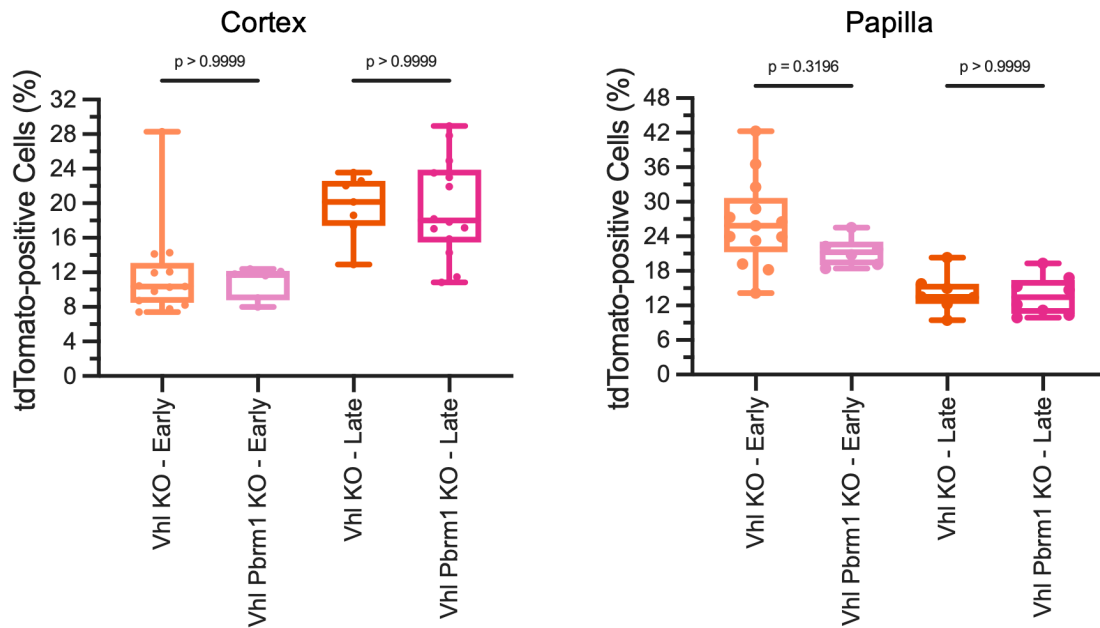


Figure 6.9: Region-specific retention of *Vhl/Pbrm1*-null and *Vhl*-null cells in the kidney.

Proportion of total cells that are tdTomato-positive in different regions of the kidney as quantified by tdTomato IHC from *Vhl* KO or *Vhl Pbrm1* KO mice given 5x 2 mg TMX and harvested at the early or late timepoints ($n = 13, 7, 6, 14$ (cortex) and $n = 13, 7, 7, 8$ (papilla) for *Vhl* KO (early), *Vhl* KO (late), *Vhl Pbrm1* KO (early) and *Vhl Pbrm1* KO (late) mice respectively). Median and inter-quartile range plotted. Pairwise comparisons using multiple Kruskal-Wallis test with Dunn's correction.

Collectively, these results demonstrate that while *Vhl/Pbrm1*-null cells are not retained in the inner medulla and papilla over time, they not only survive but also expand to almost twice their initial population in the renal cortex over a year following recombination. Concomitant *Vhl* and *Pbrm1* inactivation thus confers a strong survival advantage to RTE

cells in the cortex and a survival disadvantage to those in the inner medulla and papilla. Interestingly, concomitant *Vhl* and *Pbrm1* inactivation entrains similar survival phenotypes as those entrained by *Vhl* inactivation on its own.

6.2.4 *Vhl/Pbrm1*-null cortical RTE cells are not in cell cycle at the late timepoint

An interesting result obtained from studies of *Vhl*-null cells over time was of time-restricted proliferation; while *Vhl*-null cells were more proliferative than *Vhl*-competent cells at the early timepoint, their proliferative behaviour was not significantly different from *Vhl*-competent cells at the late timepoint (**Chapter 4** and **5**). The presence of ‘precursor’ dysplastic lesions and the renal tubules that appeared to have multi-layered epithelia, as well as the significant expansion of tdTomato-positive cells in cortex of *Vhl Pbrm1* KO mice prompted an investigation into whether *Vhl/Pbrm1*-null continue to be in cell cycle even at the late timepoints.

tdTomato/Ki67 dual IHC was performed on kidneys from *Vhl Pbrm1* KO ($n = 5$) and Control *Pbrm1* KO ($n = 4$) mice given 5x 2 mg TMX and harvested at the late timepoint. Surprisingly, no difference ($p > 0.99$) was observed in the proportion of tdTomato-positive cells that were also positive for Ki67 in the renal cortex from mice of the two genotypes (**Figure 6.10A**), indicating no increased proliferative capacity of *Vhl/Pbrm1*-null RTE cells at 10 months after *Vhl* inactivation. The proportion of *Vhl/Pbrm1*-null cells in cell cycle at 10 months after dosing ranged between 0.06%-0.32%. In comparison, the proportion of *Vhl*-null cells in cell cycle at 4-12 months after dosing ranged between 0.14%-0.20% (**Chapter 4**). These observations suggest that *Vhl/Pbrm1*-null do not as a whole proliferate more than *Vhl*-null cells at the late timepoint.

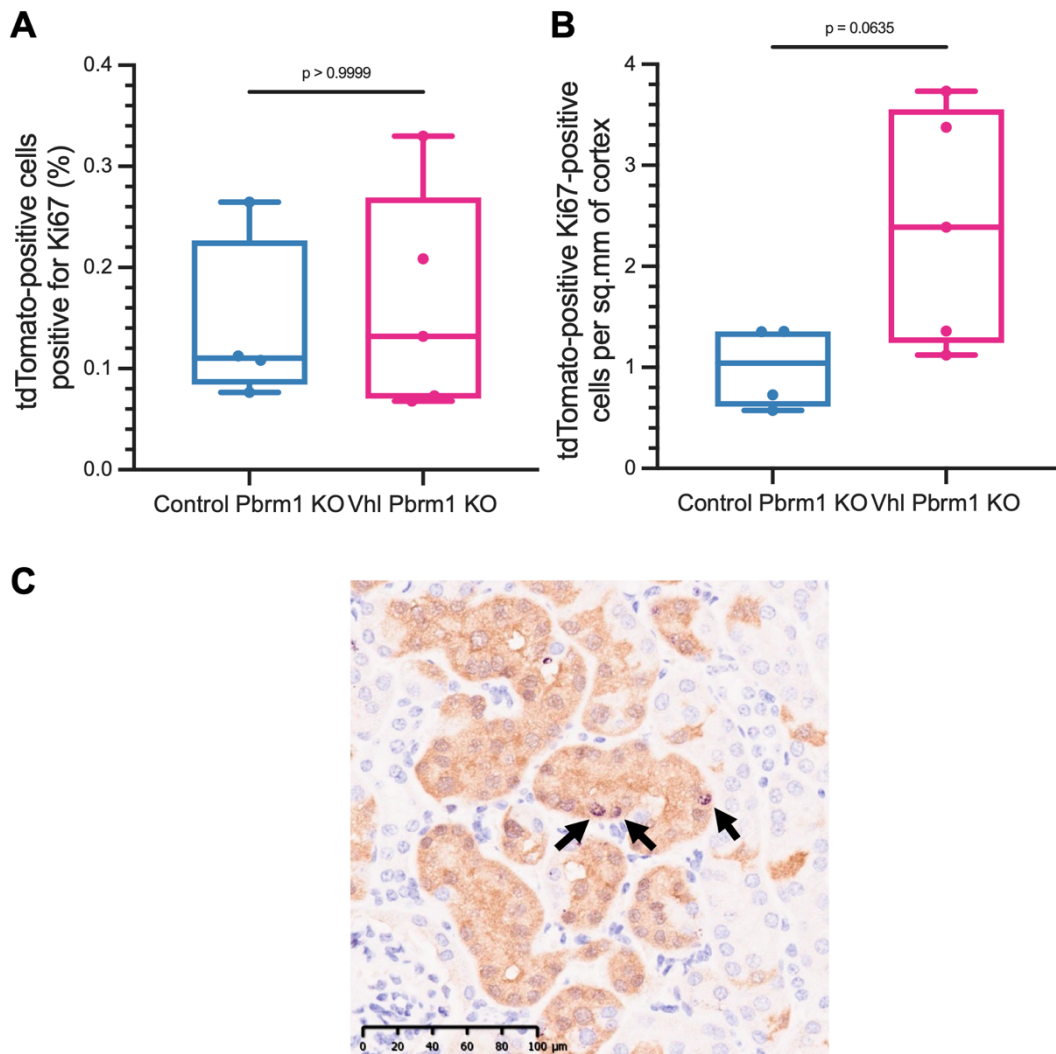


Figure 6.10: *Vhl/Pbrm1*-null cells do not exhibit increased proliferative capacity at 10 months after dosing. (A,B) Proportion of tdTomato-positive cells also positive for Ki67 signal (A), and number of tdTomato-positive Ki67-positive cells detected per mm² of renal cortex (B), as quantified by tdTomato/Ki67 dual IHC in Control *Pbrm1* KO (n = 4) and *Vhl Pbrm1* KO (n = 5) mice given 5x 2 mg TMX and harvested at 10 months after dosing. Median and inter-quartile range plotted. Pairwise comparison by Mann-Whitney test. (C) Representative tdTomato (brown) Ki67 (purple) dual IHC on kidneys of *Vhl Pbrm1* KO mice given 5x 2 mg TMX and harvested at 10 months after dosing depicting a precursor dysplasia lesion. Black arrows denote tdTomato-positive Ki67-positive cells. Scale bar denotes 100 μ m.

It should be noted here that cortices of *Vhl Pbrm1* KO mice contain almost double the proportion of cells that are tdTomato-positive cells than in Control *Pbrm1* KO mice at this timepoint. Consequently, the renal cortex of *Vhl Pbrm1* KO mice contained on average, almost twice the number of tdTomato-positive Ki67-positive cells per mm² of tissue analysed than in Control *Pbrm1* KO mice; this increase trended towards significance ($p = 0.06$) (**Figure 6.10B**). Interestingly, tdTomato-positive Ki67-positive cells were observed in lesions classified as ‘precursor dysplasia’ in *Vhl Pbrm1* KO mice (**Figure 6.10C**); however, a formal comparison of Ki67-positivity of these lesions and normal tubules could not be performed in the duration of this study.

These results suggest that *Vhl/Pbrm1*-null cells are not inherently more proliferative than *Vhl*-competent or *Vhl*-null cells in the renal cortex at 10 months after dosing, and thus do not lend support to the hypothesis that concomitant *Pbrm1* inactivation relieves the time-dependent restraint on proliferation of *Vhl*-null cells. It is possible that the sustained proliferation occurred until a slightly earlier (4-8 months after dosing) timepoint that was missed in the present analysis. Furthermore, whether cells present in disorganised tubules and precursor lesions exhibit different proliferative behaviour than those within ‘normal’ tubules remains to be analysed.

6.2.5 Effects of concomitant *Pbrm1* inactivation on gene expression in *Vhl*-null cells at the late timepoint

PBRM1 is a member of the PBAF SWI/SNF chromatin remodelling complex⁹⁹ and as such has an important function in regulating gene expression and chromatin accessibility in cells. It has been hypothesised that *Pbrm1* inactivation alters gene expression in *Vhl*-null cells, an assertion partially supported by studies showing dysregulation of HIF and NFκB-mediated gene expression following *PBRM1* inactivation in cell culture or mouse models. However, as with studies of *Vhl* inactivation on its own, the effects of concomitant *Vhl* and *Pbrm1* inactivation in cells of the RTE have not been studied *in vivo* with temporal resolution.

6.2.5.1 Differential gene expression following concomitant *Vhl* and *Pbrm1* inactivation is similar to that following *Vhl* inactivation

To analyse gene expression changes caused by concomitant *Vhl* and *Pbrm1* inactivation and to compare how these were different from those entrained over time by *Vhl* inactivation on its own, single-cell RNA sequencing (scRNA-seq) was performed on tdTomato-positive cells isolated by FACS from kidneys of *Vhl Pbrm1* KO mice at the late timepoints (n = 2 female, 1 male; 2 females at 4 and 7 months after dosing, 1 male at 17 months after dosing). This yielded data from 48,616 high-quality cells, with an average of $16,205 \pm 8892$ (SD) cells, and $2,649 \pm 989$ (SD) transcripts per cell that covered $1,025 \pm 220$ (SD) genes per cell per sample. This data was integrated with data (from **Chapter 5**) from tdTomato-positive cells isolated from *Vhl*^{wt/pjr.fl}; *Pax8-CreERT2* (“Control”) (n = 3 female, 1 male) and *Vhl*^{jiae.KO/pjr.fl}; *Pax8-CreERT2* (“*Vhl* KO”) (n = 2 female, 2 male) mice given 5x 2 mg TMX and harvested 4-12 months after TMX dosing for subsequent analysis. Cell types and PT Class were assigned to cells as was done in **Chapter 5** (also see **Chapter 8**).

Global transcriptomic profiles of cells were analysed by dimension reduction performed on these cells after batch correction for sex (see **Chapter 8**). This showed that, as with cells from *Vhl* KO mice (*Vhl*-null), cells from *Vhl Pbrm1* KO mice (*Vhl/Pbrm1*-null) occupied different positions and clusters by UMAP compared to those from Control mice (*Vhl*^{wt/KO} and *Pbrm1*^{wt/wt}, essentially *Vhl/Pbrm1*-competent) (**Figure 6.11A** and **6.11B**). Furthermore, while some *Vhl/Pbrm1*-null cells appeared to form new clusters compared to *Vhl*-null cells (**Figure 6.11B**), cells of the two genotypes showed considerable overlap in both UMAP position and clustering (**Figure 6.11B**). This suggested that the global transcriptomic profiles of *Vhl/Pbrm1*-null and *Vhl*-null RTE cells are broadly similar to each other but distinct from those of *Vhl/Pbrm1*-competent RTE cells.

To further understand these transcriptomic differences, I tested differential expression of genes in *Vhl/Pbrm1*-null cells against *Vhl*-competent cells and compared to differential expression in *Vhl*-null cells against *Vhl*-competent cells (**Figure 6.12**). Changes

in gene expression in *Vhl/Pbrm1*-null cells compared to *Vhl*-competent cells were highly correlated (Spearman coefficient = 0.79) to those in *Vhl*-null cells. 126 and 99 genes were collectively up- or down-regulated in *Vhl*-null and in *Vhl/Pbrm1*-null cells compared to *Vhl*-competent cells (**Figure 6.12** and **Appendix 8**). Of the 126 upregulated genes, only 1 and 6 of these were specifically upregulated in *Vhl/Pbrm1*-null or *Vhl*-null cells respectively (**Figure 6.12**). Of the 99 downregulated genes, 17 genes were specifically downregulated in *Vhl/Pbrm1*-null cells and none were specifically downregulated in *Vhl*-null cells (**Figure 6.12**). Genes specifically downregulated in *Vhl/Pbrm1*-null cells included the proximal tubule transcription factor²³⁸ *Maf*, which was previously shown to be downregulated in *Vhl*-null ‘PT like’ cells (**Chapter 5**). Clear associations with either the NFκB or HIF pathways were not observed.

PT Class A cells were previously identified as being highly responsive to *Vhl* inactivation compared to PT Class B cells and had shown higher upregulation of genes known to be prognostic markers in ccRCC (**Chapter 5**). It was therefore of interest to examine if *Vhl/Pbrm1*-null-specific gene expression responses were more evident in PT Class A cells compared to PT Class B cells. Differential gene expression compared to *Vhl/Pbrm1*-competent cells was highly correlated between *Vhl*-null and *Vhl/Pbrm1*-null cells in both PT Classes (Spearman coefficients = 0.81 and 0.76 for PT Class A and PT Class B respectively) (**Figure 6.13**), indicating that PT Class A cells were not particularly sensitive to additional *Pbrm1* inactivation.

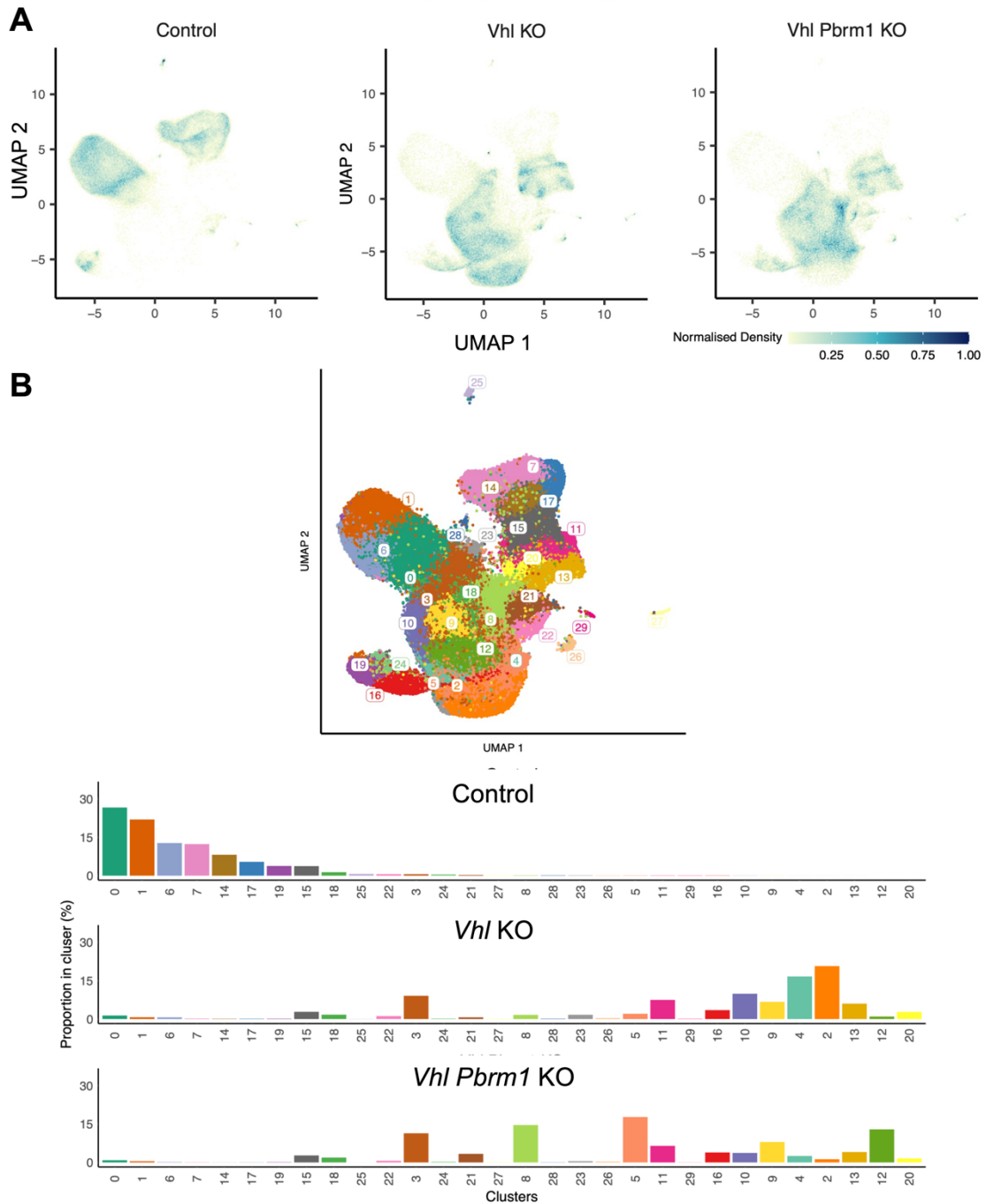


Figure 6.11: *Vhl/Pbrm1*-null cells share UMAP position and clusters with *Vhl*-null cells.

(A) Density plot depicting UMAP distribution of tdTomato-positive cells from kidneys of Control, *Vhl* KO, and *Vhl Pbrm1* KO mice harvested at the late timepoints (n = 3 female (F), 1 male (M) for Control; n = 2F, 2M for *Vhl* KO; n = 2F, 1M for *Vhl Pbrm1* KO). Each plot is divided into 250 bins and the number of cells in each bin is normalised relative to the densest bin. **(B)** UMAP plot depicting cells from Control, *Vhl* KO, and *Vhl Pbrm1* KO mice harvested at the late timepoint coloured by UMAP clusters (top). Proportion of cells from each condition belonging to any cluster (bottom).

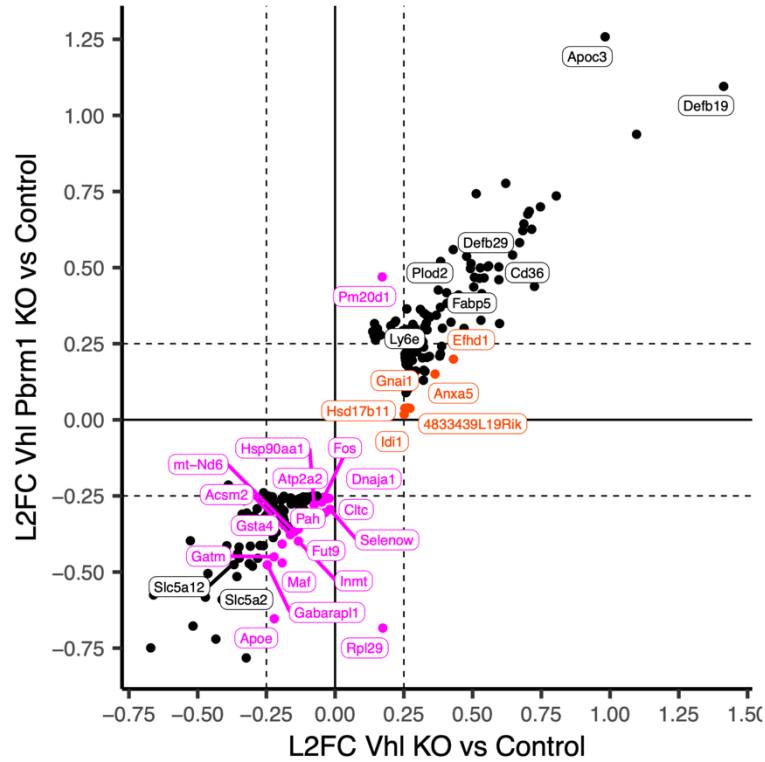


Figure 6.12: Gene expression changes entrained after concomitant *Vhl* and *Pbrm1* inactivation are correlated with those entrained after *Vhl* inactivation. Scatter plot depicting log₂-fold changes in tdTomato-positive cells from *Vhl* KO versus Control mice against those in *Vhl Pbrm1* KO versus Control mice at the late timepoint (n = 3F, 1M for Control; n = 2F, 2M for *Vhl* KO; n = 2F, 1M for *Vhl Pbrm1* KO). Genes regulated in cells from both *Vhl* KO and *Vhl Pbrm1* KO mice (black), and specifically regulated in *Vhl* KO mice (orange) or *Vhl Pbrm1* KO mice (magenta). Genes of interest regulated by *Vhl* and mentioned previously in the thesis are also labelled (black).

Taken together, these results indicate that gene expression changes entrained after concomitant *Vhl* and *Pbrm1* inactivation are similar to those entrained after *Vhl* inactivation on its own. However, *Vhl/Pbrm1*-null cells had downregulated more genes (and had not upregulated as many genes) than had *Vhl*-null cells, suggesting that *Pbrm1* inactivation might function in specifically restricting expression of genes after *Vhl* inactivation.

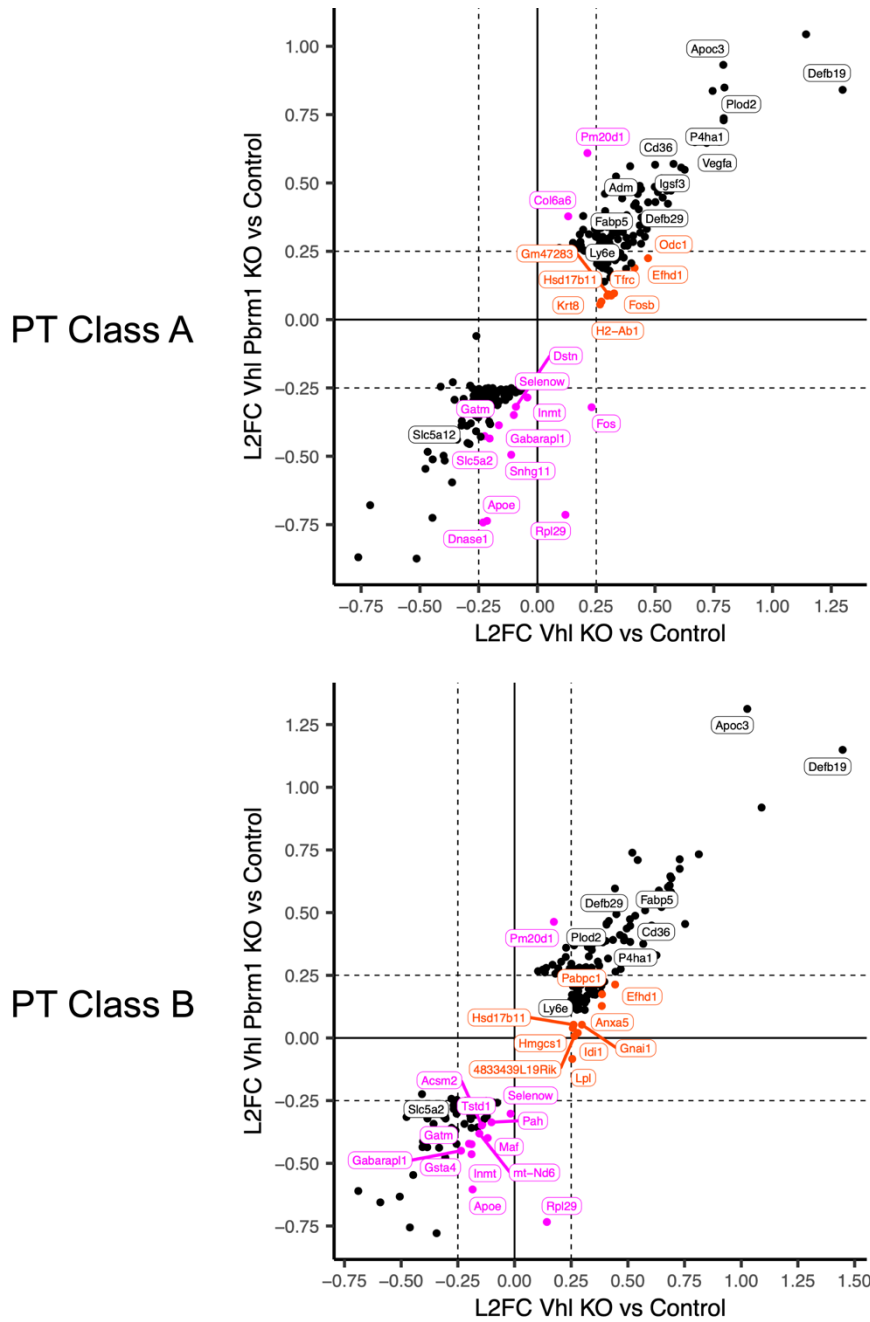


Figure 6.13: Gene expression changes entrained after concomitant *Vhl* and *Pbrm1* inactivation are correlated with those entrained after *Vhl* inactivation in both classes of PT cells. Scatter plot depicting log₂-fold changes in tdTomato-positive cells from *Vhl* KO versus Control mice against those in *Vhl Pbrm1* KO versus Control mice at the late timepoint (n = 3F, 1M for Control; n = 2F, 2M for *Vhl* KO; n = 2F, 1M for *Vhl Pbrm1* KO) calculated separately for PT Class A cells (top) and PT Class B cells (bottom). Genes regulated in cells from both *Vhl* KO and *Vhl Pbrm1* KO mice (black), and specifically regulated in *Vhl* KO mice (orange) or *Vhl Pbrm1* KO mice (magenta). Genes of interest regulated by *Vhl* and mentioned previously in the thesis are also labelled (black).

6.2.5.2 Concomitant *Pbrm1* inactivation does not impact on changes in expression of ccRCC-associated genes entrained by *Vhl* inactivation

Vhl-null cells were shown over time to enhance the expression of ccRCC-associated genes that were known to be HIF targets, but downregulate the expression of ccRCC-associated genes that were not known to be HIF targets (**Chapter 5**). I had hypothesised that gene expression in *Vhl/Pbrm1*-null cells might associate with gene expression profiles in ccRCC tumour cells more than was seen in *Vhl*-null cells. I had previously derived lists of HIF-target genes categorised by their known upregulation in ccRCC, and of genes upregulated in ccRCC categorised by their known regulation of HIF (see **Chapter 5** and **8**). Expression of genes that were: 1) known HIF targets, 2) known HIF targets and upregulated in ccRCC, 3) known HIF targets and not upregulated in ccRCC, 4) upregulated in ccRCC, and 5) upregulated in ccRCC and not known to be HIF-targets, was separately scored in *Vhl/Pbrm1*-competent, *Vhl/Pbrm1*-null, and *Vhl*-null cells.

Expression scores for genes upregulated in ccRCC, whether they were, or were not, known HIF-target genes, were similar in *Vhl/Pbrm1*-null and *Vhl*-null cells, suggesting that concomitant *Pbrm1* inactivation did not affect expression of these genes more than what occurred after *Vhl* inactivation (**Figure 6.14**). This similarity between *Vhl/Pbrm1*-null and *Vhl*-null cells was seen for every renal cell identity (**Figure 6.15**). *Vhl*-null cells showed higher expression of genes that were known to be HIF-targets, but not upregulated in ccRCC compared to *Vhl/Pbrm1*-competent cells. Interestingly, such an increase was not seen in *Vhl/Pbrm1*-null cells when compared to *Vhl*-competent cells (**Figure 6.14**).

These results indicate that concomitant *Pbrm1* inactivation does not upregulate expression of ccRCC-associated genes more than what occurs after *Vhl* inactivation on its own. However, it prevents the upregulation of HIF target genes that are not known to be upregulated in ccRCC. This lends further support to the proposition that *Pbrm1* inactivation restricts genes that are upregulated after *Vhl* inactivation in cells of the RTE.

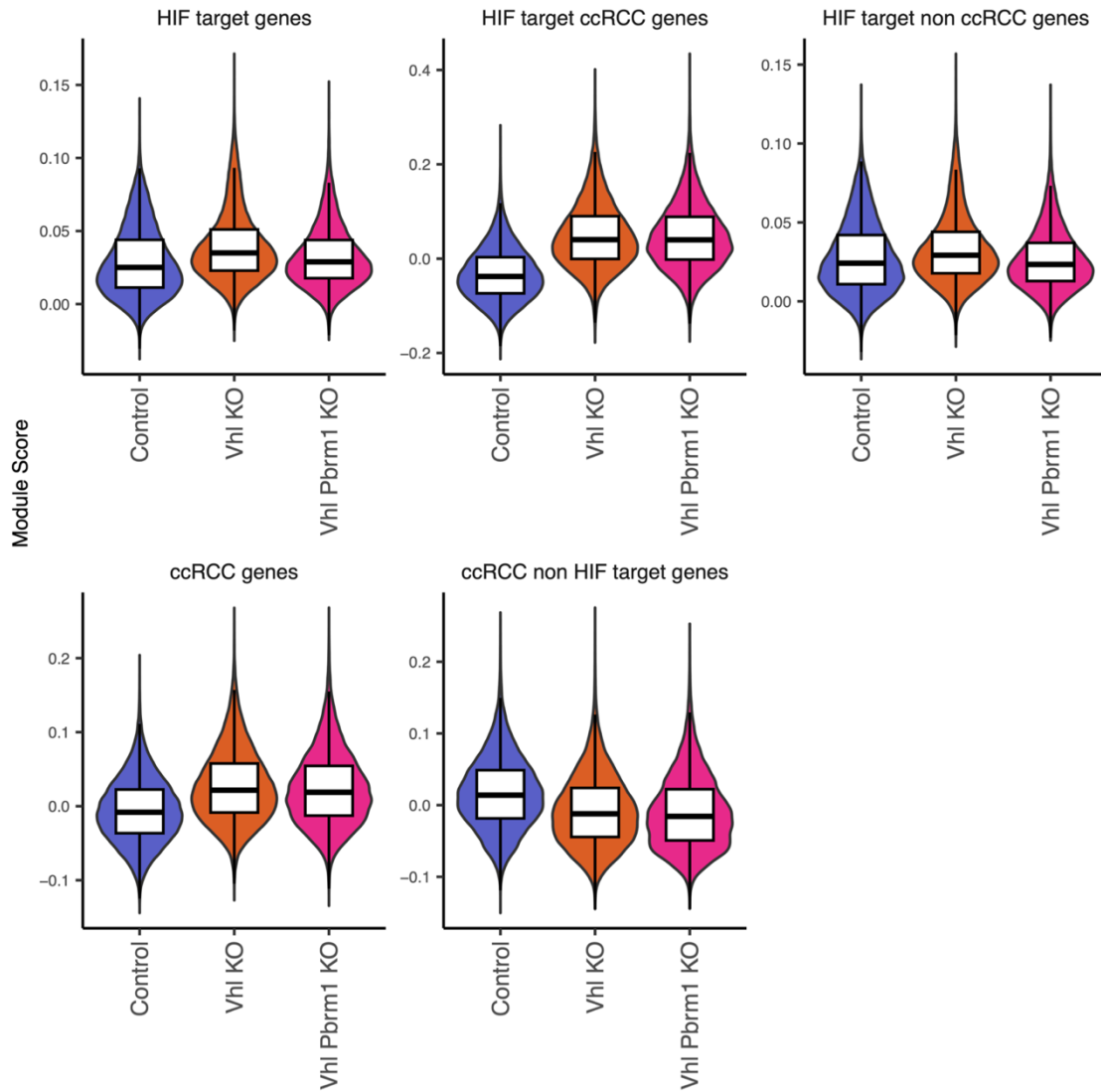


Figure 6.14: *Vhl/Pbrm1*-null cells exhibit similar expression of HIF target and ccRCC-associated genes as *Vhl*-null cells. Violin plots overlaid with boxplots depicting expression scores for different intersections of HIF target genes and genes upregulated in ccRCC in tdTomato-positive cells from Control, *Vhl* KO and *Vhl Pbrm1* KO mice harvested at the late timepoints.

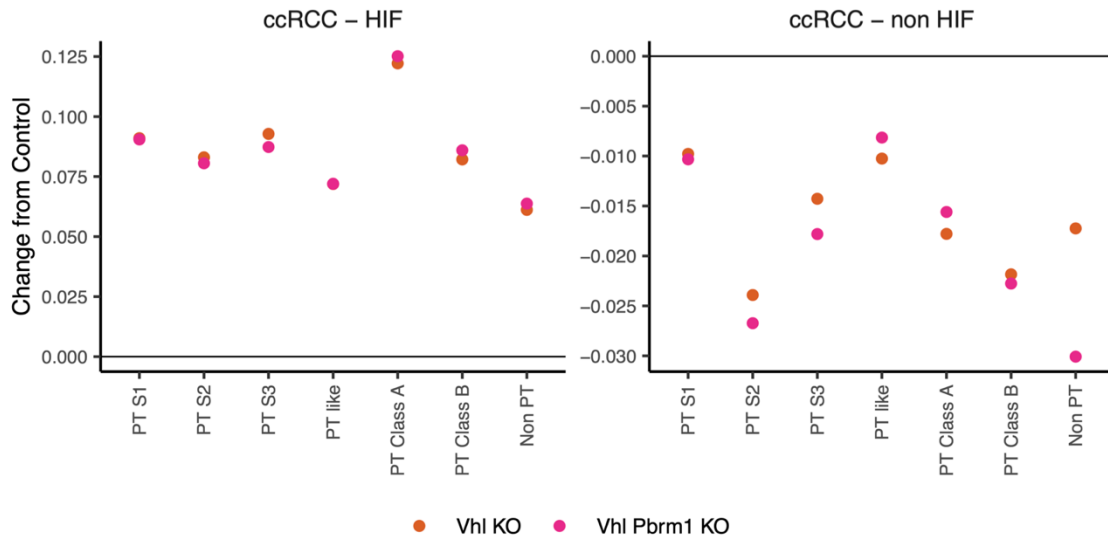


Figure 6.15: *Vhl/Pbrm1*-null and *Vhl*-null cells exhibit similar expression of ccRCC-associated genes across all renal cell identities. Scatter plot depicting change in mean expression scores compared to *Vhl*-competent cells in *Vhl*-null (orange) and *Vhl/Pbrm1*-null cells (magenta) of different renal cell identities for genes specifically upregulated in ccRCC that are known HIF-targets (left) and not known to be HIF-targets (right)

6.3 Discussion

In this chapter, I have coupled the novel reporter model of *Vhl* inactivation described in **Chapter 2** to conditional alleles of *Pbrm1* to model concomitant *Vhl* and *Pbrm1* inactivation *in vivo* in the cells of the RTE. Using this model, I could reproduce lesions reported by others at very long (17 months) timepoints following concomitant *Vhl* and *Pbrm1* inactivation in the RTE. I could also identify precursor lesions formed almost entirely of *Vhl/Pbrm1*-null cells at timepoints at which *Vhl* inactivation had not induced any morphological alterations. These results have demonstrated that additional *Pbrm1* inactivation can drive morphological transformation of *Vhl*-null cells in the RTE.

The data presented in previous chapters of this thesis (**Chapter 4** and **5**) had highlighted potential points of tumour suppression encountered by *Vhl*-null cells that might be overcome by additional *Pbrm1* inactivation. These were:

1. Region- and cell-type specific elimination of *Vhl*-null cells from tissues over time.

2. Restraints imposed on proliferation of *Vhl*-null cells by 4 months after recombination that restrict their propagation along the renal tubular boundary.
3. Time-dependent adaptations in gene expression in *Vhl*-null cells that associate with a diminution in the expression of ccRCC-associated non-HIF-target genes.
4. Upregulation of genes involved in innate immunity and associated changes in the organisation of renal macrophages in the renal cortex.

I have then performed preliminary experiments testing how concomitant *Vhl* and *Pbrm1* inactivation impacts on each of these four possible modes of tumour suppression.

6.3.1 Concomitant *Vhl* and *Pbrm1* inactivation is accurately reported by tdTomato expression

While my novel reporter model was designed to mark the recombination of the *Vhl*^{*lpr*} allele with the expression of a tdTomato reporter, I also found tdTomato-positivity to be an accurate marker of concomitant *Vhl* and *Pbrm1* inactivation in the same cells (**Figure 6.3**). The efficiency of recombination of ‘floxed’ alleles by Cre recombinase is inversely proportional to the distance between the *lox* sites in the allele¹⁹³. The *loxP* sites in the *Pbrm1* allele¹⁰⁹ used here are closer than the pairs of *loxP* and *lox2272* sites on the *Vhl*^{*lpr*} allele. It was thus expected that, in response to a certain amount of Cre activation in tissues, the *Pbrm1* allele would recombine at a greater frequency than the *Vhl*^{*lpr*} allele. However, whether *Pbrm1* would always recombine in a cell with *Vhl*^{*lpr*} recombination was not guaranteed. I observed that tdTomato-positive cells from *Vhl Pbrm1* KO mice did not contain any detectable unrecombined *Pbrm1* allele or PBRM1 protein, confirming that tdTomato expression marked both *Vhl* and *Pbrm1* recombination in cells. This also allowed for the same experimental design involving a ‘KO’ genotype and a ‘Control’ comparator, as used in **Chapters 3 – 5**, to be applied to the study of *Vhl/Pbrm1*-null cells *in situ* and *ex vivo*. It should be noted though that I could not ascertain the extent to which tdTomato-negative cells were also *Pbrm1*-null.

6.3.2 *Pbrm1* inactivation directly and specifically affects the morphology of *Vhl*-null RTE cells

The fact that concomitant *Pbrm1* and *Vhl* inactivation leads to morphological abnormalities and the development of renal cancer has been demonstrated *in vivo* by three studies – two that employed constitutively-active Cre recombinases targeted to the RTE^{104,111}, and one that used the same *Pax8-CreERT2* and 5x 2 mg TMX dose as used in this study¹⁰⁹. All three studies used mice that were homozygous for conditional *Vhl* alleles (“Homoflox”, *Vhl*^{f/f}). The data presented here reproduces the lesions reported in these studies but using mice that have inherited one inactive *Vhl* allele and in whom the second copy of *Vhl* is conditionally inactivated in adulthood in some cells (**Figures 6.1 and 6.2**). Furthermore, the tdTomato reporter allowed for the identification of smaller lesions at earlier timepoints that might have been missed by unmarked histological analyses and which may represent early stages of *Vhl* and *Pbrm1*-driven tumorigenesis.

Two types of early lesions were identified and were linked directly to concomitant *Vhl* and *Pbrm1* inactivation based on their relative infrequency in kidneys of mice that had undergone *Pbrm1* inactivation but not biallelic *Vhl* inactivation and on their overwhelming tdTomato-positivity (**Figures 6.4-6.7**). It should be noted that the use of tdTomato IHC positively biases the detection of tdTomato-positive lesions, and it is possible that small early lesions composed of tdTomato-negative cells were missed during manual examination of stained tissue sections. Nonetheless, the observation that almost all tubular cells in most lesions were tdTomato-positive indicates a direct and specific contribution of concomitant *Vhl* and *Pbrm1* inactivation to these lesions.

The first (termed ‘disorganised tubules’) involved *Vhl/Pbrm1*-null cells that appeared to have extended beyond their epithelial monolayer and thus gave the renal tubules in which they resided a contorted appearance that was distinguishable from the otherwise smooth tubular boundaries of normal renal tubules. The second (termed ‘precursor dysplasia’)

involved what appeared to be multiple renal tubules and were characterised not only by their tdTomato-positivity and disorganised appearance but also by the presence of interspersed *Vhl*-competent interstitial cells. While some of these cells were renal macrophages, the identities of others were not elucidated. Whether these interstitial cells respond to signals produced by *Vhl/Pbrm1*-null cells present in these lesions, and whether they contribute to the development of these lesions would be of great interest to investigate further.

Neither of these two classes of lesions were observed after *Vhl* inactivation on its own in this study at the same timepoint after recombination. These results thus identify 10 months after inactivation as a sufficient timeframe for the development of morphological abnormalities following somatic *Vhl* and *Pbrm1* inactivation in the RTE; further studies on the mechanisms by which these are generated may be performed at earlier timepoints (e.g., 4-8 months after TMX dosing) to capture the process contributing to the formation of these lesions.

6.3.3 *Pbrm1* inactivation does not impact the region-specific survival phenotypes of *Vhl*-null cells in the RTE

Interestingly, even though *Vhl* and *Pbrm1* recombination was induced in all anatomical regions of the kidney, all lesions associated with concomitant *Vhl* and *Pbrm1* inactivation were observed only in the renal cortex and not in any other renal region, supporting the proposition of the renal cortex as a permissive tissue for *VHL*-associated oncogenesis. Notably, I did not observe any lesions of the Bowman's capsule; this contrasts with observations of glomerular cysts following *Vhl* and *Pbrm1* inactivation made by others¹⁰⁴ and challenges the identification of parietal cells as the cell-of-origin for ccRCC.

Vhl/Pbrm1-null cells were found to have significantly increased in number in the renal cortex but were lost over time from the inner medulla and papilla (**Figure 6.8**) and mirrored the direction and magnitude of the phenotypes exhibited by *Vhl*-null cells in these

regions (**Chapter 4** and **Figure 6.9**). The tissue-specific responses entrained by *Vhl* inactivation were thus preserved even after concomitant *Pbrm1* inactivation.

Surprisingly, *Vhl/Pbrm1*-null cells did not exhibit increased proliferative capacity at least as assayed by Ki67 IHC at the 10-month timepoint (**Figure 6.10**). This result does not support the hypothesis that *Pbrm1* inactivation relieves proliferative restraints imposed on *Vhl*-null cells in the RTE at later timepoints. However, the presence of cycling cells detected in precursor dysplastic lesions in *Vhl Pbrm1* KO mice suggests that restraints on proliferation might be relieved in specific contexts that merit further investigation.

These results support the proposition that selective survival pressures acting on cells after *Vhl* inactivation on its own dictate the tissue-specificity associated with *VHL*-associated cancer, and that inactivation of *Pbrm1* contributes to oncogenesis by impacting the survival and morphological phenotypes of *Vhl*-null cells already supported in cancer-permissive tissues.

6.3.4 *Pbrm1* inactivation has specific, restrictive effects on gene expression in *Vhl*-null cells

Having established that additional *Pbrm1* inactivation affects the cellular phenotypes entrained by *Vhl* inactivation on its own particularly in the renal cortex, gene expression in *Vhl/Pbrm1*-null cells was studied and compared to that in *Vhl*-null and *Vhl*-competent cells by scRNA-seq. PBRM1 is a member of the PBAF SWI/SNF chromatin remodelling complex; *PBRM1*-deficient SWI/SNF complexes have been shown to upregulate the NKκB pathway in renal cancer cells¹⁶⁵ and *PBRM1*-mutant ccRCC cells have been shown to contain more ‘open’ regions of chromatin than cells in *PBRM1*-wild type ccRCC¹⁷⁰. Other studies, one in which gene expression in *PBRM1*- or *BAP1*-deficient renal cancer cell lines¹⁷² was compared and another in which bulk RNA sequencing was performed on renal cortices in mice carrying constitutive RTE-restricted inactivation of *Vhl* on its own or *Vhl* and *Pbrm1* together¹¹¹, have reported that *PBRM1* inactivation amplifies the expression of HIF1-target

genes and STAT3-target genes in renal cells. Paradoxically, similar analyses performed in another study reported a diminution of hypoxia-associated genes following *PBRM1* inactivation in ccRCC cancer cell lines¹⁷³.

Contrary to all these studies, and despite the cellular phenotypes observed in *Vhl* *Pbrm1* KO mice, I found surprisingly few effects of *Pbrm1* inactivation on gene expression in RTE cells beyond what occurred after *Vhl* inactivation on its own (**Figures 6.11 - 6.13**). Most genes, including the β -Defensin genes *Defb19* and *Defb29*, that were regulated after *Vhl* inactivation were regulated with similar magnitude after *Vhl* and *Pbrm1* inactivation. Instead of amplifying *Vhl*-dependent gene expression changes, concomitant inactivation of *Vhl* and *Pbrm1* resulted in the upregulation of fewer, and downregulation of more genes compared to *Vhl* inactivation on its own. Interestingly, *Vhl/Pbrm1*-null and *Vhl*-null cells exhibited similar expression of HIF target genes that were known to be upregulated in ccRCC. However, unlike *Vhl*-null cells, *Vhl/Pbrm1*-null cells did not exhibit an increase in expression of HIF target genes not associated with ccRCC (**Figures 6.14 and 6.15**). Collectively, my analyses point to *Pbrm1* inactivation having a restricting or ‘tuning’ effect on gene expression in *Vhl*-null cells and do not support amplification of the HIF response.

The discrepancies between my data and those reported by others might arise from having different definitions of HIF target genes, or from having analysed different cell types. For instance, the actions of *PBRM1* in transformed and cultured renal cancer cells might differ from those in non-cancerous renal epithelial cells *in vivo*. The RNA-sequencing study modelled *Vhl* and *Pbrm1* inactivation using *Ksp-Cre*¹¹¹ that targets the constitutive expression of Cre recombinase predominantly in the distal convoluted tubule and collecting duct and only infrequently in proximal tubules^{106,126}. In contrast, most of the tdTomato-positive analysed by scRNA-seq in my data were induced after recombination in adulthood and were of proximal tubular origin (see **Chapter 5**).

I had hypothesised that *Pbrm1* inactivation makes gene expression in *Vhl*-null more ‘oncogenic’. However, *Vhl/Pbrm1*-null cells did not exhibit any difference in expression of genes upregulated in ccRCC (that were known HIF targets or otherwise) compared to *Vhl*-null cells, suggesting that *Pbrm1* inactivation does not directly enhance the expression of ccRCC-associated genes in *Vhl*-null cells.

It should be noted here that the scRNA-seq data presented here may have some limitations. First, cells that were part of the dysplastic lesions or disorganised tubules may not have been recovered during the tissue dissociation or sorting steps, either because the lesions were not fully dissociated or because the cells were more sensitive to the stresses of the experimental procedures. Second, the average depth of sequencing for the *Vhl Pbrm1* KO mice was lower than what was obtained in data from Control and *Vhl* KO mice (**Chapter 5**), which might have underpowered some of the analyses. However, despite these limitations, the extent of positive correlation in gene expression between *Vhl Pbrm1* KO and *Vhl* KO mice supports the reliability and accuracy of data from *Vhl Pbrm1* KO mice.

6.3.5 Implications for further investigation

The relative paucity of *Pbrm1*-regulated effects on gene expression is surprising considering both, the published literature, and the extent of morphological abnormalities seen in the kidneys used for single-cell RNA sequencing. This potentially implies that the oncogenic actions of *Pbrm1* inactivation in *Vhl*-null cells do not arise from its function as a chromatin remodelling complex. PBRM1 has been reported to interact with microtubules of the mitotic spindle, specifically *via* binding to methyl groups added on microtubules by SETD2¹⁶⁶ which is also frequently mutated in ccRCC⁵⁵. Microtubular defects in *Vhl/Pbrm1*-null cells may lead to cellular disorganisation without causing specific changes in gene expression. It could also lead to errors in chromosome segregation and subsequent genomic instability, allowing further mutations to be incurred in some *Vhl/Pbrm1*-null cells that contribute to oncogenesis. *PBRM1* has also been linked to the resolution of replication fork

stalling. The molecular mechanisms underlying this function have not been resolved¹⁰⁹, and may contribute to the phenomena observed in this study.

It is possible that oncogenic effects on gene expression were not evident because *Vhl/Pbrm1*-null cells that were part of the dysplastic or disorganised lesions were specifically lost during tissue dissociation and flow cytometry and are thus not represented in the scRNA-seq analysis. If true, this implies that gene expression in morphologically ‘normal’ *Vhl/Pbrm1*-null cells is substantially different than what might be seen in ‘abnormal’ or pre-cancerous *Vhl/Pbrm1*-null cells.

Both implications are quite exciting and highlight new approaches with which *Vhl/Pbrm1*-null should be studied further with my experimental system. The tdTomato reporter can be used to perform laser-capture microdissection experiments in which tdTomato-positive cells from *Vhl Pbrm1* KO, *Vhl* KO, and Control mice that are either morphologically normal, in dysplastic lesions, or in disorganised tubules are specifically excised and analysed. Gene expression or protein expression can be measured in these types of cells to reveal if *Pbrm1* inactivation has lesion-specific effects on the profiles of *Vhl*-null cells. Beyond gene expression and protein characterisation, investigation into possible cytoskeletal defects in *Vhl/Pbrm1*-null *versus* *Vhl*-null cells merit further investigation. These studies will require careful microscopy and possibly separate analysis of cycling *versus* non-cycling cells in the RTE.

Overall, these findings have highlighted the region-specific actions of concomitant *Vhl* and *Pbrm1* inactivation that could be illuminated by the reporting ability of the novel mouse model developed in this thesis. Analyses of *Vhl/Pbrm1*-null cells have further suggested that *Pbrm1* may have minor but specific effects on gene expression in *Vhl*-null cells and have shown the need for further investigations into non-genetic and/or context-specific aspects of *Pbrm1* inactivation that promote oncogenesis in *Vhl*-null cells.

Chapter 7: Concluding Remarks

The work presented in this thesis was aimed at understanding two largely unexplained aspects of oncogenesis – the tissue-specificity of cancer development following a mutation, and the long latency between the occurrence of mutations and the formation of tumours. Focussing on cancer associated with biallelic *VHL* inactivation, I considered the following questions:

1. Why does inactivation of *VHL*, a gene with ubiquitously conserved cellular functions, associate with oncogenesis predominantly in the renal cortex? What role does *VHL* play as a tumour suppressor gene in the renal cortex?
2. How do cells in tissues respond to *VHL* inactivation *in vivo* prior to their neoplastic transformation? What aspects of transformation require additional inactivation in genes such as *PBRM1*?

To answer these questions, I validated a novel experimental strategy that allowed investigation of the earliest events following *Vhl* inactivation in different cells and the analysis of fates entrained in these cells *in vivo* over time. These investigations have demonstrated remarkable heterogeneity and tissue-specificity in the cellular and transcriptomic responses to *Vhl* inactivation between 1 and 3 weeks (“early” timepoint) after recombination, demonstrated significant and specific changes in gene expression and proliferative behaviour of *Vhl*-null cells over time, and suggested that *Pbrm1* inactivation promotes oncogenesis in some *Vhl*-null cells. Here, I discuss these aspects of my work and the insights they offer into oncogenesis associated with *VHL* inactivation and its investigation.

7.1 A novel strategy for studying *Vhl* inactivation

The new reporter mouse allele (*Vhl*^{*l^{pr}fl*}) validated in this thesis inherently links the conditional inactivation of *Vhl* to the activation of a tdTomato fluorescent reporter. Direct

coupling of *Vhl* inactivation and reporter activation avoided differential action of Cre recombinase on separate conditional alleles that limits accuracy in binary Cre-recombinase/lox-STOP-lox reporter lines¹⁷⁴. The reporter allele was then coupled either to wild-type (*Vhl*^{wt}) or constitutively inactive (*Vhl*^{jae.KO}) second *Vhl* alleles to generate mice in which the timed activation of Cre recombinase generated marked cells that were *Vhl*-haplosufficient and *Vhl*-null respectively. *Vhl*^{wt/pjr.fl} mice provided a comparator against which the evolution of changes following biallelic *Vhl* inactivation (in *Vhl*^{jae.KO/pjr.fl} mice) could be assessed.

A principal advance presented by this model was of accurate identification and characterisation of recombined cells in morphologically normal tissues. Crucially, all detected tdTomato-positive cells had undergone *Vhl* recombination. Furthermore, in mice where the reporter *Vhl*^{pjr.fl} allele was coupled to homozygous conditional *Pbrm1* alleles, all detected tdTomato-positive cells had undergone concomitant *Vhl* and *Pbrm1* recombination. This allowed two types of experiments to be performed that could not have been performed without this experimental system.

In the first, the numbers of recombined cells were quantitatively compared in equivalent tissues of *Vhl*^{wt/pjr.fl} and *Vhl*^{jae.KO/pjr.fl} mice (with or without secondary inactivation of *Pbrm1*) at different timepoints after recombination was induced. Tissue-specific survival and proliferative advantages conferred by *Vhl* and/or *Pbrm1* inactivation over time could thus be inferred. In the second, gene expression was separately assayed in recombined and unrecombined cells of the same tissue, and then compared between mice of different genotypes. Gene regulation caused directly by *Vhl* and/or *Pbrm1* inactivation and transcriptional programmes entrained specifically in *Vhl*-null or *Vhl/Pbrm1*-null cells over time could thus be inferred.

The reporter model also had its limitations. Expression of tdTomato from the endogenous *Vhl* promoter was weak and thus tdTomato could not be detected in fixed tissues

without amplification using an antibody. This introduced practical limitations on performing multiplex staining on tissues that precluded for instance, the co-localisation of tdTomato signal with cell-type specific markers to be assayed *in situ*. Initial attempts at performing spatial transcriptomic analysis of tdTomato-positive *versus* tdTomato-negative cells in kidney sections using the Nanostring nCounter platform also failed for similar reasons. Live *in vivo* imaging of tdTomato-positive cells in the mouse could also not be performed and thus the fates of individual marked cells could not be tracked *in situ* in real time. Multi-photon excitement and further optimisation of fixation techniques might improve detection of tdTomato and might be tried in the future. Another limitation was that some cells that were recombined were not detected as tdTomato-positive; some of the phenotypes seen in tdTomato-negative cells might therefore have been contributed to by undetected recombined cells. However, such cells were thought to be a rare occurrence as assayed both by genomic PCR and single-cell RNA sequencing of tdTomato-positive and -negative cells.

7.2 Selection of *Vhl*-null cells underlies tissue-specificity of *VHL*-associated oncogenesis

A striking finding of the work was that tissue-dependent effects of *Vhl* inactivation impact on the long-term survival of cells *in vivo*. Cells were considered to be positively or negatively “selected” over time if, in comparison to an early (e.g., 1-3 weeks) timepoint after recombination was induced, they were overrepresented or underrepresented amongst the total population of cells in the tissue at a later (e.g., 4-12 months) timepoint. Such selection was deemed specific to biallelic *Vhl* inactivation if it was observed in *Vhl*^{*flae.KO/pjr.fl*} mice but not in *Vhl*^{*wt/pjr.fl*} mice (with or without additional *Pbrm1*^{*fl/fl*} alleles). If no difference in representation was observed, cells were considered to be under neutral selection.

Vhl-null cells were negatively selected in the renal papilla and inner medulla. In contrast, they were under neutral or positive selection in the cortex or outer medulla. This pattern of tissue-specific selection was observed in kidneys of mice carrying *Pax8-CreERT2*

given 5x 2 mg and 1 mg doses of TMX and was also suggested (though the result was not statistically significant) by the analysis of kidneys from mice carrying *Ubc-CreERT2* activated with 0.02 mg TMX. Analysis of *Ubc-CreERT2* mice given 0.02 mg TMX also hinted at negative selection of *Vhl*-null cells in the liver. Interestingly, the loss of *Vhl*-null cells from the inner medulla and papilla was not ‘rescued’ by the concomitant inactivation of *Pbrm1*, indicating both that the consequences of *Vhl* inactivation are sufficient to confer a survival disadvantage to renal papillary cells and that additional *Pbrm1* inactivation does not support the survival of *Vhl*-null cells in all tissues.

Increases in cell numbers were associated with tissue-specific proliferative phenotypes. *Vhl*-null cells exhibited increased proliferation early after recombination in the renal cortex; such an increase was not seen in other regions of the kidney. Furthermore, while all types of proximal tubular cells (resident in the renal cortex and outer medulla) upregulated expression of the cell cycle promoting gene Cyclin D1 (*Ccnd1*) early after *Vhl* inactivation, intercalated cells of the collecting duct (resident in all renal regions; major cell population in the renal papilla) did not do so. In contrast, these cells specifically upregulated expression of the cell cycle inhibitor p21^{Cip1} (*Cdkn1a*) in response to *Vhl* inactivation.

Analysis of *Ubc-CreERT2* mice given 1 mg TMX showed widespread negative selection of *Vhl*-null cells from all tissues assayed, belying the tissue-specific responses seen with 0.02 mg TMX or in *Pax8-CreERT2* mice at any TMX dose. It is possible that either the systemic pathologies caused by widespread *Vhl* inactivation or the high recombination frequencies induced by the 1 mg TMX dose affected cellular outcomes in these mice. Nevertheless, tissue-specific proliferative phenotypes were still observed in these analyses; *Vhl*-null cells were proliferative in the renal cortex, but not in the liver at 1 to 3 weeks following recombination.

This tissue-specific pattern of selection and proliferation observed in *Vhl*-null cells is consistent with the renal cortical location and proposed proximal tubular origin of ccRCC.

My findings thus indicate that the tissue-specificity of *VHL*-associated oncogenesis might arise from tissue-dependent selective pressures acting on cells early after *Vhl* inactivation and in the absence of other cancer-associated mutations or morphological abnormalities. They illuminate a more direct role played by *Vhl* inactivation in oncogenesis than previously thought.

The role of negative selection – whether by cell autonomous mechanisms or active removal by the tissue – in dictating tissue-specific tumorigenesis associated with cancer mutations is also supported by this work. Application of such ‘reporter’ strategies in investigating early consequences of other cancer-associated mutations may reveal the role of negative selection in dictating tissue-specificity in other cancers.

7.3 *Vhl*-null cells experience time-dependent restraints on proliferation

Another notable finding in this work was that despite no further intervention performed and a fully active HIF-dependent transcriptional cascade expected within days of *Vhl* inactivation, *Vhl*-null cells significantly and specifically altered gene expression between the 1-3 week and 4-12 months timepoints. These changes were driven largely by genes not known to be HIF targets nor regulated by *Vhl* at the 1-3 week timepoint, indicating an additional adaptive process entrained in *Vhl*-null cells over time. Interestingly, transcriptomic differences between mice at 3-weeks *versus* 4-months timepoints were more substantial than between mice at 1-week *versus* 3-weeks or between mice at 4-months *versus* 8-months timepoints, indicating that *Vhl*-null cells undergo specific adaptive processes over a timeframe between 3 weeks and 4 months after *Vhl* inactivation.

These gene expression changes were associated with a remarkable change in the proliferative behaviour of *Vhl*-null cells. While *Vhl*-null proximal tubular cells exhibited increased proliferation between 1 week and 3 weeks following *Vhl* inactivation – an observation corroborated by scRNA-seq and histological analysis of *Pax8-CreERT2* mice

given 5x 2 mg TMX and histological analysis of *Pax8-CreERT2* and *Ubc-CreERT2* mice given 1 mg TMX – they did not exhibit increased cell cycle entry at the 4–12-month timepoint.

Vhl-null cells in the renal tubules, after being generated as single cells or in groups of 2-3 neighbouring cells, appeared to occupy entire profiles of the renal tubule at the 4–12-month timepoint; this behaviour was not observed for *Vhl*-competent marked cells generated in the *Vhl*^{wt/pjrf1} comparator. This likely occurred because daughter cells of *Vhl*-null cells were retained in the renal tubules after proliferation at the early timepoint, though removal of *Vhl*-competent cells interleaving *Vhl*-null cells in the tubule cannot be excluded. Notably, this did not result in tubular disorganisation or dysplasia in the tubules, suggesting that the apparent multiplication of *Vhl*-null cells was ultimately limited by the boundaries of the tubular epithelium. Factors contributing to epithelial cell architecture, mitotic spindle direction, and the organisation of the basement membrane might thus be involved in regulating *Vhl*-dependent proliferation of RTE cells.

These findings suggest that the ‘insufficiency’ of *VHL* inactivation in promoting oncogenesis^{39,185,218,219} does not arise from an inherent inability to promote proliferation in cells of the RTE but from a time-dependent adaptive process occurring in *Vhl*-null cells that restrains its proliferation and expansion beyond the confines of the tubular monolayered epithelium. While the mechanism by which this occurs remains unknown, an avenue for exploration is hinted at by some observations in the current work.

Vhl-null cells were found to upregulate the expression of β -Defensin genes – most notably *Defb19* – early after inactivation that increased over time. This was associated with upregulation of other genes involved in innate immunity and an increase in incidence of foci of renal immune cells at least some of which were comprised of resident renal macrophages. Renal macrophages in kidneys carrying *Vhl*-null cells were also found to upregulate genes involved in leukocyte activation including the chemoattractant C-C motif chemokine receptor

2 (*Ccr2*). Interestingly, a member of the β -Defensin family – human β -Defensin 3 (hBD3) has been shown by others to attract macrophages in a CCR-2 dependent manner in *in vivo* models of oral cancer^{242,246}; it is possible that *Defb19* plays similar roles after *Vhl* inactivation in the kidney. Considering that β -Defensin upregulation and immune infiltration were observed in contexts that restricted *Vhl*-null cell proliferation and did not support oncogenesis, it can be hypothesised that β -Defensin-mediated immune recruitment in response to *Vhl* inactivation in renal tubular epithelial (RTE) cells helps maintain epithelial integrity and restricts the proliferation entrained in *Vhl*-null cells over time.

7.4 PT Class A cells are possible cells-of-origin for *VHL*-associated ccRCC

Another striking finding in this work was that regardless of *Vhl* status, proximal tubular cells of all three previously characterised subtypes (PT S1, S2, and S3) could be divided into two populations based on the expression of a module of genes that includes the long noncoding RNAs *Neat1* and *Malat1* as well as transcription factors *Jun*, *Jund*, *Fos*, and *Fosb*. This dichotomy was discovered in the scRNA-seq data after noticing that cells identified as being of PT S1, S2 and S3 origin based on their expression of externally-defined cell-type-specific marker genes, each formed two populations after dimension reduction based on the most variable genes expressed in the dataset. This was possible because cell type assignment in my analysis workflow was independent of dimension reduction and cell clustering. This approach differed from most published scRNA-seq work where all cells in each cluster defined after dimension reduction are assigned a cell type identity based on genes differentially enriched in that cluster. By uncoupling cell type assignment from dimension reduction and clustering, I allowed heterogeneity beyond known cell type classifications to contribute to the annotation of cells in my dataset. Thus, while I could observe dichotomous expression of genes such as *Neat* and *Malat1* in publicly-available scRNA-seq data^{236,237}, the two PT populations defined by such dichotomous expression were not explicitly described in

reports of that data. This was because the cells with low *versus* high expression of these genes were assumed to correspond to PT S3 *versus* PT S1/S2 cell types and were annotated as such without exploring the suitability of a different classification.

Of the two PT populations, PT Class A cells exhibited 1) a greater magnitude of changes in *Vhl*-dependent genes, 2) specific regulation of genes associated with adverse prognosis of ccRCC, 3) the greatest increase in proliferation following *Vhl* inactivation, and 4) manifested the greatest increase in expression of ccRCC-associated genes that are known transcriptional targets of HIF in response to *Vhl* inactivation or concomitant *Vhl* and *Pbrm1* inactivation. These observations suggest that the PT Class A population of cells might be the cells-of-origin for ccRCC. Their presence in cancerous or dysplastic lesions associated with *Vhl* inactivation would be of interest to examine further. Whether the two classes of PT cells reflect distinct differentiation pathways or potentially interchangeable cell states has not been determined here; this too merits further investigation.

7.5 Oncogenesis in positively-selected *Vhl/Pbrm1*-null cells is possibly context specific

Based on the analysis of *Vhl*-null cells, it was hypothesised that additional *Pbrm1* inactivation might drive oncogenesis by relieving proliferative restraints at later timepoints, impairing epithelial integrity of *Vhl*-null cells, or altering the *Vhl*-dependent expression of genes involved in innate immunity. These hypotheses were preliminarily tested in this thesis.

Cells with concomitant *Vhl* and *Pbrm1* inactivation increased in number in the renal cortex. This increase was accompanied by an apparent multiplication of *Vhl/Pbrm1*-null cells along the tubular profiles that in some cases led to the formation of multi-layered epithelia and what appeared to be dysplastic lesions at 10 months after recombination. These observations provide proof-of-principle that additional *Pbrm1* inactivation drives oncogenesis in *Vhl*-null cells. However, these lesions involved only a minority of the total *Vhl/Pbrm1*-null cell population in the renal cortex, seemed to exhibit a preference for the

cortical edge of the tissue, and did not arise early after recombination was induced. Moreover, analysis of the *Vhl/Pbrm1*-null cell population as a whole in kidneys that harboured these lesions did not reveal *Vhl/Pbrm1*-null cells to be more proliferative than *Vhl*-null cells at the 10-month timepoint and revealed surprisingly few effects on gene expression that were not already observed after *Vhl* inactivation on its own. Thus, *Pbrm1* inactivation appeared to lead to multiple discrete abnormalities but not a general change in cell behaviour or gene expression of *Vhl*-null cells. It is possible that the current analysis is limited by low sequencing depth or an incorrect choice of timepoint for analysis. However, these observations do highlight that positive selection of *Vhl/Pbrm1*-null cells in the renal cortex is insufficient for oncogenesis and that *Pbrm1* inactivation possibly has oncogenic effects on *Vhl*-null cell morphology and gene expression in specific niches or cell states.

A particularly intriguing observation in this regard was of the lesions classified in the thesis as ‘precursor dysplasia’. These lesions were identified by tdTomato IHC as disorganised renal tubules that were overwhelmingly tdTomato-positive and appeared to coalesce around clusters of tdTomato-negative interstitial cells. How these lesions form, whether they represent sections of a single tubule or multiple tubules coming together, the identity of the interstitial cells, communication between the epithelial and interstitial cells in the lesions, and whether cells in these lesions harbour additional mutations, all merit further investigation and may be key to understanding context-specific oncogenesis following *Vhl* and *Pbrm1* inactivation in the RTE. Further characterisation of *Vhl/Pbrm1*-null cells in morphologically normal tubules *versus* those comprising dysplastic lesions would thus be of interest to examine.

7.6 Conclusion

Overall, this thesis presents a novel approach to elucidating the earliest events in the genesis of cancer by studying the early effects of *Vhl* inactivation in individual cells within morphological normal tissues *in vivo* and tracing the evolution of changes incurred after *Vhl*

inactivation over time with cellular resolution. It demonstrates that cell type, tissue, time, and possibly additional contexts significantly impact cellular consequences entrained by *Vhl* inactivation. It also describes a new class of proximal tubular cells with particular pro-oncogenic responses to *Vhl* inactivation. It identifies selective pressures acting on *Vhl*-null cells in different tissues consistent with tissue-specific tumorigenicity of *Vhl* inactivation. Lastly, it proposes time-restricted proliferation of *Vhl*-null cells in the RTE as a potential means of tumour suppression and invites further investigation into the contexts wherein additional *Pbrml* inactivation promotes oncogenesis by relieving these restraints.

Chapter 8: Materials and Methods

8.1 Mice

All experimental procedures were conducted following approval by the Medical Science Ethical Review Committee of the University of Oxford and authorisation under UK Home Office regulations of Animals (Scientific Procedures) Act 1986.

Mice were housed in individually ventilated cages on a 13 h light/ 11 h dark cycle with food and water provided *ad libitum*. *Vhl^{tm1.1b(tdTomato)Pjr} (Vhl^{wt/pjr.fl})* mice were commissioned from Ozgene, Australia and generated on a C57BL/6J background using goGermline technology²⁴⁷. *Vhl^{tm1jae} mice (Vhl^{wt/jae.fl})* (RRID:IMSR_JAX:012933)¹⁸⁵ were purchased from The Jackson Laboratory and were crossed with *Tg(Pgkl-cre)lLni (Pgkl-Cre)* mice (RRID:IMSR_JAX:020811)²⁴⁸ to generate mice carrying a constitutively knocked-out form of the *Vhl^{tm1jae} allele (Vhl^{jae.KO})*. Mice carrying the inducible allele of Cre-recombinase driven by the *Ubiquitin-C* promoter (*Ndori^{Tg(Ubc-cre/ERT2)1Ejb}*; termed *Ubc-CreERT2*) (RRID:IMSR_JAX:007001)¹⁹⁰ were purchased from The Jackson Laboratory. Mice carrying an inducible allele for Cre-recombinase driven by the *Pax8* promoter (*Tg(Pax8-cre/ERT2)C Amat*; termed *Pax8-CreERT2*) (RRID:IMSR_HAR:9175) were obtained *via* EMMA¹³⁷. Mice carrying conditional alleles of *Pbrm1* (*Pbrm1^{tm1c(EUCOMM)Wtsi}*; termed *Pbrm1^{fl}*) (RRID:IMSR_JAX:031875)¹⁰⁹ were purchased from The Jackson Laboratory on a C57BL/6J background. *Vhl^{wt/pjr.fl}*; *Pax8-CreERT2* mice, *Vhl^{jae.KO/pjr.fl}*; *Pax8-CreERT2* mice, *Vhl^{pjr.fl/pjr.fl}*; *Pax8-CreERT2* mice, *Vhl^{wt/pjr.fl}*; *Ubc-CreERT2* mice, *Vhl^{jae.KO/pjr.fl}*; *Ubc-CreERT2* mice, *Vhl^{wt/pjr.fl}*; *Pbrm1^{fl/fl}*; *Pax8-CreERT2* mice, and *Vhl^{jae.KO/pjr.fl}*; *Pbrm1^{fl/fl}*; *Pax8-CreERT2* mice of both sexes were used between 2-20 months of age, and all were bred on a C57BL/6J background.

8.2 Genotyping and PCR

Ear-clips, used for identification and genotyping, and tail-tips used to confirm genotypes after mouse harvest, were digested in digest buffer (100 mM EDTA (pH 8.0) (Thermo AM9261), 50 mM Tris-HCl (pH 8.0) (Merck T3253), 100 mM NaCl, 1% sodium dodecyl sulphate (SDS; Thermo AM9820) and 5% Proteinase K (Bioline BIO-37039)) shaking at 55°C overnight. DNA was extracted from the digest by first salting out proteins with 5M NaCl, then precipitating nucleic acid with isopropanol, and air drying it at 55°C before resuspending in nuclease-free water. Genomic DNA was extracted from FFPE tissues using the QiaAMP DNA FFPE Tissue Kit (Qiagen 56404) using manufacturer's protocol. Genomic DNA was genotyped by PCR using the Taq Core Kit (Qiagen 201223) according to manufacturer's instructions, and amplicons were analysed by gel electrophoresis through 2% (w/v) or 3% (w/v; to resolve bands between 100 to 300 bp) agarose in Tris-Borate-EDTA (Fischer 10130740) buffer. The final PCR reaction mix contained 1x CoralLoad PCR Buffer, 1x Q-Solution, 200 μ M dNTP mix, 300 nM primer mix, and 2.5 units of Qiagen Taq polymerase with either 1.5 mM or 2 mM MgCl₂ as required for each primer set. PCR reaction conditions are summarised in **Tables 8.1** and **8.2**. *Vhl^{l^{ae}}* and *Vhl^{l^{pjr}}* PCR conditions were used for all forms of the two alleles. Details of primers and expected bands are summarised in **Table 8.3**.

Table 8.1: PCR Reaction Protocol			
Step		Time	Temperature (°C)
Initial Denaturation		3 min	94
Cycling	Denaturation	1 min	94
	Annealing	45 sec	variable
	Extension	1 min	72
Final extension		10 min	72
Storage		<i>ad infinitum</i>	8

Table 8.2: PCR Reaction Conditions		
PCR	[Mg ²⁺] (mM)	Annealing Temperature (°C)
<i>Vhl^{l^{ae}}</i>	2	55
<i>Vhl^{p^{jr}}</i>	2	60
<i>CreERT2</i>	1.5	55
<i>Pbrm1</i>	2	60

Table 8.3: Sequences and expected amplicon lengths for each set of genotyping primers			
PCR	Primers	Amplicon	Genotype
<i>Vhl^{l^{ae}}</i>	CTGGTACCCACGAACTGTC	260bp	<i>Vhl^{l^{ae}.wt}</i> (exon 1)
	CTAGGCACCGAGCTTAGAGGTTTGCG	240bp	<i>Vhl^{l^{ae}.KO}</i>
	CTGACTTCCACTGATGCTTGTCACAG	470bp	<i>Vhl^{l^{ae}.fl}</i>
<i>Vhl^{p^{jr}}</i>	GGTGCTAATTGAAGGAAGCTACTG	357 bp	<i>Vhl^{p^{jr}.wt}</i> (exons 2 and 3)
	TCAGAGTTCTGGATCACATGAGTG		
	GGTGCTAATTGAAGGAAGCTACTG	1067 bp	<i>Vhl^{p^{jr}.fl}</i>
	CTCCTCCGAGGACAACAACATG		
	GCTTTGAGAATTTTGGGCAGTTG	521 bp	<i>Vhl^{p^{jr}.KO}</i>
	TGAAGTTAGTAGCTCCGCTTCCAC	4497 bp	<i>Vhl^{p^{jr}.inrec}</i>
	GGTGCTAATTGAAGGAAGCTACTG	433 bp	<i>Vhl^{p^{jr}.inrec}</i>
	TGAAGTTAGTAGCTCCGCTTCCAC		
<i>CreERT2</i>	CGGTCGATGCAACGAGTGATGAGG	300 bp and 600 bp	Cre +ve (<i>Pax8</i> or <i>Ubc</i>)
	CCAGAGACGGAAATCCATCGCTCG		
	CTCATACCAGTTCGCACAGGCGGC	300 bp	Cre -ve
	CCGCTAGCACTCACGTTGGTAGGC		
<i>Pbrm1</i>	ATCTCTCCAGACCCCCAAAC	243 bp	<i>Pbrm1^{wt}</i>
	AAGACCCTGCCTCAAAACAC	400 bp	<i>Pbrm1^{fl}</i>

8.3 Haematocrit Measurement

Blood was collected from mice under terminal isoflurane anaesthesia in Microvette collection tubes (Sarstedt 16.444.100) from the abdominal aorta posterior to the renal bifurcation prior to tissue harvest. A sample of blood was transferred into heparin coated capillary tubes (Hirschmann M718) and spun at high speed for 5 min in a Unico C-MH30 centrifuge to measure haematocrit levels against a calibrated scale.

8.4 Tamoxifen (TMX) Administration

TMX solution was prepared by diluting a 25% (w/v) solution of TMX (Sigma T5648) in absolute ethanol (Sigma 51976) to 20 mg/ml in corn oil (Sigma C8267). 2 mg/ml and 0.2 mg/ml solutions were prepared by serial dilution of the 20 mg/ml stock with 10% ethanol in corn oil. Single 1 mg, single 0.1 mg, single 0.02 mg, or five consecutive daily doses of 2 mg TMX were administered to mice ≥ 20 g by oral gavage using sterile, disposable plastic feeding tubes (Instech FTP-20-38). It was noted that while male mice attained 20 g body by 6-8 weeks of age, female mice did so at 10-14 weeks of age.

8.5 Bromodeoxyuridine (BrdU) Administration

BrdU was administered to mice either by intraperitoneal injection (IP) or oral administration *via* drinking water. For IP, 10 mg/ml BrdU (Sigma B5002) in sterile-filtered phosphate-buffered saline (PBS; Gibco 70011) was injected at a dose of 50 mg/kg either one day after TMX administration (for assaying cumulative proliferation) or one day before harvest (for end-point labelling experiments). For oral administration, 1 mg/ml BrdU was prepared in 1% (w/v) sucrose solution in double-distilled water. BrdU water was protected from light. Mice in which cumulative proliferation was being assayed were given BrdU water continuously for up to 4 weeks with the bottle refreshed every week.

8.6 Immunohistochemistry (IHC) and Immunofluorescence (IF) Staining

8.6.1 Tissue preparation

IHC was performed on tissues following either immersion-fixation or perfusion-fixation depending on downstream analyses. Immersion fixation was used when tissues from the same mouse were also used for immunoblotting or flow cytometry and single-cell RNA sequencing (scRNA-seq). Mice were euthanised with terminal isoflurane anaesthesia and flushed *via* the aorta with 1x PBS pH 7.4 (Gibco 70011) to clear blood from tissues. For

immersion fixation, tissues were fixed in 10% neutral buffered formalin (Sigma HT501128) with rocking for 24 h at room temperature (RT). For perfusion fixation, flushing with PBS was followed by perfusion with 4% (w/v) paraformaldehyde (Sigma P1213) in 1x PBS (Gibco) pH 7.4 at RT and tissues processed as for immersion fixation. Tissues were dehydrated through a graded ethanol series (70% to 100%) and xylene before paraffin embedding. Formalin-fixed paraffin-embedded (FFPE) tissues were cut to 4 μ m sections on a Thermo Microm HM 355S Microtome. Sections were floated on warm distilled water and mounted on poly-lysine coated slides (Fisher 10149870). Slides were dried for 3-24 h at 37°C before IHC.

8.6.2 Single-label IHC

FFPE sections were deparaffinised with xylene and ethanol and rehydrated with double-distilled water. Sections were subjected to heat-induced epitope retrieval (HIER) with TE buffer (10 mM Tris, 1 mM EDTA; pH 9.0) in a steamer (at 95°C) for 20 min or with Dako Antigen Retrieval Solution pH 6.0 (ARS; Agilent S1699) in a pressure cooker (at 120°C) for 12 min as optimised for each antibody used (**Table 8.4**). Slides were blocked for endogenous peroxidase activity using Dako Peroxidase Blocking solution (Agilent S2023) for 10 min at RT and non-specific protein binding using 5% (w/v) bovine serum albumin (BSA; Sigma 5482) in 1x TBS-T (50 mM Tris, 31.6 mM NaCl, 0.1% (v/v) Tween-20 (Promega H5151); pH 8.4) for 40 min at RT before incubation with primary antibodies diluted in Dako Antibody Diluent Solution (Agilent S3022) overnight at 4°C. Dilutions optimised for each antibody used are listed in **Table 8.4**. Sections were washed in 1x TBS-T. Signal was detected with the Dako Envision system (Agilent K4003) with a diaminobenzidine (DAB) exposure time of 1.5 min for CD45 IHC and 10 min for other IHC, and the nuclei were counterstained with modified Harris Haematoxylin (Fisher 72711) differentiated for 10 seconds in 0.25% HCl in 70% ethanol. Haematoxylin staining was blueed

by immersion in 0.06% (w/v) NH_4OH in water for 30 seconds. Slides were dehydrated and mounted DPX mountant (Merck 06522).

Table 8.4: Concentration and HIER Conditions for Primary Antibodies							
Target	Company	Catalogue No.	RRID	Host	Application	Conc.	HIER Buffer
CD45	Cell Signalling	70257	AB_2799780	Rabbit	IHC	1:200	TE pH 9.0
cleaved Caspase 3	Cell Signalling	9664	AB_2070042	Rabbit	IHC	1:2000	TE pH 9.0
CLEC4F	Invitrogen	PA5-47396	AB_2608299	Goat	IF	1:100	TE pH 9.0
F4/80	Cell Signalling	70076	AB_2799771	Rabbit	IHC	1:200	TE pH 9.0
F4/80	Cell Signalling	70076	AB_2799771	Rabbit	IF	1:100	TE pH 9.0
Ki-67	Cell Signalling	12202	AB_2620142	Rabbit	IHC	1:500	ARS pH 6.0
Ki67	Life Technologies	13569882	AB_2572796	Rabbit	Dual IHC	1:100	ARS pH 6.0
PBRM1	Proteintech	12563-1-AP	AB_2877865	Rabbit	IHC	1:1000	ARS pH 6.0
tdTomato	Rockland	600-401-379	AB_2209751	Rabbit	IHC	1:1000	TE pH 9.0
tdTomato	Rockland	600-401-379	AB_2209751	Rabbit	IF	1:50	TE pH 9.0

8.6.3 tdTomato-Ki67 and tdTomato-BrdU Dual IHC

tdTomato was visualised using the standard Envision IHC protocol as detailed above, with a 3 min DAB (brown) exposure to generate a first signal of lower intensity. Peroxidase

activity was neutralised by incubating slides in 3% (v/v) H₂O₂ (Merck H1009) in 1x PBS, and a second round of HIER performed with Dako Antigen Retrieval Solution (Agilent S1699) in a pressure cooker (at 120°C) for 12 min. Slides were then blocked for avidin, biotin and protein binding (Abcam ab64212 and Agilent X090930-2). Ki67 was detected using a biotinylated Rabbit anti-Ki67 biotin-conjugated antibody (Life Technologies 13569882, RRID:AB_2572794) and BrdU incorporation was detected by the biotinylated anti-BrdU antibody provided in the BrdU Detection Kit II (BD 551321) at RT for 2 h, before being recognised by streptavidin-HRP provided in the BrdU Detection Kit II (BD 551321) at RT for 30 min. Staining was visualised with Vector VIP (purple) substrate (Vector SK4605), Vector SG (grey) substrate (Vector SK4705) or Vector AMEC (red) substrate (Vector SK4285) with 1.5 min, 10 min, and 10 min exposure times respectively. When the SG substrate was used, sections were counterstained with Nuclear Fast Red (Abcam 65692) for 5 min at RT and with Harris haematoxylin as described above when AMEC and VIP substrate was used. When AMEC substrate was used, slides were mounted with Vectashield Vibrance Antifade Mounting Medium (Vector H170010) and with DPX as described above when SG and VIP substrates were used.

8.6.4 Trichrome Staining

Trichrome staining was performed on FFPE sections using the Trichrome stain (Connective Tissue stain) kit (Abcam ab150686) according to manufacturer's instructions. Briefly, FFPE sections were deparaffinised and rehydrated as described before treatment with pre-heated Bouin's fluid at 60°C for 1 h. Slides were washed in tap water and stained with Weigert's Iron Haematoxylin for 5 min at RT and Biebrich Scarlet/Acid Fuchsin Solution for 15 min at RT. Staining was differentiated by incubation with Phosphomolybdic/Phosphotungstic Acid for 15 min at RT; this removed the 'red' staining from collagen in the tissue section. Collagen was then stained with Aniline blue solution for

15 min at RT differentiated with 0.1% Acetic Acid Solution for 3 min at RT. Slides were dehydrated in a graded series of ethanol and xylene as described before and mounted in DPX.

8.6.5 Haematoxylin and Eosin Staining (H&E)

FFPE sections were deparaffinised and rehydrated and stained with haematoxylin as described above. Sections were rinsed twice each in 90% and 100% ethanol and then incubated in Shandon Alcoholic Eosin Y solution (Fischer 6766008) for 3 min at RT before washing in 100% ethanol, clearing with xylene, and mounting with DPX.

8.6.6 IF Staining

IF staining on FFPE tissues was performed as for IHC, but with the following modifications. After rehydration and HIER, slides were blocked with 3% BSA (w/v), 10% Donkey serum (v/v) in 1x TBS-T for 40 min at RT. Primary antibodies were diluted in 5% BSA (w/v) in 1x TBS-T at dilutions optimised for each antibody (**Table 8.4**). Slides were washed in TBS-T and then signal detected with Alexa Fluor-labelled secondary antibodies for 30 min at RT. Autofluorescence was quenched with the TrueView Autofluorescence Quenching Kit (Vector SP840015) for 5 min at RT before washing in PBS. Slides were counterstained with 1 µg/ml 4',6-diamidino-2-phenylindole (DAPI; Sigma D9542) for 5 min at RT and mounted under No 1.5 coverslips with Vectashield Vibrance Antifade Mounting Medium.

8.7 TUNEL Assays

TUNEL on FFPE sections were performed using the In Situ Cell Death Detection Kit (Roche 11684817910) using manufacturer's instructions. Briefly, after standard deparaffinisation and rehydration, tissues were subjected to Proteinase K (0.2 mg/ml in H₂O; Bioline BIO-37039) treatment for 30 min at 37°C after which endogenous peroxidase activity was blocked by incubation with 3% H₂O₂ in methanol at RT for 10 min. Tissue sections were then permeabilised by incubation in 0.1% Triton-X (Sigma X100), 0.1% Sodium Citrate

(Sigma C3434) in 1x TBS (50 mM Tris, 31.6 mM NaCl; pH 8.4) at RT for 8 min. The TUNEL Reaction Mixture as prepared in the kit was applied to sections for 1 h at 37°C, after which sections were washed with 1x PBS. Sections were then blocked using 3% (w/v) BSA, 20% (v/v) Normal Goat Serum (NGS) in 1x PBS pH 7.4 at RT for 20 min, before incubation with the Converter-POD solution for 30 min at RT. TUNEL signal was developed using DAB substrate as used in IHC, and sections were counterstained with 1% Methyl Green, provided in the kit, for 3 min at RT before dehydration and mounting with DPX.

Fluorescence-based TUNEL assays were performed using the In Situ Cell Death Detection Kit (Roche 11684817910) as above but with the following modifications. Tissues were subjected to HIER with Dako Antigen Retrieval Solution (Agilent S1699) in a microwave for 1 min and blocked with 3% BSA, 20% NGS in 0.1M Tris-HCl pH 7.4 for 30 min at RT. After incubation with the TUNEL reaction mixture as above, autofluorescence was quenched using the TrueView kit and sections were counterstained with DAPI as described for IF staining. TUNEL signal was visualised using fluorescent signals emitted by fluorescein-conjugated nucleotides incorporated into free 3'-ends of genomic DNA by the action of Terminal deoxynucleotidyl transferase in the TUNEL reaction mixture.

8.8 Microscopy

Chromogenic IHC and TUNEL sections were imaged on either a Leica DM100 LED microscope or a Hamamatsu NanoZoomer S210 slide scanner. Images were processed with LAS V4.4 or NDP Viewer software.

IF sections were imaged with a Zeiss LSM710MP or LSM980MP confocal microscope. Images were obtained with spectral unmixing and online fingerprinting. Briefly, the section was illuminated by all (405 nm, 488 nm, 561 nm, 639 nm) lasers and light collected by 32 detectors each covering around 9 nm of the visible light spectrum. Light collected from each pixel in the image was deconvoluted into individual spectral contributions from DAPI, tdTomato, Alexa Fluor 488, Alexa Fluor 546, Alexa Fluor 647, or

tissue autofluorescence. These contributions were then separated into individual channels. Images were analysed on Zeiss Zen Blue software and FIJI ImageJ.

8.9 Image Quantification

Quantification of the proportion of total cells in a tissue section that stained positive for tdTomato or Ki67 by IHC was performed using the HALO Image Analysis Software v3.5 and v3.6 (Indica Lab, Albuquerque, USA). Kidney sections were annotated manually into cortex, outer medulla, inner medulla, and papilla areas based on the well-characterised anatomical location and histological appearance of renal tubules in these areas²³² (**Figure 8.1**); liver sections were not subdivided for analysis. Nuclei were detected based on haematoxylin staining using the HALO AI v3.6 Nuclei Seq algorithm, with detected nuclei of areas $< 15 \mu\text{m}^2$ and $> 200 \mu\text{m}^2$ excluded from analysis. Cells were recorded as tdTomato-positive if the tdTomato signal passed intensity threshold in detected nuclei and in a $2 \mu\text{m}$ perimeter drawn around each nucleus. Cells were scored as positive for Ki67 if the signal passed threshold in only the nucleus. Identical analysis parameters were used for all slides stained for the same targets. Representative images of the above analysis performed on a field-of-view of tdTomato/Ki67 dual IHC in the renal cortex are provided in **Figure 8.2**. The total number of sections, average number of cells analysed per section, and average area of section analysed in this thesis for the different renal regions and livers are given in **Table 8.5**.

Table 8.5: No. of cells and section areas analysed for different renal regions and the liver using HALO					
Tissue	Sections	Cells (mean)	Cells (SD)	Area (mean)	Area (SD)
Cortex	331	96613.2	31837.8	17.4	63.9
Outer medulla	317	40722.1	12109.3	82.0	25.9
Inner medulla	274	47150.8	18544.2	62.3	23.0
Papilla	233	16493.0	6987.1	26.5	11.6
Liver	69	56128.3	21221.0	17.9	65.0

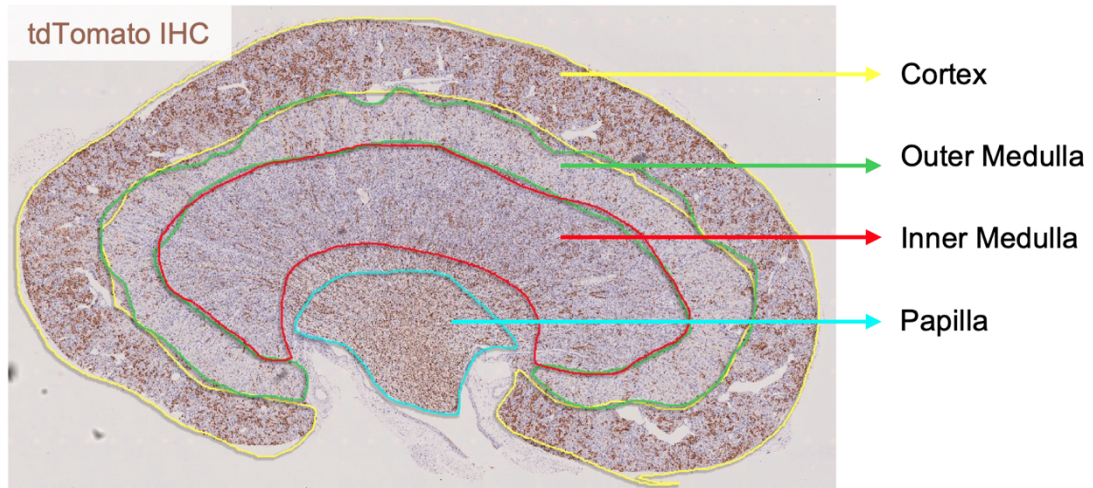


Figure 8.1: Annotation of different anatomical regions of the kidney. Representative tdTomato IHC on a *Vhl^{wt/pjr.fl}; Pax8-CreERT2* kidney harvested at the late timepoint. Anatomical regions corresponding to the renal cortex (yellow), outer medulla (green), inner medulla (red) and papilla (blue) are annotated.

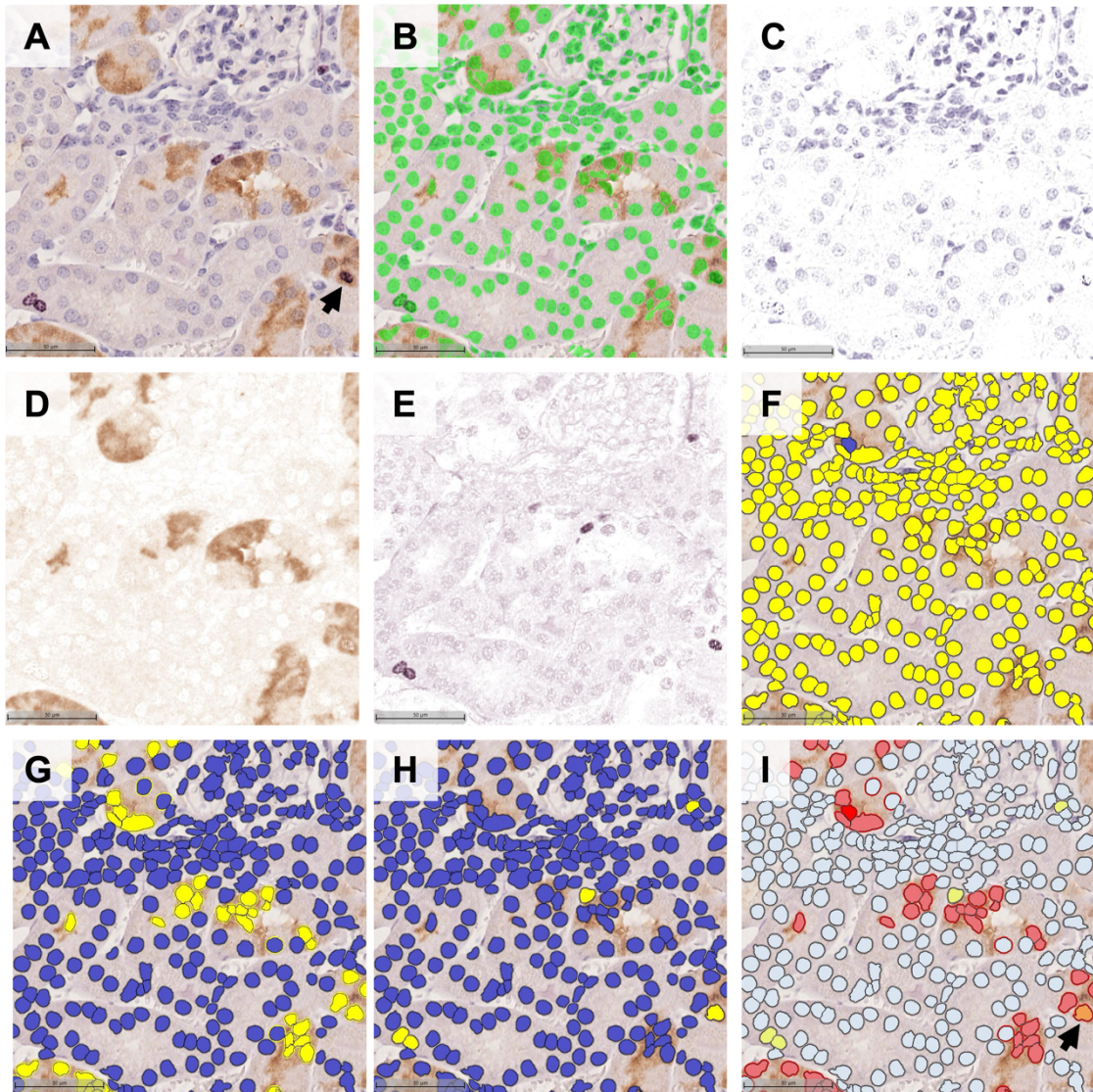


Figure 8.2: IHC Quantification by HALO. (A) Representative field-of-view of tdTomato (brown; DAB) and Ki67 (purple; VIP) dual IHC in the renal cortex of *Vhl^{wt/pjr.fl}; Pax8-CreERT2* mice given 5x 2 mg TMX and harvested 1 week after dosing. Red arrow denotes a tdTomato-positive Ki67-positive cell. (B-I) Steps performed by HALO to analyse the field-of-view shown in (A). (B) Nuclei are identified by the HALO AI Nuclei Seq algorithm. (C-E) Image deconvolution is performed to compute individual contribution of haematoxylin (C), DAB (D), and VIP (E), to the final staining. (F-H) Identification of cells that have passed thresholds for haematoxylin (F), DAB (G), and VIP (H) signal. Positive cells (yellow), negative cells (blue). (I) Identification of tdTomato-negative Ki67-negative (light blue), tdTomato-positive Ki67-negative (red), tdTomato-negative Ki67-positive (yellow), and tdTomato-positive Ki67-positive (orange) cell (also denoted by black arrow) by HALO. Scale bars denote 50 μm .

8.10 RNA *in situ* Hybridisation

RNA *in situ* hybridisation was performed on 4 µm FFPE kidney sections cut on the previous day and baked at 60°C for 1 h using either RNAscope 2.5 HD Assay – BROWN (ACD BioSciences 322300) for single assays, or RNAscope 2.5 HD Duplex Detection Kit (ACD BioSciences 322430) for dual assays, according to manufacturer's instructions. Probes used were: *Apoc3* (Cat. 518591), *Defb19* (Cat. 1245931-C1), *Neat1* (Cat. 440351) and *Fxyd2* (Cat. 572631-C2). For single assay, sections were counterstained and mounted as for IHC.

8.11 Tissue Dissociation

Kidneys were dissociated into single-cell suspension for FACS, single-cell sequencing, or primary renal cultures. Kidneys were dissociated using the Multi-Tissue Dissociation Kit 2 (Miltenyi 130-110-203). Briefly, the renal capsule was removed, and the tissue macerated for 5 min on a bed of ice. The macerated kidney was then transferred to 1.45 ml Buffer X, 30 µl of Enzyme D, 15 µl of Enzyme P, 15 µl of Buffer Y, and 6 µl of Enzyme A, all prepared according to the manufacturer's instructions. The tube was then agitated under water at 37°C in a shaking incubator at 150 rpm for 30 min. Dissociation was stopped by the addition of 150 µl Foetal Bovine Serum (FBS; Sigma F7524), and resuspension in 9 ml of RPMI-1460 medium (Merck R0883). Undigested tissue was removed using a 40 µm cell strainer and the filtrate centrifuged (300g for 10 min at 4°C). Erythrocytes were eliminated by resuspending dissociated cells in 3 ml of 1x RBC Lysis Buffer (Miltenyi 130-094-183) prepared in deionised water for 2 min at RT. Cells were centrifuged (300g for 5 min at 4°C) and resuspended in ice-cold D-PBS before being counted on a Thermo Scientific Countess II machine for total yield and viability.

8.12 FACS

Dissociated cells were resuspended in 10% FBS 2 mM EDTA in D-PBS (Thermo 14190144) to a concentration of 5 million live cells per ml. 10 µl of 10 µg/ml DAPI (Sigma

D9542) was added to stain for viability. Cells were sorted using a BD Aria Fusion Cell Sorter in the Flow Cytometry Facility at the Wellcome Trust Centre for Human Genetics by Dr Ruddy Montandon, Ghada Ben Youssef, and Mohammed Islam. tdTomato was excited with a 561 nm laser and fluorescence detected with a 582/15 band pass filter. DAPI was excited with a 405 nm laser and fluorescence detected with a 450/40 band pass filter. Live, single, tdTomato-positive and -negative cells were collected in FBS-coated polypropylene tubes and pelleted by centrifugation at 300g for 10 min. Cells were counted again for yield and viability and processed for immunoblotting, DNA extraction, or single-cell sequencing.

8.13 Mouse Embryonic Fibroblast Preparation

Mouse embryos were harvested at E13 in ice-cold HBSS (Gibco 14175). Heads were collected for genotyping, internal organs were removed, and the rest of the embryo was macerated before being digested with trypsin (Sigma T3924) for 5 minutes at 37°C to produce single cell suspensions. These were incubated in Dulbecco's Modified Eagle's Medium (Sigma D6429) containing 4500 g/L glucose supplemented with 10% FBS (Sigma), 2 mM glutamine, and 1% penicillin-streptomycin (Sigma P0781) at 37°C in humidified normoxic air containing 5% CO₂ to obtain mouse embryonic fibroblasts (MEFs). Recombination was induced by treatment with 4-hydroxytamoxifen (4-OHT) (Sigma T176) at a final concentration of 10 µM for 24 h. At 3 days after treatment, MEFs were washed with PBS pH 7.4, detached from culture plates with trypsin-EDTA solution (Sigma, T3924), and spun at 300g for 10 min at 4°C. Genomic DNA was extracted from cell pellets following SDS/Proteinase K digestion as described for mouse ear clips above.

8.14 Primary renal culture and live cell imaging

Primary renal cultures were prepared by dissociating freshly harvested kidneys as described above. Cells were plated on gelatin-coated 35 mm 'elastically supported surface' coverslip imaging dishes (Ibidi 81291) in Dulbecco's Modified Eagle's Medium (Sigma

D6429) containing 4500 g/L glucose supplemented with 10% FBS (Sigma F7524), 2 mM glutamine (Sigma G7513), 1% insulin-transferrin-selenium solution, 25 mM HEPES, 50 nM hydrocortisone and 1% penicillin-streptomycin (Sigma P0781), at 37°C in humidified normoxic air containing 5% CO₂. Recombination was induced by treatment with 4-hydroxytamoxifen (4-OHT) (Sigma T176) at a final concentration of 10 µM for 24 h. tdTomato fluorescence in live cells was imaged 3 days after finishing treatment using a Zeiss LSM710MP or LSM980MP confocal microscope as described above.

8.15 Immunoblotting

Tissues frozen after harvest in dry ice were homogenised using a chilled hammer and pestle kept over dry ice before solubilisation using Urea SDS buffer (7M Urea, 10 mM Tris, 1% SDS, 10% glycerol; pH 7.4) and boiling in 1x Laemmli Sample Buffer (BioRad 1610747) at 95°C for 5 min. Protein concentrations were determined using BioRad Protein Assay (BioRad 5000201EDU). Cells recovered by FACS were pelleted by centrifugation and frozen under dry ice. Cell pellets were first solubilised in Urea SDS buffer and boiled in 1.5x Laemmli Sample Buffer at 95°C for 5 min at a final concentration of 7,000 cells/µl.

Immunoblotting was performed as described previously²⁴⁹. Briefly, proteins were separated by SDS-polyacrylamide gel electrophoresis on a 9% polyacrylamide gel in 1x SDS running buffer (25 mM Tris, 0.19 M glycine, 0.1% SDS) at 170-180 V constant voltage, transferred to polyvinylidene difluoride membrane (Immobilon-P, Millipore IPVH00010) which were pre-hydrated in 100% methanol for 30 seconds under a constant voltage of 70 V in 1x Transfer buffer (10 mM Tris, 1 M glycine, 10% methanol, 0.001% SDS), and blocked in 4% (w/v) fat free milk (Sigma 70166), 0.1% Tween-20 in 1x PBS pH 7.4 for 1 hr at RT. Primary antibodies: tdTomato (Rockland 600-401-379, RRID:AB_2209751), HIF1A (Cayman 10009269; RRID:AB_999557), HIF2A (PM9, custom-made and characterised previously²⁵⁰), PBRM1 (Proteintech 1563-1-AP, RRID:AB_2877865) were diluted in blocking buffer (1:1000). HRP-conjugated secondary antibodies (Agilent P0448,

RRID:AB_2617138) and Clarity Max Western ECL substrate (BioRad 1705062) was used to visualise proteins using a ChemiDoc XRS+ imaging system (BioRad). After immunoblot analysis, membranes were stained with Coomassie brilliant blue to visualise separated proteins, and this was used as a reference of sample loading. Immunoblotting was performed by Dr Norma Masson in the Ratcliffe lab.

8.16 Single-cell RNA Sequencing

8.16.1 Single-cell GEM and Library Preparation

Sorted cells were prepared into single-cell droplets using the Chromium Next GEM Single-cell kit. 20,000 live cells were loaded per sample on separate Chromium Next GEM Chip G (10X Genomics PN-1000127) channels. cDNA clean-up, amplification and adaptor ligation were performed with the Chromium Next GEM Single Cell 3' Kit v3.1 (10X Genomics PN-1000268). Twelve reaction cycles were used for cDNA amplification. cDNA yield was quantified using the High Sensitivity D1000 ScreenTape Assay (Agilent 5067-5584 and 5067-5585) to optimise the number of reaction cycles for library preparation. Single cell sequencing libraries were prepared using Dual Index Kit TT Set A (10X Genomics PN-1000215) sequence indices. Prepared libraries were quantified with KAPA Library Quantification Kit (Roche KK4824) following the manufacturer's protocol. These steps were performed in collaboration with Dr Joanna Lima in the Ratcliffe lab.

8.16.2 Sequencing

Single-cell libraries were sequenced on Illumina NextSeq 2000 sequencing system with P3 200 cycle (Illumina 20040560) reagents. Equimolar pools of up to six sample libraries were prepared and diluted to 650 pM and mixed with 1% PhiX Control v3 DNA (Illumina FC-110-3001) in resuspension buffer (RSB) to a final volume of 25 µl. Sequencing was performed and preliminary sequencing results (bcl files) and FASTQ files were generated with the DRAGEN FASTQ Generation 3.8.4, or the DRAGEN BCL convert workflow optimised for Single Cell RNA Library Kit 1 library prep kit, and the Single Cell

RNA Index Adaptors 1-B index adaptor kit, with 28 and 152 Read 1 and 2 cycles respectively.

8.16.3 Data Processing and Quality Control

The mm10 reference genome was customised and the starting reference fastq files and GTF files came from [refdata-gex-mm10-2020-A.tar.gz](https://support.10xgenomics.com/single-cell-gene-expression/software/downloads) and are available at <https://support.10xgenomics.com/single-cell-gene-expression/software/downloads> or <https://cf.10xgenomics.com/supp/cell-exp/refdata-gex-mm10-2020-A.tar.gz>. The transcript sequence for *Vhl^{tpjr.KO}* that included the tdTomato sequence was then added manually to the fasta reference genome file and annotation for tdTomato transcript was added manually to the gtf file. The cellranger 6.0.1 mkref function was used to build the custom reference genome and reads taken from FASTQ files generated for each sample were aligned to the custom reference genome by CellRanger (version 6.1.1; <https://support.10xgenomics.com/single-cell-gene-expression/software/pipelines/latest/what-is-cell-ranger>) using default parameters sample by sample. After aligning, for each read pair, cell barcodes and unique molecular identifiers (UMIs) were obtained from Read 1 and read counts per feature were obtained from Read 2. Only those UMIs that could be linked to a valid cell barcode and a gene exon region were included to create the cell by gene count matrix. Reads from *Vhl* exon 1 were excluded from analysis to prevent ambiguous alignment. Cells were subjected to the following filters: detected genes > 200, fraction of mitochondrially-encoded reads < 0.5, and detected genes < 3 x median for each sample. The threshold for mitochondrially-encoded reads was set to this value in line with published kidney scRNA-seq studies to account for the high mitochondrial content in RTE cells¹⁷⁹. Information and quality control metrics for all sequenced samples are provided in **Appendix 9**. Cells from different samples and sequencing runs were then aggregated, which sometimes required manual renaming of duplicate cell ‘names’. Downstream analysis was conducted using the R package Seurat 4.0.3: Read counts were log-normalised and

scaled, principal component (PC) analysis was run using the scaled data and uniform manifold approximation and projection (UMAP) was generated using the top 30 PCs. These steps were performed by Dr Ran Li in the Ratcliffe lab.

8.16.4 Batch Correction and Dimension Reduction

Gene expression in renal tubular epithelial (RTE) cells, and proximal tubular (PT) cells in particular, has been reported to be highly sex-specific^{182,233,234}. To account for this difference batch correction was performed based on sex using Seurat following “Tips for integrating large datasets” from the Vignettes of Seurat. Samples were split by sex. Briefly, for each sex, read counts were log-normalised and the top 2000 varying features were identified and analysed using the `FindVariableFeatures` command. Next, features were selected for downstream integration using `SelectIntegrationFeatures` and for each sex, normalised read counts were scaled using these selected features and PCA were run afterwards. Then anchoring features between the two sexes were calculated using `FindIntegrationAnchors` functions with parameter “reduction='rpca'”, which used reciprocal PCA (top 30 PCs) to identify an effective space in which to find anchors. Finally, samples of the two sexes were integrated with the `IntegrateData` function using the identified anchoring features. For the integrated data, standard Seurat workflow was performed: Expression of these genes was scaled to have a mean at 0 and standard deviation of 1 using the `ScaleData` function. Principal components were analysed for the expression of these genes using the `RunPCA` function. The first 30 components were then included for shared nearest neighbour modularity optimisation with the Louvain algorithm using the `FindClusters` function and resolution set to 1. Finally, UMAP dimension reduction was performed using the `RunUMAP` function with default parameters and 30 dimensions. These steps were performed by Dr Ran Li in the Ratcliffe lab.

8.16.5 Inferring Cell Types in scRNA-seq

Lists of genes differentially expressed across renal cell types in wild type C57BL/6J mice were obtained from previous work¹⁷⁹⁻¹⁸². For each cell type described in these papers, marker genes were selected as those with log₂-fold change (compared to other cells in the dataset) > 1.0, and which were expressed in >50% of cells they marked, and <20% of the cells they did not mark. Separate markers were listed for the S2 and S3 subtypes of proximal tubular cells from male and female mice, based on reported sex-specific expression of markers¹⁸². The four papers to which we referred did not always divide renal cell types in the same manner. Conflicts were resolved with reference to histological and anatomical literature on the kidney^{235,251}. Marker gene lists were prepared for the following cell types: three segments of the proximal tubule S1, S2, and S3; Loop of Henle epithelium (LoH), Distal Convoluted Tubule (DCT), Principal and Intercalated cells of the Collecting Duct (CDPC and CDIC respectively), Parietal cells (PEC), Podocytes, Endothelial cells, Fibroblasts, Vascular Smooth Muscle Cells (VSMC), Pericytes, Macrophages, Monocytes, Neutrophils, NK cells, Granulocytes, B Lymphocytes, and T Lymphocytes and are provided in **Appendix 10**.

Genes present in this list, but not detected in any cells in our dataset, were excluded from analysis. Marker gene lists were then converted into ‘gene module scores’ using Seurat’s AddModuleScore²⁵² function, with 100 bins and 50 control genes used per bin. Individual cells were assigned the cell type that attained the highest module score. Only one of the four papers to which we referred¹⁸² reported data from mice of both sexes and found sex-specific cell type marker gene expression in PT S2 and S3 cells. Accordingly, sex-specific modules for S2 and S3 PT cells were used in this assessment for male and female cells.

8.16.6 Identifying Anti-Correlated Gene Sets and Annotating PT Classes

The top 1200 varying genes in PT cells from tdTomato-positive and -negative Control samples were identified by calculating the variance in normalised expression for every detected gene across cells using the `var` function in R. Pairwise Spearman correlation coefficients were calculated for each combination of genes, resulting in a 1200 x 1200 correlation matrix. Hierarchical clustering was performed on this matrix using `'hclust'`, and heatmap plotted using the `'heatmap.2'` function in R. Two 'sets' of anti-correlated genes were visually distinguished, and genes contributing to each set were identified from the dendrogram using `'cutree'`.

Each gene set was converted to a module and used to score cells using the `AddModuleScore` function with 100 bins and 50 controls per bin. Class A PT cells were identified as those with Module A expression score > 0.135 , and the others were identified as PT Class B cells.

8.16.7 Differential Expression Testing

Differential expression (DE) testing was performed for various combinations of cells using the `FindMarkers` function of Seurat in R using Wilcoxon test and Bonferroni correction for multiple testing. Normalized, unscaled expression was used for calculating $\log_2$ -fold changes between conditions. Genes were considered regulated if robustly altered ($|\log_2\text{-fold change}| > 0.25$), and statistically significant (adjusted p value < 0.01). DE testing was performed in individual cell identities if > 100 cells of that identity were present in each condition. All types and classes of PT cells, and CDIC cells passed this threshold in tdTomato-positive cells. In addition to these identities, Macrophage, Monocyte, Neutrophil, NK Cell, Granulocyte, B Lymphocyte and T Lymphocyte cell types passed this threshold in tdTomato-negative cells.

8.16.7.1 Genes regulated by *Vhl* at the early timepoint

Differential expression (DE) was first tested in all tdTomato-positive cells from KO mice *versus* Control mice harvested at the early timepoint, and genes that were regulated were identified. The same analysis was then performed comparing tdTomato-negative cells in KO mice *versus* Control mice at the early timepoint, and genes regulated identified. Genes regulated in tdTomato-positive cells but not regulated in the same direction in tdTomato-negative cells were considered to be specifically regulated by *Vhl* inactivation at the early timepoint.

8.16.7.2 Genes regulated with time in *Vhl*-null cells

Differential expression was first tested in tdTomato-positive cells from KO mice at the late *versus* early timepoints, and genes regulated identified. The same analysis was then performed comparing tdTomato-positive cells in Control mice at the late *versus* early timepoints, and genes regulated identified. Genes regulated in cells from KO mice but not regulated in the same direction in cells from Control mice were considered to be regulated over time specifically in *Vhl*-null cells.

8.16.7.3 Genes regulated by concomitant *Vhl* and *Pbrm1* inactivation

Genes regulated when tdTomato-positive cells (either all, or just PT Class A or PT Class B) from *Vhl Pbrm1* KO mice were compared to those from Control mice at the late timepoint were considered to be specifically regulated by concomitant *Vhl* and *Pbrm1* inactivation at the late timepoint. Genes specifically regulated after *Vhl* inactivation but not after concomitant *Vhl Pbrm1* inactivation (and *vice versa*) were identified as those that were significantly regulated in cells of *Vhl* KO mice but not in the same direction in *Vhl Pbrm1* KO mice compared to cells of Control mice (and *vice versa*) and those for which the absolute difference in log₂-fold change between *Vhl* KO and *Vhl Pbrm1* KO when compared to Control was higher than 0.2.

8.16.7.4 Comparing *Vhl*-dependent gene expression across cell identities

DE testing was performed as described above but for subsets of cells of individual cell identities, and a list of upregulated and downregulated genes across all identities prepared. Log₂-fold changes for these genes were calculated for cells of each identity in tdTomato-positive KO samples compared to Control at the early timepoint. Principal component analysis on these changes was performed using the `prcomp` function in R, after scaling and centring data.

8.16.7.5 Sex-specific *Vhl*-regulated genes

DE testing was performed as described above separately on cells from male or female mice. Male-specific genes were identified as those that were significantly regulated in cells from male mice but not from female mice, and for whom $\Delta|\log_2\text{-fold change}| > 0.2$ in males compared to females; and *vice versa*.

8.16.7.6 Cell-type specific *Vhl*-regulated genes

DE testing was performed as described above, but for subsets of cells of individual cell identities. CDIC-specific or PT S3-specific genes were identified as those that were regulated in CDICs or PT S3 respectively but no other cell identity in the same direction, and for which $\Delta|\log_2\text{-fold change}| > 0.2$ in CDICs or PT S3 respectively compared to all other identities. PT Class A specific genes were identified as those that were significantly regulated in PT Class A cells but not PT Class B, and for whom $\Delta|\log_2\text{-fold change}| > 0.2$ in PT Class A than PT Class B.

8.16.7.7 Genes regulated in renal interstitial cells

DE testing was performed on tdTomato-negative KO *versus* Control samples at the late timepoint for cells of the following cell types separately: Macrophage, Monocyte, Neutrophil, NK Cell, Granulocyte, B Lymphocyte and T Lymphocyte. GO term annotation was performed for genes upregulated in each cell type using gProfiler²⁵³ (<https://biit.cs.ut.ee/gprofiler/gost>).

8.16.7.8 Reproducibility between replicates

Log₂-fold changes were calculated between individual tdTomato-positive KO samples *versus* all sex-matched tdTomato-positive Control samples at the early timepoint, for *Vhl*-regulated genes identified as above. Spearman correlation coefficients were calculated using the `cor` function in R for log₂-fold changes for matched genes between individual KO samples and plotted as a heatmap.

8.16.8 Gene Set Enrichment Analysis (GSEA)

GSEA was performed using the `fgsea` package in R, and barcode plots created using the `PlotEnrichment` function in R. Genes specifically expressed in PT like cells compared to PT were ranked according to log₂-fold change. Cell type marker gene lists prepared above were used as gene sets to test enrichment, or otherwise, of particular cell type marker genes.

8.16.9 Definition of HIF target genes

Canonical HIF target genes have been previously defined in six human cancer cell lines based on HIF genome binding (ChIP-seq) and hypoxic regulation (RNA-seq)¹⁴⁹. Briefly, for each cell line, a HIF target gene was defined as being induced by hypoxia and having a HIF-1 (HIF1A and HIF1B) and/or HIF-2 (HIF2A and HIF1B) binding site in proximity to that gene (gene must be in the three closest transcriptional start sites to the binding site). Merging the HIF target genes identified in each cell line provides a list of 1301 known, *bona fide* HIF target genes; encompassing both cell-type-conserved and cell-type-specific targets. Mouse orthologues for these genes were obtained using the `babelgene` package in R. 1226 genes were obtained and are given in **Appendix 11**.

8.16.10 Definition of ccRCC prognosis genes

Pathology data in The Human Protein Atlas (<https://www.proteinatlas.org/>, RRID:SCR_006710) was searched on 27 July 2023 for genes specifically regulated in PT Class A but not PT Class B cells to determine if any were identified as prognostic markers for renal cancer and relevant Kaplan-Meier plots for relevant genes were exported.

8.16.11 Definition of genes upregulated in ccRCC

A recent scRNA-seq study on human ccRCC samples reported genes upregulated in ccRCC tumour cells compared to normal proximal tubular cells¹⁷⁰. Genes that were upregulated in at least 25 of the 30 tumours analysed were considered to be “upregulated in ccRCC” in our analysis. Mouse orthologues for these genes were obtained using the *babelgene* package in R. These were compared to the list of HIF target genes as defined above to identify HIF-driven and non-HIF driven genes upregulated in ccRCC (**Appendix 12**). These were then converted to modules and expression scored using the *AddModuleScore*²⁵² function in R, with 100 bins and 50 control genes per bin.

8.16.12 Identification of PT like cells

Cell clusters were determined after batch correction for sex using Seurat for tdTomato-positive KO and Control samples harvested at the early or late timepoints. Clusters in which >80% cells were of S1, S2, or S3 PT cell type were considered “PT clusters”. Cells assigned to non-PT cell types that were present within PT-clusters were considered to be PT like cells.

8.16.13 Visualising anti-correlated gene expression in public datasets

Single-cell and single-nucleus RNA sequencing data from renal cells derived from healthy mice^{236,237} are available for interrogation at <http://humphreyslab.com/SingleCell/>. Expression of *Neat1* and *Fxyd2* was visualised as t-SNE plots using the online tool on the website and exported as screenshots.

8.14 Statistical Analysis

Differences in retention of tdTomato-positive cells in Control or KO animals over time were analysed by comparing linear fitted models using the *lm* function in R. *t*-testing between intercept and slope of fitted models was used to assess significance of differences in

initial recombination and survival over time respectively. Associated p values were adjusted by Bonferroni correction for multiple testing.

For comparison of means, data was first subjected to D'Agostino-Pearson omnibus normality test. If the data passed the normality test, differences between groups were tested by one-way ANOVA (data grouped by one variable) or two-way ANOVA (data grouped by two variables) with Holm-Šídák multiple testing correction. If normality was not passed, differences were tested by Mann-Whitney test (data grouped by one variable), or Kruskal-Wallis test (data grouped by two variables) with Dunn's multiple testing correction.

For DE testing, p values calculated by Wilcoxon tests by the FindMarkers function were adjusted by Bonferroni correction for multiple testing.

8.15 Data Visualisation

Single-cell sequencing data was visualised either with in-built functions of Seurat or using the ggplot2 and cowplot packages in R. Statistical analyses and relevant plots were also generated in Prism 10 for macOS (GraphPad Software, www.graphpad.com).

Appendices

Appendix 1: PT Modules

List of genes that are part of PT Module A and PT Module B

Genes in PT Module A				
<i>Abcc2</i>	<i>Col4a4</i>	<i>Igfbp4</i>	<i>Prss8</i>	<i>Spag9</i>
<i>Abcg2</i>	<i>Cpeb4</i>	<i>Itgb8</i>	<i>Psap</i>	<i>Spp1</i>
<i>Acsn2</i>	<i>Csnk1a1</i>	<i>Itpr1</i>	<i>Psmc7</i>	<i>Spp2</i>
<i>Actb</i>	<i>Ctsb</i>	<i>Itpr2</i>	<i>Ptpr</i>	<i>Sptbn1</i>
<i>Actg1</i>	<i>Ctsd</i>	<i>Ivns1abp</i>	<i>Rab11fip3</i>	<i>Sqstm1</i>
<i>Akap9</i>	<i>Ctsl</i>	<i>Jun</i>	<i>Rbm25</i>	<i>Srrm2</i>
<i>Alpl</i>	<i>Cubn</i>	<i>Jund</i>	<i>Rbm39</i>	<i>Srsf11</i>
<i>Amn</i>	<i>Cyp2d26</i>	<i>Kcnj15</i>	<i>Rbm47</i>	<i>Srsf2</i>
<i>Ank3</i>	<i>Dcaf8</i>	<i>Kcnj16</i>	<i>Rbpms</i>	<i>Srsf3</i>
<i>Apob</i>	<i>Ddx24</i>	<i>Kcnq1ot1</i>	<i>Retreg1</i>	<i>Srsf5</i>
<i>Apoe</i>	<i>Ddx3x</i>	<i>Kidins220</i>	<i>Rgl1</i>	<i>Syne2</i>
<i>App</i>	<i>Ddx5</i>	<i>Kl</i>	<i>Rrbp1</i>	<i>Tcn2</i>
<i>Arhgap5</i>	<i>Dlst</i>	<i>Klf6</i>	<i>Rsrc2</i>	<i>Tgoln1</i>
<i>Arl6ip1</i>	<i>Dnase1</i>	<i>Klf9</i>	<i>Rsrp1</i>	<i>Timp3</i>
<i>Ash1l</i>	<i>Dusp1</i>	<i>Krt8</i>	<i>Rufy3</i>	<i>Tinag</i>
<i>Aspa</i>	<i>Dync1h1</i>	<i>Lgmn</i>	<i>Samd9l</i>	<i>Tmem174</i>
<i>Atp1a1</i>	<i>Eif3a</i>	<i>Lipa</i>	<i>Scaf11</i>	<i>Tmem252</i>
<i>Atp1b1</i>	<i>Eif4a1</i>	<i>Lrp2</i>	<i>Sdc4</i>	<i>Tns1</i>
<i>Atp2a2</i>	<i>Eif4a2</i>	<i>Luc7l2</i>	<i>Sec62</i>	<i>Top1</i>
<i>Atp6v0a4</i>	<i>Eif5</i>	<i>Malat1</i>	<i>Selenop</i>	<i>Tpr</i>
<i>Atp6v0b</i>	<i>Enpp2</i>	<i>Mettl7a1</i>	<i>Selenot</i>	<i>Tprkb</i>
<i>Atp6v0c</i>	<i>Errfi1</i>	<i>Mia2</i>	<i>Sephs2</i>	<i>Tra2b</i>
<i>Atrx</i>	<i>Fam107b</i>	<i>Myo6</i>	<i>Sf3b1</i>	<i>Trpm7</i>
<i>Atxn7l3b</i>	<i>Fos</i>	<i>Ncoa7</i>	<i>Sfpq</i>	<i>Tsc22d1</i>
<i>B2m</i>	<i>Fosb</i>	<i>Ncor1</i>	<i>Slc13a1</i>	<i>Tubb4b</i>
<i>BC005537</i>	<i>Fus</i>	<i>Ndel1</i>	<i>Slc16a2</i>	<i>Txnip</i>
<i>BC005561</i>	<i>Fut9</i>	<i>Neat1</i>	<i>Slc1a1</i>	<i>U2surp</i>
<i>Birc6</i>	<i>G6pc</i>	<i>Neu1</i>	<i>Slc22a8</i>	<i>Ubap2l</i>
<i>Brd2</i>	<i>Galnt11</i>	<i>Nisch</i>	<i>Slc25a36</i>	<i>Ubc</i>
<i>Ccn1</i>	<i>Gas6</i>	<i>Nox4</i>	<i>Slc2a2</i>	<i>Ube2d3</i>
<i>Cd2ap</i>	<i>Gcc2</i>	<i>Npc2</i>	<i>Slc34a1</i>	<i>Wwc2</i>
<i>Cdh16</i>	<i>Gcnt1</i>	<i>Nus1</i>	<i>Slc3a2</i>	<i>Xist</i>
<i>Ces1d</i>	<i>Gm26917</i>	<i>Osgin1</i>	<i>Slc47a1</i>	<i>Xylb</i>
<i>Cfl1</i>	<i>Golga4</i>	<i>Pabpn1</i>	<i>Slc4a4</i>	<i>Zbtb20</i>
<i>Cgnl1</i>	<i>Gpx3</i>	<i>Pdzk1ip1</i>	<i>Slc5a12</i>	<i>Zfand5</i>
<i>Chd3</i>	<i>Grina</i>	<i>Phldb2</i>	<i>Slc5a2</i>	<i>Zfp36l1</i>
<i>Chd4</i>	<i>H3f3b</i>	<i>Picalm</i>	<i>Slc6a19</i>	<i>Zfp503</i>
<i>Chka</i>	<i>Herpud1</i>	<i>Pim3</i>	<i>Slc6a6</i>	<i>Zfp871</i>
<i>Cited2</i>	<i>Hnrnpa2b1</i>	<i>Plpbp</i>	<i>Slc7a7</i>	<i>Zfp91</i>
<i>Cldn2</i>	<i>Hnrnpc</i>	<i>Pnrc1</i>	<i>Slc7a8</i>	
<i>Clec2h</i>	<i>Hnrnp1</i>	<i>Ppp1r10</i>	<i>Slc7a9</i>	
<i>Clk1</i>	<i>Hnrnpu</i>	<i>Prkaa2</i>	<i>Smpdl3a</i>	
<i>Cltrn</i>	<i>Hsp90aa1</i>	<i>Prpf38b</i>	<i>Snmp70</i>	
<i>Col4a3</i>	<i>Hspa8</i>	<i>Prpf4b</i>	<i>Snx29</i>	
	<i>Igf1r</i>	<i>Prrc2c</i>	<i>Son</i>	

Genes in PT Module B					
<i>Adh5</i>	<i>Cox7b</i>	<i>Micos13</i>	<i>Nme2</i>	<i>Rpl24</i>	<i>Rps24</i>
<i>Akr1a1</i>	<i>Cox7c</i>	<i>Miox</i>	<i>Nop10</i>	<i>Rpl26</i>	<i>Rps25</i>
<i>Akr1c21</i>	<i>Cox8a</i>	<i>Mpc1</i>	<i>Oaz1</i>	<i>Rpl27</i>	<i>Rps26</i>
<i>Akr7a5</i>	<i>Cyb5a</i>	<i>Mpc2</i>	<i>Park7</i>	<i>Rpl27a</i>	<i>Rps27</i>
<i>Aldoa</i>	<i>Cyc1</i>	<i>Mrpl20</i>	<i>Pcbd1</i>	<i>Rpl28</i>	<i>Rps27a</i>
<i>Anapc13</i>	<i>Cycs</i>	<i>Mrpl42</i>	<i>Pdcd5</i>	<i>Rpl29</i>	<i>Rps27l</i>
<i>Arpp19</i>	<i>D8Ert738e</i>	<i>Mrpl51</i>	<i>Pebp1</i>	<i>Rpl3</i>	<i>Rps28</i>
<i>Atox1</i>	<i>Dbi</i>	<i>Mrpl52</i>	<i>Pepd</i>	<i>Rpl30</i>	<i>Rps29</i>
<i>Atp5a1</i>	<i>Dcxr</i>	<i>Mrps14</i>	<i>Pfdn5</i>	<i>Rpl31</i>	<i>Rps3</i>
<i>Atp5b</i>	<i>Ddt</i>	<i>Mrps21</i>	<i>Pgam1</i>	<i>Rpl32</i>	<i>Rps3a1</i>
<i>Atp5c1</i>	<i>Eef1a1</i>	<i>Msrb1</i>	<i>Pgk1</i>	<i>Rpl34</i>	<i>Rps4x</i>
<i>Atp5d</i>	<i>Eef1b2</i>	<i>Myl6</i>	<i>Phyh</i>	<i>Rpl35</i>	<i>Rps5</i>
<i>Atp5e</i>	<i>Eif1</i>	<i>Naca</i>	<i>Pnkd</i>	<i>Rpl35a</i>	<i>Rps6</i>
<i>Atp5f1</i>	<i>Eif3k</i>	<i>Ndufa11</i>	<i>Polr2f</i>	<i>Rpl36</i>	<i>Rps7</i>
<i>Atp5g1</i>	<i>Elob</i>	<i>Ndufa12</i>	<i>Ppia</i>	<i>Rpl36a</i>	<i>Rps8</i>
<i>Atp5g2</i>	<i>Esd</i>	<i>Ndufa13</i>	<i>Prdx1</i>	<i>Rpl36al</i>	<i>Rps9</i>
<i>Atp5g3</i>	<i>Etfa</i>	<i>Ndufa2</i>	<i>Prdx2</i>	<i>Rpl37</i>	<i>Rpsa</i>
<i>Atp5h</i>	<i>Etfb</i>	<i>Ndufa3</i>	<i>Prdx5</i>	<i>Rpl37a</i>	<i>Rtraf</i>
<i>Atp5j</i>	<i>Fau</i>	<i>Ndufa4</i>	<i>Psm7</i>	<i>Rpl38</i>	<i>S100a1</i>
<i>Atp5j2</i>	<i>Fbp1</i>	<i>Ndufa5</i>	<i>Psmb1</i>	<i>Rpl39</i>	<i>Scand1</i>
<i>Atp5k</i>	<i>Fmc1</i>	<i>Ndufa6</i>	<i>Psmb3</i>	<i>Rpl41</i>	<i>Scp2</i>
<i>Atp5l</i>	<i>Fth1</i>	<i>Ndufa7</i>	<i>Psmb4</i>	<i>Rpl5</i>	<i>Sdhb</i>
<i>Atp5md</i>	<i>Ftl1</i>	<i>Ndufa8</i>	<i>Psmb5</i>	<i>Rpl6</i>	<i>Sdhc</i>
<i>Atp5mpl</i>	<i>Fxyd2</i>	<i>Ndufab1</i>	<i>Psmb6</i>	<i>Rpl7</i>	<i>Sec61g</i>
<i>Atp5o</i>	<i>Gabarap</i>	<i>Ndufb1-ps</i>	<i>Qdpr</i>	<i>Rpl7a</i>	<i>Selenbp1</i>
<i>Atp6v1f</i>	<i>Gapdh</i>	<i>Ndufb10</i>	<i>Rack1</i>	<i>Rpl8</i>	<i>Sem1</i>
<i>Atp6v1g1</i>	<i>Gm10076</i>	<i>Ndufb11</i>	<i>Rbx1</i>	<i>Rpl9</i>	<i>Serf2</i>
<i>Atpif1</i>	<i>Gng5</i>	<i>Ndufb2</i>	<i>Rida</i>	<i>Rplp0</i>	<i>Slc25a3</i>
<i>Bola3</i>	<i>Gpx1</i>	<i>Ndufb3</i>	<i>Romo1</i>	<i>Rplp1</i>	<i>Slc25a39</i>
<i>Btf3</i>	<i>Gpx4</i>	<i>Ndufb4</i>	<i>Rpl10</i>	<i>Rplp2</i>	<i>Slc25a5</i>
<i>Calm3</i>	<i>Grcc10</i>	<i>Ndufb5</i>	<i>Rpl10a</i>	<i>Rps10</i>	<i>Smdt1</i>
<i>Calml4</i>	<i>Grhpr</i>	<i>Ndufb6</i>	<i>Rpl11</i>	<i>Rps11</i>	<i>Sod2</i>
<i>Cenpx</i>	<i>H2afj</i>	<i>Ndufb7</i>	<i>Rpl12</i>	<i>Rps12</i>	<i>Spr</i>
<i>Chchd10</i>	<i>H2afz</i>	<i>Ndufc1</i>	<i>Rpl13</i>	<i>Rps13</i>	<i>Stard10</i>
<i>Chchd2</i>	<i>Higd2a</i>	<i>Ndufc2</i>	<i>Rpl13a</i>	<i>Rps14</i>	<i>Swi5</i>
<i>Cisd1</i>	<i>Hint1</i>	<i>Ndufs2</i>	<i>Rpl14</i>	<i>Rps15</i>	<i>Taldo1</i>
<i>Clta</i>	<i>Hsbp1</i>	<i>Ndufs4</i>	<i>Rpl15</i>	<i>Rps15a</i>	<i>Tbca</i>
<i>Cops9</i>	<i>Hspe1</i>	<i>Ndufs5</i>	<i>Rpl17</i>	<i>Rps16</i>	<i>Timm13</i>
<i>Cox17</i>	<i>Lamtor4</i>	<i>Ndufs6</i>	<i>Rpl18</i>	<i>Rps17</i>	<i>Timm8b</i>
<i>Cox4i1</i>	<i>Ldha</i>	<i>Ndufs7</i>	<i>Rpl18a</i>	<i>Rps18</i>	<i>Tle5</i>
<i>Cox5a</i>	<i>Ldhb</i>	<i>Ndufs8</i>	<i>Rpl19</i>	<i>Rps19</i>	<i>Tma7</i>
<i>Cox5b</i>	<i>Mdh1</i>	<i>Ndufv2</i>	<i>Rpl21</i>	<i>Rps2</i>	<i>Tmem256</i>
<i>Cox6b1</i>	<i>Mdh2</i>	<i>Ndufv3</i>	<i>Rpl22</i>	<i>Rps20</i>	<i>Tomm7</i>
<i>Cox6c</i>	<i>Mettl26</i>	<i>Nedd8</i>	<i>Rpl23</i>	<i>Rps21</i>	<i>Tpi1</i>
<i>Cox7a2</i>	<i>Micos10</i>	<i>Nme1</i>	<i>Rpl23a</i>	<i>Rps23</i>	<i>Tpt1</i>
<i>Ttc36</i>	<i>Uba52</i>	<i>Uqcr10</i>	<i>Uqcrrs1</i>	<i>Vamp8</i>	
<i>Txn1</i>	<i>Ubl5</i>	<i>Uqcr11</i>	<i>Uqcrrh</i>	<i>Vps29</i>	
<i>Txndc17</i>	<i>Uqcc2</i>	<i>Uqcrb</i>	<i>Uqcrrq</i>	<i>Ybx1</i>	

Appendix 2: Differentially expressed genes early after *Vhl* inactivation

List of genes differentially expressed in tdTomato-positive or -negative cells in KO *versus* Control mice at the early timepoint. L2FC – log₂-fold change. Detection – Fraction of cells in which the gene is detected.

Gene	L2FC Pos	Detection KO pos	p positive	L2FC Neg	Detection KO neg	p negative
<i>Defb19</i>	0.983	0.478	0.000	0.023	0.022	0.000
<i>Gpi1</i>	0.706	0.777	0.000	0.043	0.381	0.000
<i>Aldoa</i>	0.618	0.924	0.000	0.047	0.611	0.000
<i>Ldha</i>	0.612	0.920	0.000	-0.004	0.548	1.000
<i>Bnip3</i>	0.595	0.741	0.000	0.026	0.307	0.000
<i>Pgam1</i>	0.582	0.839	0.000	0.023	0.468	0.000
<i>Kap</i>	0.576	0.764	0.000	-0.087	0.696	0.000
<i>Acmsd</i>	0.566	0.678	0.000	0.022	0.238	0.000
<i>Ly6a</i>	0.545	0.537	0.000	0.037	0.377	0.000
<i>Pgk1</i>	0.544	0.874	0.000	0.016	0.512	1.000
<i>Eno1</i>	0.532	0.718	0.000	0.078	0.339	0.000
<i>Adh1</i>	0.529	0.553	0.000	0.040	0.254	0.000
<i>Odc1</i>	0.486	0.847	0.000	0.014	0.530	0.071
<i>Ugt2b37</i>	0.484	0.318	0.000	0.038	0.169	0.000
<i>Tpi1</i>	0.477	0.917	0.000	-0.010	0.596	1.000
<i>Ggt1</i>	0.475	0.725	0.000	-0.048	0.425	0.000
<i>Fam162a</i>	0.472	0.575	0.000	0.025	0.225	0.000
<i>Bnip3l</i>	0.461	0.633	0.000	0.025	0.293	0.000
<i>Defb29</i>	0.451	0.656	0.000	-0.029	0.308	1.000
<i>P4ha1</i>	0.441	0.361	0.000	0.031	0.102	0.000
<i>Pfkl</i>	0.438	0.326	0.000	0.014	0.045	0.000
<i>Ankrd37</i>	0.428	0.270	0.000	0.007	0.012	0.000
<i>Napsa</i>	0.404	0.635	0.000	-0.100	0.453	0.000
<i>Plod2</i>	0.403	0.324	0.000	0.020	0.082	0.000
<i>Mif</i>	0.397	0.951	0.000	0.032	0.693	0.000
<i>Cyp4b1</i>	0.364	0.396	0.000	0.094	0.268	0.000
<i>Cyp2e1</i>	0.358	0.418	0.000	-0.001	0.205	1.000
<i>Higd1a</i>	0.353	0.599	0.000	0.046	0.338	0.000
<i>Acy3</i>	0.350	0.745	0.000	-0.053	0.489	0.000
<i>Dhx29</i>	0.349	0.308	0.000	0.011	0.066	0.000
<i>S100a11</i>	0.321	0.458	0.000	0.100	0.338	0.000
<i>Angptl3</i>	0.313	0.219	0.000	0.030	0.048	0.000
<i>Me1</i>	0.305	0.653	0.000	-0.052	0.360	0.000
<i>Mep1a</i>	0.303	0.624	0.000	-0.090	0.391	0.000
<i>Fuca1</i>	0.295	0.589	0.000	0.042	0.377	0.000
<i>Pfkfb</i>	0.294	0.185	0.000	0.036	0.050	0.000
<i>Ldc1</i>	0.284	0.200	0.000	-0.026	0.044	0.000
<i>Ctnnbip1</i>	0.282	0.368	0.000	0.011	0.131	0.000
<i>Prelid2</i>	0.281	0.181	0.000	0.010	0.017	0.000
<i>Ccnd1</i>	0.276	0.476	0.000	0.129	0.298	0.000
<i>Bsg</i>	0.274	0.625	0.000	0.030	0.449	0.000
<i>Gls</i>	0.272	0.410	0.000	0.064	0.282	0.000
<i>Eno1b</i>	0.272	0.232	0.000	-0.015	0.040	1.000
<i>Them7</i>	0.270	0.427	0.000	-0.028	0.189	1.000
<i>Ces1f</i>	0.265	0.313	0.000	-0.025	0.187	1.000
<i>Acox1</i>	0.265	0.662	0.000	-0.013	0.430	1.000
<i>Ugt2b38</i>	0.259	0.466	0.000	0.006	0.286	0.009
<i>Cndp2</i>	0.256	0.534	0.000	-0.137	0.371	0.000
<i>Apoc3</i>	0.253	0.150	0.000	-0.005	0.029	1.000
<i>Nat8</i>	0.252	0.466	0.000	-0.021	0.275	1.000

Gene	L2FC Pos	Detection KO pos	p positive	L2FC Neg	Detection KO neg	p negative
<i>4833439L19R1k</i>	0.251	0.828	0.000	0.045	0.551	0.000
<i>Fos</i>	-0.253	0.137	0.000	-0.024	0.217	1.000
<i>Apob</i>	-0.254	0.122	0.000	0.082	0.233	0.000
<i>Ass1</i>	-0.255	0.879	0.000	-0.033	0.693	0.000
<i>Clec2h</i>	-0.255	0.271	0.000	0.018	0.222	0.000
<i>Fosb</i>	-0.259	0.169	0.000	-0.118	0.195	0.000
<i>Osgin1</i>	-0.261	0.145	0.000	-0.037	0.157	1.000
<i>Slc6a19</i>	-0.262	0.247	0.000	-0.019	0.205	1.000
<i>G6pc</i>	-0.280	0.413	0.000	0.066	0.387	0.000
<i>Slc22a8</i>	-0.281	0.422	0.000	-0.010	0.375	1.000
<i>Spp2</i>	-0.286	0.472	0.000	0.085	0.389	0.000
<i>Jun</i>	-0.286	0.345	0.000	-0.079	0.417	0.000
<i>D630023F18R1k</i>	-0.299	0.140	0.000	0.028	0.150	0.000
<i>Apoe</i>	-0.316	0.311	0.000	0.087	0.467	0.000
<i>Slc13a1</i>	-0.318	0.538	0.000	-0.078	0.441	0.000
<i>Hpd</i>	-0.320	0.109	0.000	-0.083	0.150	0.000
<i>Dnase1</i>	-0.339	0.317	0.000	-0.039	0.321	1.000
<i>Xist</i>	-0.368	0.181	0.000	-0.014	0.335	1.000
<i>Gsta3</i>	-0.485	0.249	0.000	-0.032	0.291	1.000
<i>Gsta4</i>	-0.490	0.232	0.000	-0.066	0.257	0.000
<i>Gsta2</i>	-0.508	0.611	0.000	-0.059	0.579	0.000

Appendix 3: Reproducibility of gene expression changes following *Vhl* inactivation

Differential expression of *Vhl*-regulated genes in tdTomato-positive cells from individual KO mice at the early timepoint.

Gene	L2FC Female 1	L2FC Female 2	L2FC Male 1	L2FC Male 2
<i>Defb19</i>	1.283	1.401	0.501	0.581
<i>Gpi1</i>	0.628	0.871	0.634	0.860
<i>Aldoa</i>	0.607	0.746	0.522	0.672
<i>Bnip3</i>	0.593	0.648	0.583	0.621
<i>Ldha</i>	0.671	0.734	0.381	0.558
<i>Pgam1</i>	0.608	0.738	0.391	0.608
<i>Pgk1</i>	0.541	0.676	0.454	0.614
<i>Eno1</i>	0.668	0.609	0.388	0.499
<i>Acmsd</i>	0.704	0.513	0.433	0.482
<i>Ly6a</i>	0.448	0.707	0.367	0.483
<i>Fam162a</i>	0.463	0.595	0.371	0.574
<i>Bnip3l</i>	0.449	0.611	0.357	0.521
<i>Pfkl</i>	0.450	0.595	0.327	0.557
<i>Ankrd37</i>	0.351	0.610	0.334	0.622
<i>Ggt1</i>	0.409	0.515	0.388	0.553
<i>P4ha1</i>	0.552	0.610	0.272	0.394
<i>Tpi1</i>	0.488	0.592	0.303	0.434
<i>Mif</i>	0.390	0.458	0.447	0.482
<i>Adh1</i>	0.377	0.392	0.460	0.466
<i>Odc1</i>	0.532	0.689	0.156	0.286
<i>Napsa</i>	0.423	0.743	0.156	0.273
<i>Plod2</i>	0.418	0.439	0.346	0.384
<i>Dhx29</i>	0.312	0.365	0.314	0.476
<i>Defb29</i>	0.478	0.467	0.253	0.227
<i>Angptl3</i>	0.508	0.477	0.134	0.157
<i>Higd1a</i>	0.373	0.364	0.222	0.313

Gene	L2FC Female 1	L2FC Female 2	L2FC Male 1	L2FC Male 2
<i>Eno1b</i>	0.103	0.338	0.258	0.564
<i>Bsg</i>	0.324	0.537	0.098	0.275
<i>Pfkfb</i>	0.182	0.476	0.275	0.291
<i>Ldc1</i>	0.138	0.287	0.227	0.563
<i>Fuca1</i>	0.253	0.317	0.265	0.378
<i>Gls</i>	0.271	0.529	0.112	0.283
<i>Cttnbip1</i>	0.197	0.322	0.229	0.441
<i>S100a11</i>	0.244	0.413	0.198	0.303
<i>Prelid2</i>	0.311	0.411	0.205	0.230
<i>Mep1a</i>	0.235	0.365	0.231	0.296
<i>Apoc3</i>	0.349	0.445	0.091	0.148
<i>4833439L19Rik</i>	0.358	0.092	0.322	0.254
<i>Ccnd1</i>	0.128	0.319	0.372	0.205
<i>Me1</i>	0.237	0.249	0.180	0.347
<i>Kap</i>	0.041	0.730	0.103	0.113
<i>Them7</i>	0.196	0.292	0.086	0.357
<i>Acox1</i>	0.003	0.037	0.412	0.444
<i>Ugt2b37</i>	0.022	0.026	0.354	0.480
<i>Cyp2e1</i>	0.063	0.105	0.221	0.419
<i>Nat8</i>	0.149	0.125	0.212	0.305
<i>Acy3</i>	0.200	0.281	0.078	0.183
<i>Cyp4b1</i>	0.078	-0.033	0.189	0.282
<i>Ces1f</i>	0.071	0.087	0.015	0.205
<i>Cndp2</i>	0.132	-0.019	-0.045	0.142
<i>Ugt2b38</i>	-0.049	0.051	-0.096	0.109
<i>Xist</i>	-0.288	0.070	0.000	0.000
<i>Apoe</i>	-0.147	-0.197	-0.006	-0.071
<i>Fosb</i>	-0.286	-0.113	-0.153	0.019
<i>Osgin1</i>	-0.361	-0.209	-0.047	0.057
<i>Hpd</i>	-0.291	-0.318	0.002	0.008
<i>Slc22a8</i>	-0.193	-0.228	-0.128	-0.070
<i>Fos</i>	-0.160	-0.157	-0.237	-0.114
<i>Jun</i>	-0.181	-0.125	-0.319	-0.074
<i>Spp2</i>	-0.209	-0.195	-0.214	-0.202
<i>Apob</i>	-0.251	-0.434	-0.004	-0.134
<i>Slc6a19</i>	-0.436	-0.175	-0.182	-0.039
<i>Gsta4</i>	-0.338	-0.421	-0.045	-0.043
<i>Slc13a1</i>	-0.261	-0.429	-0.132	-0.122
<i>Ass1</i>	-0.224	-0.367	-0.265	-0.113
<i>G6pc</i>	-0.238	-0.474	-0.170	-0.108
<i>D630023F18Rik</i>	-0.246	-0.250	-0.321	-0.189
<i>Clec2h</i>	-0.309	-0.293	-0.254	-0.255
<i>Dnase1</i>	-0.490	-0.405	-0.290	-0.069
<i>Gsta2</i>	-0.199	-0.309	-0.493	-0.321
<i>Gsta3</i>	-0.440	-0.617	-0.270	-0.156

Appendix 4: Cell-type specific early responses to *Vhl* inactivation

Differentially expressed genes early after *Vhl* inactivation in different renal cell identities

Gene	PT all L2FC	PT S1 L2FC	PT S2 L2FC	PT S3 L2FC	PT Class A L2FC	PT Class B L2FC	CDIC L2FC	PT all <i>p</i>	PT S1 <i>p</i>	PT S2 <i>p</i>	PT S3 <i>p</i>	PT Class A <i>p</i>	PT Class B <i>p</i>	CDIC <i>p</i>
<i>Defb19</i>	0.987	0.946	0.944	1.337	0.979	0.990	0.825	0.000	0.000	0.000	0.000	0.000	0.000	0.000
<i>Gpi1</i>	0.708	0.698	0.722	0.785	0.650	0.709	0.704	0.000	0.000	0.000	0.000	0.000	0.000	0.000
<i>Aldoa</i>	0.622	0.603	0.643	0.784	0.595	0.593	0.270	0.000	0.000	0.000	0.000	0.000	0.000	0.000
<i>Ldha</i>	0.612	0.598	0.627	0.692	0.636	0.577	0.850	0.000	0.000	0.000	0.000	0.000	0.000	0.000
<i>Bnip3</i>	0.595	0.601	0.553	0.573	0.537	0.591	0.816	0.000	0.000	0.000	0.000	0.000	0.000	0.000
<i>Pgam1</i>	0.584	0.570	0.595	0.600	0.557	0.565	0.530	0.000	0.000	0.000	0.000	0.000	0.000	0.000
<i>Kap</i>	0.575	0.562	0.783	-0.205	0.650	0.499	0.562	0.000	0.000	0.000	–	0.000	0.000	0.000
<i>Acmsd</i>	0.568	0.566	0.590	0.619	0.572	0.557	0.552	0.000	0.000	0.000	0.000	0.000	0.000	0.000
<i>Ly6a</i>	0.546	0.527	0.604	0.591	0.511	0.541	0.549	0.000	0.000	0.000	0.000	0.000	0.000	0.000
<i>Pgk1</i>	0.545	0.526	0.556	0.771	0.510	0.524	0.473	0.000	0.000	0.000	0.000	0.000	0.000	0.000
<i>Eno1</i>	0.533	0.529	0.499	0.682	0.548	0.503	0.671	0.000	0.000	0.000	0.000	0.000	0.000	0.000
<i>Adh1</i>	0.531	0.473	0.759	0.373	0.405	0.533	0.298	0.000	0.000	0.000	0.000	0.000	0.000	0.089
<i>Odc1</i>	0.486	0.432	0.678	0.554	0.443	0.480	0.633	0.000	0.000	0.000	0.000	0.000	0.000	0.000
<i>Ugt2b37</i>	0.485	0.380	0.889	-0.008	0.511	0.453	0.182	0.000	0.000	0.000	–	0.000	0.000	–
<i>Tpi1</i>	0.477	0.462	0.503	0.610	0.502	0.442	0.489	0.000	0.000	0.000	0.000	0.000	0.000	0.000
<i>Ggt1</i>	0.476	0.513	0.422	0.281	0.504	0.476	0.483	0.000	0.000	0.000	0.000	0.000	0.000	0.000
<i>Fam162a</i>	0.473	0.417	0.577	0.734	0.364	0.487	0.415	0.000	0.000	0.000	0.000	0.000	0.000	0.000
<i>Bnip3l</i>	0.462	0.463	0.431	0.406	0.447	0.460	0.455	0.000	0.000	0.000	0.000	0.000	0.000	0.000
<i>Defb29</i>	0.452	0.440	0.490	0.235	0.420	0.434	0.507	0.000	0.000	0.000	–	0.000	0.000	0.000
<i>P4ha1</i>	0.442	0.424	0.453	0.642	0.685	0.376	0.379	0.000	0.000	0.000	0.000	0.000	0.000	0.000
<i>Pfkl</i>	0.439	0.452	0.370	0.479	0.451	0.433	0.515	0.000	0.000	0.000	0.000	0.000	0.000	0.000
<i>Ankrd37</i>	0.429	0.454	0.359	0.358	0.639	0.360	0.274	0.000	0.000	0.000	0.000	0.000	0.000	0.000
<i>Napsa</i>	0.405	0.502	0.244	-0.057	0.440	0.375	0.389	0.000	0.000	–	–	0.000	0.000	0.319
<i>Plod2</i>	0.405	0.392	0.471	0.391	0.621	0.343	0.165	0.000	0.000	0.000	0.000	0.000	0.000	–
<i>Mif</i>	0.399	0.370	0.423	0.852	0.352	0.379	0.229	0.000	0.000	0.000	0.000	0.000	0.000	–
<i>Cyp4b1</i>	0.365	0.290	0.719	0.037	0.396	0.359	0.171	0.000	0.000	0.000	–	0.000	0.000	–
<i>Cyp2e1</i>	0.359	0.337	0.545	0.133	0.382	0.345	0.204	0.000	0.000	0.000	–	0.000	0.000	–
<i>Higd1a</i>	0.353	0.331	0.426	0.316	0.320	0.334	0.280	0.000	0.000	0.000	0.000	0.000	0.000	0.547

Gene	PT all L2FC	PT S1 L2FC	PT S2 L2FC	PT S3 L2FC	PT Class A L2FC	PT Class B L2FC	CDIC L2FC	PT all <i>p</i>	PT S1 <i>p</i>	PT S2 <i>p</i>	PT S3 <i>p</i>	PT Class A <i>p</i>	PT Class B <i>p</i>	CDIC <i>p</i>
<i>Dhx29</i>	0.351	0.351	0.360	0.310	0.322	0.362	0.147	0.000	0.000	0.000	0.000	0.000	0.000	—
<i>Acy3</i>	0.350	0.337	0.487	-0.112	0.324	0.304	0.356	0.000	0.000	0.000	—	0.000	0.000	0.270
<i>S100a11</i>	0.326	0.309	0.388	0.096	0.252	0.318	-0.111	0.000	0.000	0.000	—	0.000	0.000	—
<i>Angptl3</i>	0.315	0.360	0.130	0.232	0.367	0.306	0.153	0.000	0.000	—	—	0.000	0.000	—
<i>Me1</i>	0.306	0.252	0.438	0.371	0.206	0.301	0.475	0.000	0.000	0.000	0.000	—	0.000	0.000
<i>Mep1a</i>	0.303	0.332	0.291	-0.013	0.322	0.306	0.363	0.000	0.000	0.000	—	0.000	0.000	0.020
<i>Fuca1</i>	0.296	0.281	0.372	0.223	0.311	0.307	0.314	0.000	0.000	0.000	—	0.000	0.000	0.496
<i>Pfkip</i>	0.293	0.280	0.298	0.403	0.329	0.282	0.509	0.000	0.000	0.000	0.000	0.000	0.000	0.000
<i>Ldc1</i>	0.285	0.227	0.454	0.270	0.324	0.270	0.139	0.000	—	0.000	0.000	0.000	0.000	—
<i>Ctnnbip1</i>	0.283	0.276	0.328	0.285	0.264	0.290	0.168	0.000	0.000	0.000	0.000	0.000	0.000	—
<i>Prelid2</i>	0.281	0.263	0.278	0.432	0.256	0.288	0.442	0.000	0.000	0.000	0.000	0.000	0.000	0.000
<i>Ccnd1</i>	0.278	0.292	0.207	0.366	0.278	0.301	0.220	0.000	0.000	—	0.000	0.000	0.000	—
<i>Gls</i>	0.275	0.292	0.243	0.112	0.326	0.280	0.079	0.000	0.000	—	—	0.000	0.000	—
<i>Bsg</i>	0.274	0.249	0.285	0.445	0.294	0.302	0.288	0.000	—	0.000	0.000	0.000	0.000	0.041
<i>Eno1b</i>	0.273	0.265	0.311	0.266	0.208	0.288	0.163	0.000	0.000	0.000	0.000	—	0.000	—
<i>Them7</i>	0.271	0.250	0.349	0.169	0.184	0.269	0.226	0.000	0.000	0.000	—	—	0.000	—
<i>Ces1f</i>	0.266	0.184	0.607	-0.052	0.336	0.241	0.114	0.000	—	0.000	—	0.000	—	—
<i>Acox1</i>	0.265	0.257	0.359	0.231	0.166	0.332	0.331	0.000	0.000	0.000	—	—	0.000	0.052
<i>Ugt2b38</i>	0.260	0.158	0.736	-0.170	0.328	0.245	0.188	0.000	—	0.000	—	0.000	0.000	—
<i>Cndp2</i>	0.256	0.205	0.491	-0.192	0.208	0.213	0.256	0.000	—	0.000	—	—	—	1.000
<i>Apoc3</i>	0.254	0.198	0.301	0.526	0.225	0.261	0.175	—	—	0.000	0.000	—	0.000	—
<i>Nat8</i>	0.253	0.277	0.257	0.171	0.282	0.235	0.258	0.000	0.000	0.000	—	0.000	—	1.000
<i>4833439L19Ri</i>	0.251	0.261	0.214	0.307	0.234	0.225	0.476	0.000	0.000	—	0.000	—	—	0.000
<i>Slco1a1</i>	0.251	0.096	0.711	0.003	0.201	0.247	0.076	0.000	—	0.000	—	—	0.000	—
<i>Cd36</i>	0.249	0.153	0.603	-0.109	0.236	0.241	0.093	0.000	—	0.000	—	0.000	—	—
<i>Bhlhe40</i>	0.240	0.298	0.050	0.229	0.353	0.236	0.287	—	0.000	—	—	0.000	—	0.013
<i>Pdk1</i>	0.237	0.245	0.219	0.205	0.277	0.226	0.162	—	—	—	—	0.000	—	—
<i>Slc22a30</i>	0.237	0.200	0.442	0.039	0.488	0.173	0.082	—	—	0.000	—	0.000	—	—
<i>Prelid1</i>	0.237	0.229	0.257	0.195	0.218	0.211	0.479	—	—	0.000	—	—	—	0.000
<i>Slc17a3</i>	0.235	0.218	0.335	0.023	0.315	0.252	0.114	—	—	0.000	—	0.000	0.000	—
<i>Gas5</i>	0.235	0.248	0.120	0.366	0.191	0.259	0.354	—	—	—	0.000	—	0.000	0.026
<i>Itgav</i>	0.235	0.244	0.225	0.233	0.338	0.231	-0.013	—	—	—	—	0.000	—	—
<i>Hsp90b1</i>	0.234	0.233	0.250	0.127	0.268	0.257	0.259	—	—	0.000	—	0.000	0.000	1.000
<i>Hspa5</i>	0.233	0.243	0.242	0.127	0.255	0.315	0.070	—	—	—	—	0.000	0.000	—
<i>Trappc6a</i>	0.233	0.209	0.276	0.317	0.175	0.235	0.159	—	—	0.000	0.000	—	—	—
<i>Atp11a</i>	0.232	0.185	0.463	-0.129	0.348	0.211	0.076	—	—	0.000	—	0.000	—	—
<i>Gapdh</i>	0.231	0.213	0.259	0.396	0.214	0.200	0.331	—	—	0.000	0.000	—	—	0.000

Gene	PT all L2FC	PT S1 L2FC	PT S2 L2FC	PT S3 L2FC	PT Class A L2FC	PT Class B L2FC	CDIC L2FC	PT all <i>p</i>	PT S1 <i>p</i>	PT S2 <i>p</i>	PT S3 <i>p</i>	PT Class A <i>p</i>	PT Class B <i>p</i>	CDIC <i>p</i>
<i>Atp1a1</i>	0.229	0.216	0.319	0.173	0.242	0.278	0.092	—	—	0.000	—	—	0.000	—
<i>Rpl22l1</i>	0.228	0.220	0.178	0.416	0.191	0.235	0.218	—	—	—	0.000	—	—	—
<i>Nat8f1</i>	0.224	0.263	0.122	0.308	0.228	0.248	0.425	—	0.000	—	0.000	—	—	0.004
<i>Agps</i>	0.224	0.172	0.443	-0.022	0.183	0.230	0.072	—	—	0.000	—	—	—	—
<i>Dynl1</i>	0.224	0.204	0.279	0.129	0.196	0.240	0.082	—	—	0.000	—	—	—	—
<i>Wwp1</i>	0.224	0.220	0.273	0.082	0.219	0.228	0.159	—	—	0.000	—	—	—	—
<i>Ybx1</i>	0.223	0.203	0.323	0.136	0.219	0.178	0.293	—	—	0.000	—	—	—	0.000
<i>Glb1</i>	0.223	0.187	0.329	0.251	0.250	0.220	0.097	—	—	0.000	0.000	0.000	—	—
<i>Rpl37</i>	0.218	0.218	0.173	0.302	0.225	0.174	0.271	—	—	—	0.000	—	—	0.000
<i>Lars2</i>	0.218	0.206	0.328	0.132	0.222	0.219	0.165	—	—	0.000	—	—	—	—
<i>Hmgcs1</i>	0.218	0.208	0.289	0.043	0.199	0.222	0.108	—	—	0.000	—	—	—	—
<i>Ndufv3</i>	0.217	0.198	0.292	0.178	0.194	0.179	0.304	—	—	0.000	—	—	—	0.010
<i>Mpv17l</i>	0.217	0.162	0.373	-0.151	0.153	0.198	0.122	—	—	0.000	—	—	—	—
<i>Smim4</i>	0.215	0.158	0.448	0.175	0.137	0.212	-0.012	—	—	0.000	—	—	—	—
<i>Adm</i>	0.214	0.247	0.118	0.132	0.481	0.133	0.295	—	—	—	—	0.000	—	1.000
<i>Psap</i>	0.210	0.164	0.443	-0.026	0.221	0.243	0.166	—	—	0.000	—	—	0.000	—
<i>Nudt19</i>	0.206	0.166	0.382	-0.195	0.118	0.187	0.056	—	—	0.000	—	—	—	—
<i>Chdh</i>	0.205	0.224	0.161	0.178	0.255	0.212	0.168	—	—	—	—	0.000	—	—
<i>Gm10076</i>	0.203	0.193	0.158	0.333	0.204	0.165	0.259	—	—	—	0.000	—	—	1.000
<i>Egln1</i>	0.201	0.200	0.182	0.262	0.202	0.200	0.288	—	—	—	0.000	—	—	0.033
<i>Samd9l</i>	0.200	0.230	0.100	0.243	0.285	0.215	0.288	—	—	—	—	0.000	—	1.000
<i>Slc7a13</i>	0.199	0.082	0.574	-0.111	0.208	0.178	0.057	—	—	0.000	—	—	—	—
<i>Fabp5</i>	0.198	0.196	0.157	0.172	0.115	0.215	0.306	—	—	—	—	—	—	0.002
<i>Rpl36</i>	0.197	0.196	0.162	0.263	0.176	0.160	0.143	—	—	—	0.000	—	—	—
<i>Pafah1b3</i>	0.194	0.190	0.157	0.295	0.143	0.203	0.141	—	—	—	0.000	—	—	—
<i>Tmem189</i>	0.193	0.186	0.223	0.116	0.255	0.189	0.047	—	—	—	—	0.000	—	—
<i>Uba52</i>	0.191	0.186	0.181	0.283	0.177	0.159	0.223	—	—	—	0.000	—	—	—
<i>Acsn3</i>	0.190	0.080	0.567	-0.170	0.143	0.174	0.022	—	—	0.000	—	—	—	—
<i>Rpl38</i>	0.189	0.189	0.146	0.281	0.199	0.143	0.235	—	—	—	0.000	—	—	—
<i>Rps28</i>	0.185	0.182	0.144	0.341	0.184	0.150	0.340	—	—	—	0.000	—	—	0.000
<i>Dusp6</i>	0.183	0.208	0.096	0.192	0.321	0.166	0.113	—	—	—	—	0.000	—	—
<i>Hsd17b11</i>	0.182	0.140	0.383	-0.039	0.219	0.176	-0.015	—	—	0.000	—	—	—	—
<i>Tmem178</i>	0.182	0.187	0.188	0.131	0.280	0.154	0.158	—	—	—	—	0.000	—	—
<i>Selenbp1</i>	0.181	0.173	0.193	0.316	0.131	0.183	0.430	—	—	—	0.000	—	—	0.000
<i>Rpl39</i>	0.179	0.174	0.141	0.281	0.183	0.138	0.268	—	—	—	0.000	—	—	0.000
<i>Igsf3</i>	0.179	0.205	0.115	0.090	0.426	0.099	0.102	—	—	—	—	0.000	—	—
<i>Csad</i>	0.178	0.155	0.297	-0.116	0.187	0.179	-0.017	—	—	0.000	—	—	—	—

Gene	PT all L2FC	PT S1 L2FC	PT S2 L2FC	PT S3 L2FC	PT Class A L2FC	PT Class B L2FC	CDIC L2FC	PT all <i>p</i>	PT S1 <i>p</i>	PT S2 <i>p</i>	PT S3 <i>p</i>	PT Class A <i>p</i>	PT Class B <i>p</i>	CDIC <i>p</i>
<i>Ldhd</i>	0.178	0.153	0.337	-0.132	0.161	0.166	0.178	—	—	0.000	—	—	—	—
<i>Rps21</i>	0.178	0.169	0.176	0.265	0.182	0.138	0.178	—	—	—	0.000	—	—	—
<i>Msra</i>	0.174	0.191	0.089	0.190	0.141	0.153	0.422	—	—	—	—	—	—	0.008
<i>Rps15a</i>	0.169	0.160	0.149	0.262	0.151	0.136	0.179	—	—	—	0.000	—	—	—
<i>Spink1</i>	0.167	0.154	0.193	0.145	0.152	0.164	0.731	—	—	—	—	—	—	0.000
<i>2410006H16Ri</i>	0.166	0.167	0.130	0.278	0.166	0.187	0.222	—	—	—	0.000	—	—	—
<i>Rps27</i>	0.163	0.157	0.125	0.301	0.154	0.134	0.180	—	—	—	0.000	—	—	—
<i>Rps29</i>	0.162	0.153	0.146	0.278	0.155	0.122	0.219	—	—	—	0.000	—	—	—
<i>Nhp2</i>	0.161	0.128	0.290	0.002	0.107	0.151	0.094	—	—	0.000	—	—	—	—
<i>Rpl37a</i>	0.158	0.155	0.123	0.267	0.149	0.123	0.176	—	—	—	0.000	—	—	—
<i>Fuca2</i>	0.157	0.118	0.315	0.065	0.185	0.180	0.125	—	—	0.000	—	—	—	—
<i>Rpl35</i>	0.154	0.153	0.082	0.298	0.149	0.112	0.226	—	—	—	0.000	—	—	—
<i>Gm44785</i>	0.154	0.133	0.267	0.051	0.087	0.170	0.063	—	—	0.000	—	—	—	—
<i>Ghr</i>	0.154	0.126	0.268	-0.158	0.164	0.166	0.119	—	—	0.000	—	—	—	—
<i>Cyp4a12a</i>	0.153	0.078	0.454	-0.062	0.138	0.148	0.040	—	—	0.000	—	—	—	—
<i>Eef1d</i>	0.151	0.156	0.072	0.278	0.104	0.132	0.096	—	—	—	0.000	—	—	—
<i>Rpl36a</i>	0.150	0.155	0.052	0.339	0.136	0.116	0.259	—	—	—	0.000	—	—	0.446
<i>Vegfa</i>	0.149	0.197	-0.007	0.242	0.379	0.163	0.233	—	—	—	—	0.000	—	—
<i>Car2</i>	0.147	0.143	0.135	0.468	0.146	0.136	-0.189	—	—	—	0.000	—	—	—
<i>Enpp2</i>	0.147	0.133	0.288	0.140	0.184	0.194	0.053	—	—	0.000	—	—	—	—
<i>Selenop</i>	0.146	0.101	0.442	-0.259	0.114	0.187	0.151	—	—	0.000	0.000	—	—	—
<i>Cyp51</i>	0.143	0.109	0.277	-0.264	0.170	0.130	0.007	—	—	0.000	0.000	—	—	—
<i>Acat1</i>	0.142	0.110	0.266	0.097	0.088	0.111	0.048	—	—	0.000	—	—	—	—
<i>Slc22a28</i>	0.140	0.130	0.233	0.040	0.330	0.104	0.078	—	—	—	—	0.000	—	—
<i>Tfr</i>	0.138	0.113	0.261	0.039	0.254	0.113	0.135	—	—	0.000	—	—	—	—
<i>Cyp2j13</i>	0.138	0.118	0.270	0.003	0.232	0.108	-0.004	—	—	0.000	—	—	—	—
<i>Ddah1</i>	0.138	0.158	0.075	0.139	0.092	0.133	0.504	—	—	—	—	—	—	0.000
<i>Nat8f5</i>	0.138	0.114	0.268	0.006	0.157	0.128	0.053	—	—	0.000	—	—	—	—
<i>Cdkn1a</i>	0.137	0.128	0.159	0.159	0.161	0.133	0.394	—	—	—	—	—	—	0.000
<i>Rpl12</i>	0.135	0.147	0.023	0.283	0.121	0.103	0.224	—	—	—	0.000	—	—	—
<i>Hsd11b1</i>	0.134	0.068	0.360	0.123	0.089	0.144	0.050	—	—	0.000	—	—	—	—
<i>Slc4a4</i>	0.133	0.142	0.134	0.442	0.138	0.218	0.477	—	—	—	0.000	—	—	0.000
<i>Ddit4</i>	0.133	0.144	0.116	0.066	0.322	0.079	0.100	—	—	—	—	0.000	—	—
<i>Igfbp5</i>	0.133	0.121	0.165	0.294	0.290	0.130	-0.209	—	—	—	0.000	0.000	—	—
<i>Igfbp1</i>	0.129	0.160	-0.019	0.273	0.217	0.132	0.149	—	—	—	0.000	—	—	—
<i>Rpsa</i>	0.125	0.128	0.064	0.251	0.102	0.093	0.145	—	—	—	0.000	—	—	—
<i>Ly6e</i>	0.124	0.062	0.359	0.030	0.096	0.125	0.203	—	—	0.000	—	—	—	—

Gene	PT all L2FC	PT S1 L2FC	PT S2 L2FC	PT S3 L2FC	PT Class A L2FC	PT Class B L2FC	CDIC L2FC	PT all <i>p</i>	PT S1 <i>p</i>	PT S2 <i>p</i>	PT S3 <i>p</i>	PT Class A <i>p</i>	PT Class B <i>p</i>	CDIC <i>p</i>
<i>Eef1b2</i>	0.123	0.125	0.034	0.297	0.113	0.096	0.172	—	—	—	0.000	—	—	—
<i>Fgl1</i>	0.121	0.096	0.144	0.300	0.141	0.115	0.080	—	—	—	0.000	—	—	—
<i>Slc7a12</i>	0.120	0.065	0.257	-0.201	0.088	0.117	-0.007	—	—	0.000	—	—	—	—
<i>Rpl3</i>	0.118	0.126	0.007	0.289	0.096	0.087	0.101	—	—	—	0.000	—	—	—
<i>Prdx4</i>	0.110	0.105	0.070	0.330	0.128	0.112	0.171	—	—	—	0.000	—	—	—
<i>Npm1</i>	0.104	0.108	0.012	0.329	0.068	0.092	0.103	—	—	—	0.000	—	—	—
<i>Rps5</i>	0.101	0.107	0.015	0.252	0.076	0.071	0.200	—	—	—	0.000	—	—	—
<i>Slco1a6</i>	0.097	0.080	0.253	-0.393	0.134	0.088	0.034	—	—	0.000	0.000	—	—	—
<i>Atp6v1a</i>	0.096	0.084	0.178	-0.010	0.057	0.117	-0.255	—	—	—	—	—	—	0.006
<i>Pepd</i>	0.095	0.084	0.096	0.259	0.074	0.073	0.181	—	—	—	0.000	—	—	—
<i>Aadat</i>	0.095	0.107	0.047	-0.307	0.051	0.074	0.042	—	—	—	0.000	—	—	—
<i>Rps19</i>	0.090	0.101	0.009	0.159	0.074	0.058	0.300	—	—	—	—	—	—	0.000
<i>Akr1b3</i>	0.089	0.043	0.274	0.016	0.041	0.101	0.003	—	—	0.000	—	—	—	—
<i>Ces1d</i>	0.084	0.134	-0.106	0.405	0.138	0.106	0.265	—	—	—	0.000	—	—	1.000
<i>Ero1l</i>	0.083	0.083	0.081	0.116	0.161	0.075	0.298	—	—	—	—	—	—	0.000
<i>Ifitm2</i>	0.079	0.087	-0.014	0.300	0.054	0.086	0.143	—	—	—	0.000	—	—	—
<i>Lpl</i>	0.078	0.010	0.312	-0.185	0.021	0.104	0.077	—	—	0.000	—	—	—	—
<i>Lrp2</i>	0.072	0.084	0.060	0.052	0.081	0.119	0.478	—	—	—	—	—	—	0.001
<i>Soat1</i>	0.071	0.075	0.058	0.059	0.138	0.055	0.367	—	—	—	—	—	—	0.000
<i>Mep1b</i>	0.067	0.019	0.246	-0.293	0.056	0.059	0.024	—	—	—	0.000	—	—	—
<i>Fbp1</i>	0.060	0.056	0.079	0.053	0.036	0.035	0.573	—	—	—	—	—	—	0.000
<i>Gm26917</i>	0.054	0.095	-0.062	0.010	0.316	0.082	0.130	—	—	—	—	0.000	—	—
<i>Cyp2a4</i>	0.051	0.020	0.181	-0.280	0.047	0.041	0.020	—	—	—	0.000	—	—	—
<i>4931406C07Ri</i>	0.047	0.062	0.028	-0.041	0.044	0.039	0.381	—	—	—	—	—	—	0.008
<i>Pkm</i>	0.039	0.019	0.023	0.265	0.024	0.043	0.335	—	—	—	0.000	—	—	0.022
<i>Hspd1</i>	0.032	0.038	0.001	0.067	0.038	0.010	0.339	—	—	—	—	—	—	0.000
<i>Actb</i>	0.026	0.051	-0.071	0.086	-0.076	0.113	0.253	—	—	—	—	—	—	0.000
<i>Ugt3a1</i>	0.024	0.013	0.105	-0.275	0.003	0.036	0.025	—	—	—	0.000	—	—	—
<i>Ociad2</i>	0.017	0.011	0.049	-0.002	0.007	0.020	-0.430	—	—	—	—	—	—	0.000
<i>Kng2</i>	0.013	0.022	0.004	-0.305	0.029	0.006	-0.210	—	—	—	0.000	—	—	—
<i>Atp6v1e1</i>	0.012	-0.001	0.068	0.115	0.003	-0.012	-0.271	—	—	—	—	—	—	0.000
<i>Slc25a4</i>	0.011	0.007	-0.027	0.287	0.021	-0.005	-0.142	—	—	—	0.000	—	—	—
<i>Ckap4</i>	0.003	0.002	0.003	0.008	0.005	0.002	0.337	—	—	—	—	—	—	0.000
<i>Wnk1</i>	0.002	0.023	-0.060	0.006	0.044	0.039	-0.394	—	—	—	—	—	—	0.002
<i>Serpina1f</i>	0.002	0.002	-0.011	-0.345	0.011	-0.006	-0.027	—	—	—	0.000	—	—	—
<i>Emb</i>	0.001	0.001	0.004	0.004	0.002	0.001	-0.411	—	—	—	—	—	—	0.008
<i>Tmem213</i>	0.001	0.001	0.002	0.000	0.000	0.001	-0.458	—	—	—	—	—	—	0.000

Gene	PT all L2FC	PT S1 L2FC	PT S2 L2FC	PT S3 L2FC	PT Class A L2FC	PT Class B L2FC	CDIC L2FC	PT all <i>p</i>	PT S1 <i>p</i>	PT S2 <i>p</i>	PT S3 <i>p</i>	PT Class A <i>p</i>	PT Class B <i>p</i>	CDIC <i>p</i>
<i>Glyat</i>	0.000	-0.003	0.069	0.114	-0.021	-0.003	0.422	—	—	—	—	—	—	0.001
<i>Ptges</i>	0.000	0.000	0.001	0.000	0.000	0.000	0.361	—	—	—	—	—	—	0.000
<i>Spink8</i>	0.000	0.000	0.001	0.001	0.000	0.000	-0.453	—	—	—	—	—	—	0.000
<i>Atp6v1c2</i>	0.000	0.000	-0.001	0.000	0.000	0.000	-0.495	—	—	—	—	—	—	0.000
<i>Atp6v1g3</i>	0.000	0.000	-0.001	0.001	0.000	-0.001	-0.411	—	—	—	—	—	—	0.000
<i>Ehd3</i>	0.000	0.000	0.000	-0.002	0.001	-0.001	0.392	—	—	—	—	—	—	0.000
<i>Atp6v0d2</i>	-0.001	-0.001	-0.002	0.001	0.000	-0.001	-0.309	—	—	—	—	—	—	0.001
<i>Car15</i>	-0.001	-0.001	0.000	0.001	0.000	-0.001	-0.742	—	—	—	—	—	—	0.000
<i>Kcnk1</i>	-0.002	0.001	-0.004	-0.417	0.004	-0.010	-0.011	—	—	—	0.000	—	—	—
<i>Ces2c</i>	-0.002	-0.021	0.143	-0.385	0.010	-0.019	0.055	—	—	—	0.000	—	—	—
<i>Scd1</i>	-0.006	0.010	-0.027	-0.414	0.005	-0.012	-0.020	—	—	—	0.000	—	—	—
<i>Zbtb38</i>	-0.008	-0.004	-0.073	0.252	0.013	0.030	0.135	—	—	—	0.000	—	—	—
<i>Slc22a7</i>	-0.011	0.000	0.003	-0.510	0.003	-0.022	-0.034	—	—	—	0.000	—	—	—
<i>Slc22a13</i>	-0.013	0.000	-0.053	-0.279	-0.010	-0.013	0.012	—	—	—	0.000	—	—	—
<i>Ndrp1</i>	-0.024	-0.009	-0.031	0.101	-0.037	0.003	0.421	—	—	—	—	—	—	0.004
<i>Ttr</i>	-0.031	-0.057	0.150	-0.293	0.039	-0.043	0.021	—	—	—	0.000	—	—	—
<i>Cltn</i>	-0.047	-0.059	-0.001	0.084	-0.013	-0.029	0.492	—	—	—	—	—	—	0.000
<i>Miox</i>	-0.050	-0.040	-0.103	0.206	-0.069	-0.072	0.486	—	—	—	—	—	—	0.000
<i>Cbr1</i>	-0.051	0.012	-0.417	0.074	-0.096	-0.061	0.185	—	—	0.000	—	—	—	—
<i>Errfi1</i>	-0.059	-0.001	-0.250	0.118	-0.023	0.092	0.303	—	—	0.000	—	—	—	0.289
<i>Bhmt</i>	-0.061	0.013	-0.314	0.076	-0.080	-0.058	0.028	—	—	0.000	—	—	—	—
<i>Abcg2</i>	-0.061	-0.050	-0.071	-0.020	-0.011	-0.022	0.437	—	—	—	—	—	—	0.000
<i>Ppp1r10</i>	-0.062	0.005	-0.271	-0.010	0.030	0.050	-0.011	—	—	0.000	—	—	—	—
<i>Timp3</i>	-0.070	-0.059	-0.069	0.316	-0.055	0.086	0.329	—	—	—	0.000	—	—	1.000
<i>Hao2</i>	-0.072	0.011	-0.435	0.059	-0.079	-0.099	0.364	—	—	0.000	—	—	—	0.528
<i>Eci3</i>	-0.075	-0.034	-0.152	-0.266	-0.091	-0.088	0.100	—	—	—	0.000	—	—	—
<i>Papss2</i>	-0.078	-0.009	-0.266	0.007	-0.120	-0.060	0.124	—	—	0.000	—	—	—	—
<i>Rdh16f2</i>	-0.079	-0.010	-0.351	0.141	-0.144	-0.040	0.358	—	—	0.000	—	—	—	0.451
<i>Aldob</i>	-0.081	-0.076	-0.098	-0.040	-0.121	-0.090	0.451	—	—	—	—	—	—	0.003
<i>Gatm</i>	-0.081	-0.078	-0.055	0.309	-0.046	-0.089	0.432	—	—	—	0.000	—	—	0.001
<i>Aldh11l1</i>	-0.086	-0.052	-0.179	-0.124	-0.110	-0.085	-0.373	—	—	—	—	—	—	0.000
<i>mt-Nd3</i>	-0.090	-0.092	-0.019	-0.158	-0.065	-0.081	-0.257	—	—	—	—	—	—	0.000
<i>Glul</i>	-0.098	-0.042	-0.307	0.013	-0.114	-0.043	0.080	—	—	0.000	—	—	—	—
<i>Pdxk</i>	-0.099	-0.050	-0.278	0.003	-0.121	-0.072	-0.099	—	—	0.000	—	—	—	—
<i>Fam107b</i>	-0.099	-0.055	-0.252	0.032	-0.132	0.016	-0.076	—	—	0.000	—	—	—	—
<i>Cela1</i>	-0.102	-0.053	-0.342	0.275	-0.080	-0.138	0.336	—	—	0.000	0.000	—	—	0.126
<i>Ddx5</i>	-0.103	-0.055	-0.280	0.004	-0.036	-0.014	-0.014	—	—	0.000	—	—	—	—

Gene	PT all L2FC	PT S1 L2FC	PT S2 L2FC	PT S3 L2FC	PT Class A L2FC	PT Class B L2FC	CDIC L2FC	PT all <i>p</i>	PT S1 <i>p</i>	PT S2 <i>p</i>	PT S3 <i>p</i>	PT Class A <i>p</i>	PT Class B <i>p</i>	CDIC <i>p</i>
<i>Pdk4</i>	-0.104	-0.022	-0.350	-0.062	-0.112	-0.009	-0.018	—	—	0.000	—	—	—	—
<i>Hacl1</i>	-0.110	-0.070	-0.305	0.064	-0.119	-0.114	0.109	—	—	0.000	—	—	—	—
<i>Cpeb4</i>	-0.113	-0.066	-0.263	0.002	-0.063	0.013	0.042	—	—	0.000	—	—	—	—
<i>Cyp2d26</i>	-0.114	-0.079	-0.268	0.118	-0.054	-0.061	0.090	—	—	0.000	—	—	—	—
<i>Gclc</i>	-0.115	-0.086	-0.187	-0.273	-0.117	-0.086	-0.202	—	—	—	0.000	—	—	—
<i>Kcnq1ot1</i>	-0.116	-0.071	-0.263	-0.033	0.018	0.016	0.016	—	—	0.000	—	—	—	—
<i>Clk1</i>	-0.119	-0.064	-0.299	-0.010	-0.038	0.035	-0.019	—	—	0.000	—	—	—	—
<i>Srsf5</i>	-0.121	-0.084	-0.271	-0.017	-0.032	-0.063	-0.028	—	—	0.000	—	—	—	—
<i>Gm10639</i>	-0.124	-0.088	-0.274	-0.064	-0.106	-0.139	-0.093	—	—	0.000	—	—	—	—
<i>Txnip</i>	-0.127	-0.013	-0.453	-0.061	-0.114	0.007	-0.319	—	—	0.000	—	—	—	0.230
<i>Prodh2</i>	-0.131	-0.102	-0.256	0.072	-0.112	-0.074	0.021	—	—	0.000	—	—	—	—
<i>Akr1c19</i>	-0.134	-0.104	-0.259	-0.041	-0.149	-0.128	-0.172	—	—	0.000	—	—	—	—
<i>Dpys</i>	-0.145	-0.099	-0.302	0.024	-0.164	-0.140	0.011	—	—	0.000	—	—	—	—
<i>Tln2</i>	-0.147	-0.120	-0.251	0.032	-0.194	-0.072	0.143	—	—	0.000	—	—	—	—
<i>Aldh2</i>	-0.152	-0.124	-0.285	-0.004	-0.201	-0.164	0.177	—	—	0.000	—	—	—	—
<i>Cited2</i>	-0.153	-0.085	-0.411	-0.004	-0.139	0.001	0.073	—	—	0.000	—	—	—	—
<i>ldh1</i>	-0.154	-0.112	-0.305	0.111	-0.121	-0.176	0.239	—	—	0.000	—	—	—	—
<i>Oat</i>	-0.155	-0.108	-0.421	0.066	-0.153	-0.161	0.077	—	—	0.000	—	—	—	—
<i>S100g</i>	-0.162	-0.099	-0.394	0.053	-0.154	-0.170	-0.116	—	—	0.000	—	—	—	—
<i>Cyp4a10</i>	-0.163	-0.060	-0.396	-0.052	-0.260	-0.092	0.046	—	—	0.000	—	0.000	—	—
<i>Slc13a3</i>	-0.168	-0.096	-0.311	-0.046	-0.216	-0.094	0.035	—	—	0.000	—	—	—	—
<i>Brd2</i>	-0.170	-0.115	-0.344	-0.062	-0.193	0.028	0.043	—	—	0.000	—	—	—	—
<i>Pah</i>	-0.171	-0.135	-0.285	0.159	-0.177	-0.169	-0.092	—	—	0.000	—	—	—	—
<i>Itpr2</i>	-0.174	-0.145	-0.288	0.034	-0.155	-0.065	-0.191	—	—	0.000	—	—	—	—
<i>Nox4</i>	-0.175	-0.141	-0.294	0.164	-0.171	-0.099	0.178	—	—	0.000	—	—	—	—
<i>Mfsd4b5</i>	-0.180	-0.140	-0.270	-0.120	-0.193	-0.088	0.068	—	—	0.000	—	—	—	—
<i>Snhg11</i>	-0.187	-0.228	-0.026	-0.139	-0.282	-0.031	0.001	—	—	—	—	0.000	—	—
<i>Gabarapl1</i>	-0.191	-0.148	-0.322	-0.086	-0.188	-0.158	-0.323	—	—	0.000	—	—	—	0.007
<i>Fmo1</i>	-0.197	-0.155	-0.355	-0.071	-0.197	-0.194	0.288	—	—	0.000	—	—	—	1.000
<i>Prlr</i>	-0.198	-0.040	-0.579	-0.200	-0.222	-0.117	-0.010	—	—	0.000	—	—	—	—
<i>Jund</i>	-0.198	-0.129	-0.432	-0.090	-0.129	0.015	0.052	—	—	0.000	—	—	—	—
<i>Klf6</i>	-0.198	-0.124	-0.453	-0.006	-0.149	0.002	0.075	—	—	0.000	—	—	—	—
<i>Acaa2</i>	-0.199	-0.174	-0.327	0.134	-0.196	-0.219	-0.024	—	—	0.000	—	—	—	—
<i>Spp1</i>	-0.209	-0.156	-0.529	0.331	-0.142	-0.170	-0.109	—	—	0.000	0.000	—	—	—
<i>Acaa1b</i>	-0.210	-0.135	-0.443	-0.002	-0.234	-0.222	0.064	—	—	0.000	—	—	—	—
<i>Gstm1</i>	-0.224	-0.234	-0.217	-0.235	-0.330	-0.233	-0.173	—	—	—	—	0.000	—	—
<i>Abcc4</i>	-0.226	-0.187	-0.373	-0.027	-0.338	-0.136	0.026	—	—	0.000	—	0.000	—	—

Gene	PT all L2FC	PT S1 L2FC	PT S2 L2FC	PT S3 L2FC	PT Class A L2FC	PT Class B L2FC	CDIC L2FC	PT all <i>p</i>	PT S1 <i>p</i>	PT S2 <i>p</i>	PT S3 <i>p</i>	PT Class A <i>p</i>	PT Class B <i>p</i>	CDIC <i>p</i>
<i>Acsf1</i>	-0.227	-0.168	-0.431	-0.075	-0.195	-0.176	0.028	—	—	0.000	—	—	—	—
<i>Ccn1</i>	-0.233	-0.159	-0.476	-0.062	-0.207	-0.012	0.054	—	—	0.000	—	—	—	—
<i>Dusp1</i>	-0.242	-0.195	-0.414	-0.032	-0.193	0.001	0.095	—	—	0.000	—	—	—	—
<i>Cyp4a14</i>	-0.245	-0.080	-0.641	0.062	-0.355	-0.152	-0.027	—	—	0.000	—	0.000	—	—
<i>Mgst1</i>	-0.246	-0.176	-0.480	-0.021	-0.264	-0.215	-0.112	—	—	0.000	—	0.000	—	—
<i>Neat1</i>	-0.252	-0.183	-0.484	-0.098	-0.092	-0.022	0.081	—	—	0.000	—	—	—	—
<i>Fos</i>	-0.255	-0.218	-0.410	-0.050	-0.268	-0.021	0.054	0.000	—	0.000	—	0.000	—	—
<i>Apob</i>	-0.255	-0.265	-0.207	-0.354	-0.256	-0.223	-0.033	0.000	0.000	—	0.000	0.000	—	—
<i>Clec2h</i>	-0.256	-0.294	-0.056	-0.030	-0.267	-0.191	0.046	0.000	0.000	—	—	0.000	—	—
<i>Ass1</i>	-0.256	-0.228	-0.331	-0.144	-0.255	-0.283	0.251	0.000	—	0.000	—	0.000	0.000	1.000
<i>Fosb</i>	-0.260	-0.197	-0.478	-0.050	-0.232	-0.011	-0.109	0.000	—	0.000	—	—	—	—
<i>Osgin1</i>	-0.262	-0.192	-0.476	-0.120	-0.410	-0.060	0.000	0.000	—	0.000	—	0.000	—	—
<i>Slc6a19</i>	-0.263	-0.283	-0.156	-0.022	-0.274	-0.134	-0.009	0.000	0.000	—	—	0.000	—	—
<i>G6pc</i>	-0.282	-0.272	-0.268	-0.084	-0.282	-0.155	0.047	0.000	0.000	0.000	—	0.000	—	—
<i>Slc22a8</i>	-0.282	-0.196	-0.521	0.149	-0.282	-0.219	0.265	0.000	—	0.000	—	0.000	—	0.655
<i>Spp2</i>	-0.287	-0.297	-0.230	0.111	-0.226	-0.243	0.147	0.000	0.000	—	—	—	—	—
<i>Jun</i>	-0.288	-0.192	-0.611	-0.117	-0.168	0.006	0.085	0.000	—	0.000	—	—	—	—
<i>D630023F18Rik</i>	-0.301	-0.357	-0.063	-0.038	-0.274	-0.286	0.014	0.000	0.000	—	—	0.000	0.000	—
<i>Slc13a1</i>	-0.319	-0.287	-0.399	-0.009	-0.265	-0.248	0.103	0.000	0.000	0.000	—	0.000	—	—
<i>Apoe</i>	-0.319	-0.245	-0.652	0.223	-0.324	-0.270	0.114	0.000	—	0.000	—	0.000	0.000	—
<i>Hpd</i>	-0.322	-0.149	-0.757	-0.057	-0.358	-0.315	-0.072	0.000	—	0.000	—	0.000	0.000	—
<i>Dnase1</i>	-0.341	-0.368	-0.207	-0.116	-0.407	-0.264	-0.043	0.000	0.000	—	—	0.000	—	—
<i>Xist</i>	-0.370	-0.277	-0.734	-0.008	-0.302	-0.021	-0.032	0.000	0.000	0.000	—	0.000	—	—
<i>Gsta3</i>	-0.486	-0.458	-0.626	-0.082	-0.480	-0.512	-0.227	0.000	0.000	0.000	—	0.000	0.000	—
<i>Gsta4</i>	-0.492	-0.427	-0.812	-0.053	-0.466	-0.519	-0.122	0.000	0.000	0.000	—	0.000	0.000	—
<i>Gsta2</i>	-0.509	-0.438	-0.891	-0.151	-0.475	-0.540	0.055	0.000	0.000	0.000	—	0.000	0.000	—

Appendix 5: Time-dependent gene regulation in *Vhl*-null cells

Differentially expressed genes in tdTomato-positive cells from KO or Control mice at the late *versus* early timepoints.

Gene	L2FC KO	Detection KO	KO <i>p</i>	L2FC Control	Detection Control	Control <i>p</i>
<i>Pfkfb</i>	0.801	0.666	0.000	0.006	0.013	—
<i>Apoc3</i>	0.719	0.453	0.000	0.029	0.040	—
<i>Hsd11b1</i>	0.639	0.393	0.000	-0.005	0.016	—
<i>Tm6gd1</i>	0.497	0.520	0.000	0.194	0.275	—
<i>Ly6e</i>	0.442	0.321	0.000	0.122	0.101	—
<i>Napsa</i>	0.422	0.846	0.000	0.103	0.434	—
<i>Kap</i>	0.420	0.938	0.000	-0.058	0.432	—
<i>Cd36</i>	0.413	0.417	0.000	0.027	0.075	—
<i>Defb19</i>	0.408	0.714	0.000	-0.010	0.017	—
<i>Slc7a13</i>	0.379	0.335	0.000	0.150	0.121	—
<i>Pkm</i>	0.373	0.245	0.000	0.015	0.016	—
<i>Slco1a1</i>	0.371	0.324	0.000	-0.031	0.046	—
<i>Cyp4b1</i>	0.347	0.521	0.000	0.405	0.393	0.000
<i>Angptl3</i>	0.342	0.364	0.000	0.004	0.053	—
<i>Anxa5</i>	0.327	0.358	0.000	0.005	0.106	—
<i>Efh1</i>	0.325	0.298	0.000	0.028	0.035	—
<i>Ly6a</i>	0.313	0.711	0.000	-0.007	0.242	—
<i>Pigr</i>	0.304	0.205	0.000	0.090	0.073	—
<i>Ldc1</i>	0.303	0.392	0.000	0.000	0.033	—
<i>AY036118</i>	0.300	0.936	0.000	0.561	0.933	0.000
<i>Dnase1</i>	0.297	0.436	0.000	0.052	0.457	—
<i>Cyp2a4</i>	0.283	0.245	0.000	0.165	0.131	—
<i>Fabp5</i>	0.282	0.320	0.000	-0.024	0.039	—
<i>Wfdc2</i>	0.265	0.289	0.000	-0.036	0.120	—
<i>Azgp1</i>	0.261	0.189	0.000	0.130	0.090	—
<i>Csad</i>	0.255	0.460	0.000	0.047	0.199	—
<i>Lars2</i>	0.214	0.846	—	0.542	0.883	0.000
<i>mt-Nd6</i>	0.214	0.437	—	0.425	0.513	0.000
<i>Gm42418</i>	0.119	1.000	—	0.348	1.000	0.000
<i>Cyp2d12</i>	0.096	0.084	—	0.386	0.217	0.000
<i>Rarres2</i>	0.031	0.063	—	0.343	0.240	0.000
<i>mt-Atp8</i>	-0.034	0.542	—	0.404	0.708	0.000
<i>Apob</i>	-0.085	0.084	—	0.399	0.431	0.000
<i>Keg1</i>	-0.139	0.731	—	-0.277	0.687	0.000
<i>Ndufc2</i>	-0.148	0.702	—	-0.275	0.596	0.000
<i>Lyplal1</i>	-0.246	0.300	—	-0.301	0.249	0.000
<i>Gsta2</i>	-0.251	0.467	0.000	-0.005	0.886	—
<i>Acs1</i>	-0.252	0.282	0.000	-0.081	0.457	—
<i>Idh1</i>	-0.254	0.566	0.000	-0.053	0.727	—
<i>Xist</i>	-0.256	0.101	0.000	-0.139	0.269	—
<i>Mdk</i>	-0.257	0.108	0.000	-0.049	0.183	—
<i>Gpx3</i>	-0.264	0.948	0.000	0.014	0.990	—
<i>Ass1</i>	-0.270	0.794	0.000	-0.086	0.916	—
<i>Cryl1</i>	-0.272	0.492	0.000	-0.194	0.533	—
<i>Slc5a2</i>	-0.274	0.212	0.000	-0.110	0.334	—
<i>Rsrp1</i>	-0.275	0.380	0.000	-0.112	0.481	—
<i>Clec2h</i>	-0.321	0.087	0.000	-0.156	0.292	—
<i>Slc5a12</i>	-0.324	0.161	0.000	-0.110	0.323	—
<i>Spp2</i>	-0.334	0.296	0.000	-0.064	0.550	—
<i>Ftl1-ps1</i>	-0.341	0.284	0.000	-0.197	0.332	—
<i>Spp1</i>	-0.374	0.413	0.000	-0.134	0.646	—
<i>Gatm</i>	-0.380	0.645	0.000	-0.150	0.783	—
<i>Oat</i>	-0.467	0.166	0.000	-0.262	0.354	0.000

Appendix 6: Reproducibility of time-dependent gene expression changes in *Vhl*-null cells

Differential expression of time-regulated genes in tdTomato-positive cells of individual KO mice over time

Gene	L2FC Female 6	L2FC Female 7	L2FC Male 4	L2FC Male 5
<i>Pfkfb</i>	0.843	0.832	0.838	0.701
<i>Apoc3</i>	0.796	1.210	0.421	0.449
<i>Hsd11b1</i>	0.134	0.098	0.815	0.998
<i>Tm6gd1</i>	0.192	0.339	0.661	0.706
<i>Defb19</i>	0.237	0.398	0.555	0.694
<i>Kap</i>	0.710	0.673	0.211	0.247
<i>Napsa</i>	0.289	0.437	0.515	0.508
<i>Pkm</i>	0.397	0.251	0.572	0.224
<i>Cd36</i>	0.240	0.225	0.395	0.522
<i>Angptl3</i>	0.507	0.503	0.216	0.152
<i>Efh1</i>	0.412	0.443	0.236	0.249
<i>Ly6e</i>	0.045	0.024	0.769	0.498
<i>Ly6a</i>	0.186	0.282	0.346	0.379
<i>Fabp5</i>	0.268	0.346	0.344	0.217
<i>Pigr</i>	0.086	0.126	0.502	0.399
<i>Anxa5</i>	0.123	-0.009	0.624	0.355
<i>Ldc1</i>	0.162	0.158	0.362	0.392
<i>Slc7a13</i>	0.007	-0.001	0.483	0.528
<i>Wfdc2</i>	0.384	0.618	0.008	0.000
<i>Slco1a1</i>	0.000	0.000	0.339	0.618
<i>Cyp2a4</i>	0.003	0.047	0.521	0.357
<i>Csad</i>	0.148	0.143	0.300	0.315
<i>Azgp1</i>	0.028	0.032	0.428	0.407
<i>Dnase1</i>	-0.105	-0.111	0.492	0.523
<i>Xist</i>	-0.232	-0.493	0.001	0.000
<i>Gsta2</i>	-0.111	-0.169	-0.316	-0.329
<i>Acsf1</i>	-0.177	-0.162	-0.276	-0.344
<i>Mdk</i>	-0.198	-0.211	-0.262	-0.345
<i>ldh1</i>	-0.143	-0.313	-0.245	-0.325
<i>Rsrp1</i>	-0.334	-0.298	-0.220	-0.241
<i>Cryl1</i>	-0.175	-0.264	-0.367	-0.297
<i>Ass1</i>	-0.307	-0.372	-0.164	-0.286
<i>Gpx3</i>	-0.290	-0.383	-0.156	-0.309
<i>Slc5a2</i>	-0.263	-0.332	-0.190	-0.364
<i>Clec2h</i>	-0.224	-0.251	-0.370	-0.435
<i>Spp2</i>	-0.246	-0.315	-0.354	-0.376
<i>Slc5a12</i>	-0.248	-0.333	-0.318	-0.418
<i>Spp1</i>	-0.343	-0.282	-0.227	-0.469
<i>Ftl1-ps1</i>	-0.077	-0.498	-0.534	-0.362
<i>Gatm</i>	-0.215	-0.409	-0.391	-0.526

Appendix 7: Differentially expressed genes in *Vhl*-null PT like cells *versus* PT cells at the late timepoint

Gene	L2FC PT like	Detection PT like	Detection PT	<i>p</i>
<i>Cbr1</i>	0.687	0.688	0.342	0.000
<i>Wfdc2</i>	0.674	0.662	0.256	0.000
<i>Rdh16f2</i>	0.643	0.712	0.315	0.000
<i>Apoc3</i>	0.615	0.714	0.432	0.000
<i>Gsta4</i>	0.440	0.484	0.209	0.000
<i>Hao2</i>	0.416	0.833	0.674	0.000
<i>Uba52</i>	0.405	0.845	0.674	0.000
<i>Gsta2</i>	0.372	0.697	0.448	0.000
<i>Gm10076</i>	0.371	0.681	0.540	0.000
<i>Defb19</i>	0.359	0.836	0.706	0.000
<i>Gas5</i>	0.356	0.851	0.708	0.000
<i>Rpl35</i>	0.356	0.879	0.753	0.000
<i>Npm1</i>	0.356	0.889	0.763	0.000
<i>Tmsb10</i>	0.353	0.298	0.083	0.000
<i>Rpl36a</i>	0.349	0.913	0.806	0.000
<i>ccdc198</i>	0.345	0.667	0.472	0.000
<i>Rps4x</i>	0.336	0.957	0.878	0.000
<i>Eef1d</i>	0.332	0.755	0.588	0.000
<i>Rpl31</i>	0.329	0.876	0.756	0.000
<i>Serpina1a</i>	0.328	0.268	0.073	0.000
<i>Rpl3</i>	0.328	0.940	0.850	0.000
<i>Eef1b2</i>	0.326	0.896	0.785	0.000
<i>Rpl12</i>	0.326	0.954	0.879	0.000
<i>Acaa1b</i>	0.324	0.710	0.548	0.000
<i>Rps26</i>	0.321	0.950	0.879	0.000
<i>Fabp5</i>	0.318	0.490	0.306	0.000
<i>Rpl10</i>	0.316	0.953	0.875	0.000
<i>Gsto1</i>	0.309	0.367	0.154	0.000
<i>Zfas1</i>	0.306	0.437	0.247	0.000
<i>Fabp1</i>	0.304	0.244	0.074	0.000
<i>Rps5</i>	0.304	0.945	0.870	0.000
<i>Naca</i>	0.295	0.922	0.842	0.000
<i>Rpl10a</i>	0.294	0.942	0.869	0.000
<i>Rps9</i>	0.293	0.957	0.893	0.000
<i>S100g</i>	0.293	0.347	0.165	0.000
<i>Acadl</i>	0.293	0.745	0.613	0.000
<i>S100a10</i>	0.292	0.656	0.488	0.000
<i>Rps7</i>	0.291	0.953	0.887	0.000
<i>Cth</i>	0.291	0.611	0.445	0.000
<i>Rps18</i>	0.289	0.962	0.906	0.000
<i>Rpl18</i>	0.289	0.936	0.859	0.000
<i>Rpl22l1</i>	0.288	0.864	0.754	0.000
<i>Rpl15</i>	0.287	0.947	0.873	0.000
<i>Rpl7</i>	0.285	0.938	0.860	0.000
<i>Rps3</i>	0.282	0.938	0.868	0.000
<i>Ifitm2</i>	0.282	0.500	0.329	0.000
<i>Rps13</i>	0.282	0.942	0.872	0.000
<i>Rpl23a</i>	0.282	0.937	0.868	0.000
<i>Nap1l1</i>	0.281	0.612	0.448	0.000
<i>Rpl37</i>	0.281	0.957	0.896	0.000
<i>Apoe</i>	0.280	0.448	0.290	0.000
<i>Rps6</i>	0.280	0.921	0.839	0.000
<i>Rpl22</i>	0.279	0.923	0.846	0.000
<i>Rps17</i>	0.279	0.950	0.891	0.000
<i>Rpl9</i>	0.278	0.945	0.886	0.000
<i>Rack1</i>	0.277	0.934	0.855	0.000
<i>Rpl34</i>	0.277	0.934	0.862	0.000

Gene	L2FC PT like	Detection PT like	Detection PT	p
<i>Rps27</i>	0.275	0.914	0.834	0.000
<i>2410006H16Rik</i>	0.274	0.439	0.271	0.000
<i>Rps28</i>	0.274	0.942	0.870	0.000
<i>Rps3a1</i>	0.273	0.966	0.917	0.000
<i>Al314278</i>	0.272	0.780	0.663	0.000
<i>Rpl14</i>	0.272	0.929	0.854	0.000
<i>Mrpl52</i>	0.272	0.657	0.496	0.000
<i>Rpl36</i>	0.271	0.934	0.872	0.000
<i>Car2</i>	0.269	0.807	0.666	0.000
<i>Rps27a</i>	0.268	0.971	0.917	0.000
<i>Rpl39</i>	0.267	0.940	0.874	0.000
<i>Rps16</i>	0.267	0.964	0.910	0.000
<i>Rps15a</i>	0.267	0.952	0.892	0.000
<i>Rpl29</i>	0.266	0.910	0.819	0.000
<i>Eif3f</i>	0.266	0.655	0.503	0.000
<i>Gnai1</i>	0.266	0.523	0.362	0.000
<i>Cox7a2l</i>	0.265	0.737	0.593	0.000
<i>Rpl6</i>	0.264	0.956	0.902	0.000
<i>Gstm5</i>	0.264	0.717	0.589	0.000
<i>Rpl18a</i>	0.262	0.961	0.902	0.000
<i>Rpl7a</i>	0.261	0.936	0.868	0.000
<i>Acot1</i>	0.261	0.358	0.180	0.000
<i>Rpl11</i>	0.259	0.960	0.912	0.000
<i>Atp5g2</i>	0.259	0.856	0.756	0.000
<i>Rps24</i>	0.258	0.973	0.927	0.000
<i>Rps8</i>	0.256	0.982	0.953	0.000
<i>Rpl38</i>	0.256	0.952	0.891	0.000
<i>Rps20</i>	0.255	0.962	0.908	0.000
<i>Rpl5</i>	0.255	0.941	0.883	0.000
<i>Rps10</i>	0.254	0.962	0.916	0.000
<i>Lyplal1</i>	0.253	0.453	0.288	0.000
<i>Rpl32</i>	0.253	0.972	0.932	0.000
<i>Rpl30</i>	0.252	0.951	0.891	0.000
<i>Rps19</i>	0.250	0.965	0.919	0.000
<i>Rpl21</i>	0.250	0.959	0.909	0.000
<i>Slco3a1</i>	-0.250	0.053	0.203	0.000
<i>Atp2a2</i>	-0.251	0.301	0.456	0.000
<i>Gcnt1</i>	-0.251	0.337	0.485	0.000
<i>Azgp1</i>	-0.252	0.056	0.202	0.000
<i>Slc4a4</i>	-0.252	0.772	0.873	0.000
<i>BC005561</i>	-0.252	0.095	0.221	0.000
<i>Por</i>	-0.252	0.220	0.379	0.000
<i>Syne2</i>	-0.252	0.179	0.327	0.000
<i>Cgnl1</i>	-0.254	0.130	0.278	0.000
<i>Nox4</i>	-0.256	0.313	0.435	0.000
<i>Ass1</i>	-0.256	0.706	0.805	0.000
<i>Chd3</i>	-0.257	0.395	0.536	0.000
<i>Dusp1</i>	-0.258	0.110	0.225	0.000
<i>Agps</i>	-0.258	0.244	0.386	0.000
<i>Ank3</i>	-0.259	0.120	0.275	0.000
<i>Slc13a1</i>	-0.260	0.360	0.509	0.000
<i>Ugt3a2</i>	-0.262	0.500	0.625	0.000
<i>Abcc2</i>	-0.262	0.240	0.396	0.000
<i>Nudt19</i>	-0.264	0.481	0.570	0.000
<i>Cltn</i>	-0.271	0.767	0.856	0.000
<i>Smpdl3a</i>	-0.272	0.387	0.536	0.000
<i>Idh1</i>	-0.273	0.471	0.577	0.000
<i>Neu1</i>	-0.273	0.353	0.508	0.000
<i>mt-Nd4l</i>	-0.279	0.647	0.767	0.000
<i>Car12</i>	-0.280	0.035	0.195	0.000
<i>Fgf1</i>	-0.280	0.164	0.320	0.000
<i>mt-Nd5</i>	-0.284	0.805	0.880	0.000
<i>Slc47a1</i>	-0.285	0.326	0.478	0.000
<i>Slc17a1</i>	-0.286	0.311	0.475	0.000
<i>Ghr</i>	-0.286	0.417	0.558	0.000
<i>Cyp2j5</i>	-0.286	0.601	0.731	0.000

Gene	L2FC PT like	Detection PT like	Detection PT	p
<i>Hsd3b2</i>	-0.289	0.064	0.249	0.000
<i>Slc22a18</i>	-0.290	0.238	0.419	0.000
<i>Slc22a28</i>	-0.301	0.043	0.228	0.000
<i>Jun</i>	-0.301	0.203	0.304	0.000
<i>AC149090.1</i>	-0.301	0.083	0.229	0.000
<i>Cyp4a12a</i>	-0.303	0.026	0.185	0.000
<i>Atp1a1</i>	-0.305	0.742	0.850	0.000
<i>Tcn2</i>	-0.306	0.472	0.641	0.000
<i>G6pc</i>	-0.307	0.210	0.384	0.000
<i>Cndp2</i>	-0.315	0.483	0.599	0.000
<i>Kcnj15</i>	-0.315	0.428	0.594	0.000
<i>Acox1</i>	-0.316	0.570	0.722	0.000
<i>Arl6ip1</i>	-0.316	0.530	0.680	0.000
<i>Ldhd</i>	-0.319	0.405	0.590	0.000
<i>Atp1b1</i>	-0.320	0.592	0.738	0.000
<i>Lgmn</i>	-0.323	0.209	0.419	0.000
<i>Slco1a6</i>	-0.323	0.065	0.265	0.000
<i>Comt</i>	-0.326	0.345	0.533	0.000
<i>Hsd17b11</i>	-0.330	0.160	0.350	0.000
<i>Cyp2a4</i>	-0.332	0.096	0.260	0.000
<i>Slc5a12</i>	-0.335	0.012	0.175	0.000
<i>Cyp2j13</i>	-0.335	0.039	0.243	0.000
<i>Serpinf2</i>	-0.335	0.061	0.256	0.000
<i>Slc22a12</i>	-0.337	0.262	0.449	0.000
<i>Slc6a6</i>	-0.338	0.164	0.371	0.000
<i>Slc22a6</i>	-0.339	0.320	0.485	0.000
<i>Igfbp4</i>	-0.341	0.256	0.466	0.000
<i>Enpp2</i>	-0.346	0.133	0.341	0.000
<i>Slc17a3</i>	-0.356	0.404	0.570	0.000
<i>Lipa</i>	-0.358	0.173	0.384	0.000
<i>Gm26917</i>	-0.363	0.061	0.212	0.000
<i>Hsd11b1</i>	-0.370	0.244	0.408	0.000
<i>Fuca2</i>	-0.371	0.242	0.464	0.000
<i>Errfi1</i>	-0.381	0.361	0.542	0.000
<i>Psap</i>	-0.402	0.264	0.502	0.000
<i>Ly6e</i>	-0.404	0.136	0.336	0.000
<i>Ugt2b37</i>	-0.404	0.072	0.253	0.000
<i>Fut9</i>	-0.405	0.513	0.705	0.000
<i>Neat1</i>	-0.407	0.113	0.270	0.000
<i>Slc5a2</i>	-0.409	0.026	0.229	0.000
<i>Maf</i>	-0.418	0.502	0.676	0.000
<i>mt-Nd3</i>	-0.422	0.696	0.836	0.000
<i>Acsm3</i>	-0.436	0.084	0.312	0.000
<i>Cd36</i>	-0.466	0.209	0.437	0.000
<i>Gatm</i>	-0.473	0.513	0.658	0.000
<i>Gpx3</i>	-0.476	0.862	0.957	0.000
<i>Slc22a30</i>	-0.477	0.080	0.324	0.000
<i>Cyp2e1</i>	-0.479	0.355	0.555	0.000
<i>Atp11a</i>	-0.486	0.109	0.368	0.000
<i>Inmt</i>	-0.500	0.221	0.498	0.000
<i>Zbtb20</i>	-0.501	0.400	0.628	0.000
<i>Malat1</i>	-0.518	0.799	0.919	0.000
<i>Slc34a1</i>	-0.520	0.683	0.860	0.000
<i>Timp3</i>	-0.522	0.548	0.773	0.000
<i>Lpl</i>	-0.576	0.125	0.388	0.000
<i>Selenop</i>	-0.581	0.462	0.713	0.000
<i>Ces1f</i>	-0.583	0.178	0.473	0.000
<i>Dnase1</i>	-0.603	0.173	0.461	0.000
<i>Slc7a13</i>	-0.635	0.076	0.360	0.000
<i>Slco1a1</i>	-0.654	0.069	0.348	0.000
<i>Ugt2b38</i>	-0.710	0.082	0.447	0.000
<i>Acsm2</i>	-0.734	0.492	0.819	0.000
<i>Cyp4b1</i>	-0.836	0.181	0.552	0.000

Appendix 8: Differential expression in *Vhl*-null cells or *Vhl/Pbrm1*-null cells at the late timepoint

Differentially expressed genes in tdTomato-positive cells from *Vhl* KO or *Vhl Pbrm1* KO mice compared to Control mice at the late timepoint

Gene	L2FC <i>Vhl</i> KO	Detection <i>Vhl</i> KO	p <i>Vhl</i> KO	L2FC <i>Vhl Pbrm1</i> KO	Detection <i>Vhl Pbrm1</i> KO	p <i>Vhl Pbrm1</i> KO
<i>Defb19</i>	1.413	0.719	0.000	1.095	0.490	0.000
<i>Apoc3</i>	0.982	0.466	0.000	1.258	0.540	0.000
<i>Pfklp</i>	1.096	0.668	0.000	0.938	0.535	0.000
<i>Gpi1</i>	0.804	0.825	0.000	0.736	0.752	0.000
<i>Ldha</i>	0.747	0.930	0.000	0.700	0.909	0.000
<i>Napsa</i>	0.621	0.846	0.000	0.777	0.849	0.000
<i>Angptl3</i>	0.706	0.384	0.000	0.685	0.327	0.000
<i>Aldoa</i>	0.701	0.936	0.000	0.676	0.911	0.000
<i>Ly6a</i>	0.715	0.707	0.000	0.626	0.622	0.000
<i>Pgam1</i>	0.686	0.841	0.000	0.644	0.791	0.000
<i>Pgk1</i>	0.683	0.885	0.000	0.621	0.833	0.000
<i>Fam162a</i>	0.513	0.595	0.000	0.743	0.647	0.000
<i>Bnip3</i>	0.671	0.711	0.000	0.582	0.622	0.000
<i>Bnip3l</i>	0.614	0.665	0.000	0.580	0.596	0.000
<i>Eno1</i>	0.646	0.757	0.000	0.541	0.674	0.000
<i>Hsd11b1</i>	0.725	0.371	0.000	0.438	0.224	0.000
<i>Kap</i>	0.595	0.931	0.000	0.502	0.774	0.000
<i>Wfdc2</i>	0.558	0.318	0.000	0.506	0.267	0.000
<i>Rpl22l1</i>	0.555	0.767	0.000	0.503	0.704	0.000
<i>Adh1</i>	0.596	0.624	0.000	0.460	0.509	0.000
<i>Defb29</i>	0.528	0.705	0.000	0.499	0.633	0.000
<i>Mif</i>	0.478	0.949	0.000	0.537	0.949	0.000
<i>Ldc1</i>	0.542	0.378	0.000	0.466	0.300	0.000
<i>Plod2</i>	0.494	0.355	0.000	0.513	0.330	0.000
<i>Cd36</i>	0.525	0.395	0.000	0.465	0.338	0.000
<i>Ankrd37</i>	0.492	0.312	0.000	0.497	0.275	0.000
<i>Bsg</i>	0.429	0.699	0.000	0.559	0.703	0.000
<i>Fabp5</i>	0.507	0.324	0.000	0.468	0.254	0.000
<i>P4ha1</i>	0.533	0.428	0.000	0.414	0.324	0.000
<i>Me1</i>	0.558	0.762	0.000	0.388	0.630	0.000
<i>Tpi1</i>	0.504	0.907	0.000	0.437	0.862	0.000
<i>Acmsd</i>	0.542	0.627	0.000	0.375	0.484	0.000
<i>Odc1</i>	0.598	0.885	0.000	0.316	0.752	0.000
<i>Pfkl</i>	0.510	0.385	0.000	0.398	0.276	0.000
<i>Tmigd1</i>	0.384	0.511	0.000	0.520	0.529	0.000
<i>Mpv17l</i>	0.449	0.536	0.000	0.410	0.479	0.000
<i>Slco1a1</i>	0.530	0.297	0.000	0.327	0.235	0.000
<i>Ggt1</i>	0.406	0.779	0.000	0.417	0.720	0.000
<i>Gas5</i>	0.375	0.725	0.000	0.426	0.704	0.000
<i>Pkm</i>	0.407	0.244	0.000	0.383	0.208	0.000
<i>Gls</i>	0.469	0.473	0.000	0.300	0.346	0.000
<i>Dhx29</i>	0.383	0.342	0.000	0.370	0.291	0.000
<i>Mep1a</i>	0.421	0.735	0.000	0.321	0.634	0.000
<i>Ggct</i>	0.454	0.524	0.000	0.277	0.392	0.000
<i>Acsm3</i>	0.449	0.270	0.000	0.273	0.205	0.000
<i>S100a11</i>	0.368	0.522	0.000	0.343	0.433	0.000
<i>Egln3</i>	0.390	0.262	0.000	0.301	0.183	0.000
<i>Rpl12</i>	0.342	0.889	0.000	0.339	0.862	0.000
<i>Dynl1</i>	0.344	0.605	0.000	0.337	0.540	0.000
<i>Ctnnbip1</i>	0.329	0.385	0.000	0.346	0.349	0.000
<i>Prelid2</i>	0.322	0.213	0.000	0.351	0.192	0.000
<i>Trappc6a</i>	0.310	0.356	0.000	0.363	0.337	0.000

Gene	L2FC Vhl KO	Detection Vhl KO	p Vhl KO	L2FC Vhl Pbrm1 KO	Detection Vhl Pbrm1 KO	p Vhl Pbrm1 KO
<i>Fuca1</i>	0.332	0.672	0.000	0.318	0.601	0.000
<i>Pm20d1</i>	0.171	0.177	—	0.469	0.316	0.000
<i>Higd1a</i>	0.326	0.584	0.000	0.306	0.518	0.000
<i>Them7</i>	0.334	0.458	0.000	0.296	0.392	0.000
<i>Efh1</i>	0.430	0.309	0.000	0.199	0.125	—
<i>Gm10076</i>	0.388	0.555	0.000	0.241	0.483	—
<i>Mt1</i>	0.261	0.235	0.000	0.364	0.218	0.000
<i>Uba52</i>	0.383	0.698	0.000	0.217	0.594	—
<i>Rpl7a</i>	0.283	0.877	0.000	0.313	0.862	0.000
<i>Nudt19</i>	0.291	0.541	0.000	0.301	0.510	0.000
<i>Agps</i>	0.381	0.358	0.000	0.209	0.248	—
<i>Csad</i>	0.314	0.448	0.000	0.263	0.376	0.000
<i>Atp11a</i>	0.319	0.325	0.000	0.256	0.276	0.000
<i>Cystm1</i>	0.280	0.684	0.000	0.294	0.634	0.000
<i>Tomm6</i>	0.322	0.574	0.000	0.239	0.475	—
<i>Nhp2</i>	0.257	0.382	0.000	0.297	0.361	0.000
<i>Btf3</i>	0.280	0.743	0.000	0.272	0.689	0.000
<i>Ipo5</i>	0.343	0.298	0.000	0.208	0.185	—
<i>Eef1d</i>	0.272	0.605	0.000	0.278	0.554	0.000
<i>Glb1</i>	0.298	0.330	0.000	0.250	0.267	—
<i>Egln1</i>	0.272	0.356	0.000	0.275	0.310	0.000
<i>Gm44785</i>	0.220	0.166	—	0.324	0.204	0.000
<i>Eef1b2</i>	0.247	0.796	—	0.293	0.778	0.000
<i>Msra</i>	0.257	0.738	0.000	0.282	0.702	0.000
<i>Pdk1</i>	0.334	0.242	0.000	0.205	0.136	—
<i>Cdkn1a</i>	0.217	0.143	—	0.320	0.164	0.000
<i>Rps15a</i>	0.258	0.898	0.000	0.271	0.888	0.000
<i>Acy3</i>	0.286	0.789	0.000	0.242	0.719	—
<i>Tmem178</i>	0.279	0.257	0.000	0.248	0.204	—
<i>Rpl36a</i>	0.266	0.820	0.000	0.257	0.781	0.000
<i>Slc17a3</i>	0.279	0.540	0.000	0.243	0.484	—
<i>Ly6e</i>	0.317	0.300	0.000	0.203	0.242	—
<i>Rpl39</i>	0.297	0.881	0.000	0.223	0.832	—
<i>Anxa5</i>	0.363	0.342	0.000	0.150	0.191	—
<i>Slc6a18</i>	0.285	0.406	0.000	0.227	0.341	—
<i>Itm2b</i>	0.202	0.813	—	0.309	0.809	0.000
<i>Rpl10a</i>	0.237	0.876	—	0.272	0.865	0.000
<i>Fdx1</i>	0.276	0.619	0.000	0.232	0.542	—
<i>Gapdh</i>	0.227	0.977	—	0.274	0.978	0.000
<i>Pafah1b3</i>	0.246	0.236	—	0.252	0.206	0.000
<i>Mep1b</i>	0.246	0.177	—	0.250	0.167	0.000
<i>Rpl9</i>	0.223	0.893	—	0.271	0.890	0.000
<i>Hprt</i>	0.277	0.339	0.000	0.209	0.259	—
<i>Lypla1</i>	0.327	0.495	0.000	0.159	0.351	—
<i>Chdh</i>	0.322	0.438	0.000	0.163	0.298	—
<i>Ostf1</i>	0.259	0.365	0.000	0.223	0.294	—
<i>Cndp2</i>	0.281	0.570	0.000	0.195	0.499	—
<i>Rpl35</i>	0.276	0.767	0.000	0.196	0.701	—
<i>Rpl27</i>	0.218	0.877	—	0.253	0.860	0.000
<i>Rpl4</i>	0.254	0.794	0.000	0.216	0.730	—
<i>Rps18</i>	0.215	0.912	—	0.253	0.909	0.000
<i>Ndufb8</i>	0.143	0.844	—	0.316	0.868	0.000
<i>Prelid1</i>	0.254	0.657	0.000	0.205	0.576	—
<i>Ifitm2</i>	0.293	0.350	0.000	0.161	0.227	—
<i>Hsd17b2</i>	0.154	0.137	—	0.299	0.201	0.000
<i>Rpl23a</i>	0.256	0.876	0.000	0.197	0.815	—
<i>Pabpc1</i>	0.321	0.618	0.000	0.130	0.458	—
<i>Tmem176b</i>	0.165	0.726	—	0.279	0.708	0.000
<i>Rpl31</i>	0.258	0.771	0.000	0.183	0.699	—
<i>Igfbp1</i>	0.266	0.185	0.000	0.175	0.119	—
<i>Itgav</i>	0.273	0.321	0.000	0.159	0.220	—
<i>Cryab</i>	0.137	0.163	—	0.290	0.195	0.000
<i>Car9</i>	0.142	0.109	—	0.276	0.174	0.000
<i>Cyp2e1</i>	0.281	0.510	0.000	0.136	0.410	—

Gene	L2FC Vhl KO	Detection Vhl KO	p Vhl KO	L2FC Vhl Pbrm1 KO	Detection Vhl Pbrm1 KO	p Vhl Pbrm1 KO
<i>Imem176a</i>	0.147	0.708	—	0.262	0.692	0.000
<i>Vegfa</i>	0.254	0.359	0.000	0.146	0.285	—
<i>Ptma</i>	0.254	0.901	0.000	0.140	0.836	—
<i>Samd9l</i>	0.274	0.623	0.000	0.111	0.483	—
<i>Far1</i>	0.265	0.485	0.000	0.101	0.343	—
<i>Gphn</i>	0.258	0.394	0.000	0.089	0.247	—
<i>4833439L19Rik</i>	0.272	0.832	0.000	0.038	0.676	—
<i>Gnai1</i>	0.265	0.380	0.000	0.039	0.217	—
<i>Hsd17b11</i>	0.253	0.320	0.000	0.038	0.184	—
<i>Idi1</i>	0.251	0.332	0.000	0.018	0.175	—
<i>Cltc</i>	-0.022	0.485	—	-0.258	0.296	0.000
<i>Dnaja1</i>	-0.029	0.742	—	-0.259	0.588	0.000
<i>Fos</i>	-0.040	0.128	—	-0.254	0.052	0.000
<i>Selenow</i>	-0.018	0.184	—	-0.294	0.020	0.000
<i>Abat</i>	-0.066	0.427	—	-0.250	0.272	0.000
<i>Atp2a2</i>	-0.049	0.440	—	-0.270	0.276	0.000
<i>Ddx5</i>	-0.086	0.685	—	-0.252	0.559	0.000
<i>Fut9</i>	-0.037	0.688	—	-0.305	0.499	0.000
<i>Hsp90aa1</i>	-0.076	0.753	—	-0.279	0.615	0.000
<i>Tgoln1</i>	-0.095	0.337	—	-0.261	0.210	0.000
<i>Dynll2</i>	-0.103	0.468	—	-0.254	0.332	0.000
<i>Zfp871</i>	-0.116	0.289	—	-0.253	0.181	0.000
<i>Spns3</i>	-0.107	0.177	—	-0.266	0.068	0.000
<i>Snx29</i>	-0.117	0.235	—	-0.257	0.136	0.000
<i>Ccn1</i>	-0.112	0.176	—	-0.267	0.125	0.000
<i>Cldn2</i>	-0.129	0.425	—	-0.259	0.310	0.000
<i>Fam151a</i>	-0.130	0.169	—	-0.267	0.069	0.000
<i>Abcg2</i>	-0.119	0.653	—	-0.282	0.519	0.000
<i>Cgnl1</i>	-0.137	0.269	—	-0.267	0.169	0.000
<i>Sqstm1</i>	-0.113	0.470	—	-0.295	0.319	0.000
<i>Ptms</i>	-0.119	0.674	—	-0.293	0.524	0.000
<i>Fmo2</i>	-0.135	0.580	—	-0.278	0.449	0.000
<i>Hpd</i>	-0.162	0.135	—	-0.257	0.068	0.000
<i>Tstd1</i>	-0.116	0.228	—	-0.305	0.084	0.000
<i>Lipa</i>	-0.163	0.363	—	-0.264	0.274	0.000
<i>Cotl1</i>	-0.160	0.265	—	-0.277	0.158	0.000
<i>Chka</i>	-0.186	0.112	—	-0.251	0.070	0.000
<i>Sord</i>	-0.130	0.821	—	-0.311	0.690	0.000
<i>Nox4</i>	-0.189	0.431	—	-0.260	0.343	0.000
<i>Sgk2</i>	-0.196	0.163	—	-0.264	0.103	0.000
<i>Apom</i>	-0.228	0.220	—	-0.253	0.174	0.000
<i>0610005C13Rik</i>	-0.186	0.524	—	-0.299	0.407	0.000
<i>Itpr2</i>	-0.196	0.167	—	-0.291	0.094	0.000
<i>mt-Nd4</i>	-0.242	0.954	—	-0.250	0.946	0.000
<i>Pah</i>	-0.134	0.644	—	-0.359	0.471	0.000
<i>Abcc4</i>	-0.236	0.023	—	-0.257	0.008	0.000
<i>Osgin1</i>	-0.225	0.119	—	-0.274	0.079	0.000
<i>Oat</i>	-0.260	0.177	0.000	-0.240	0.162	—
<i>Tln2</i>	-0.199	0.202	—	-0.303	0.115	0.000
<i>Slc13a3</i>	-0.232	0.262	—	-0.271	0.212	0.000
<i>Abcc2</i>	-0.189	0.378	—	-0.319	0.271	0.000
<i>Car14</i>	-0.226	0.124	—	-0.281	0.076	0.000
<i>Hmgcs2</i>	-0.257	0.019	0.000	-0.253	0.017	0.000
<i>Rpl29</i>	0.173	0.828	—	-0.684	0.295	0.000
<i>Snhg11</i>	-0.185	0.112	—	-0.331	0.036	0.000
<i>mt-Nd6</i>	-0.148	0.431	—	-0.368	0.278	0.000
<i>Gstm1</i>	-0.264	0.414	0.000	-0.256	0.372	0.000
<i>Fmo1</i>	-0.171	0.652	—	-0.351	0.501	0.000
<i>Acss1</i>	-0.243	0.110	—	-0.280	0.074	0.000
<i>Cryl1</i>	-0.194	0.491	—	-0.330	0.361	0.000
<i>Slc7a8</i>	-0.230	0.120	—	-0.296	0.068	0.000
<i>Spag9</i>	-0.201	0.418	—	-0.326	0.299	0.000
<i>mt-Nd3</i>	-0.183	0.815	—	-0.347	0.730	0.000
<i>Inmt</i>	-0.133	0.447	—	-0.399	0.286	0.000

Gene	L2FC <i>Vhl</i> KO	Detection <i>Vhl</i> KO	p <i>Vhl</i> KO	L2FC <i>Vhl Pbrm1</i> KO	Detection <i>Vhl Pbrm1</i> KO	p <i>Vhl Pbrm1</i> KO
<i>Prodh</i>	-0.243	0.248	—	-0.292	0.185	0.000
<i>Acsm2</i>	-0.163	0.774	—	-0.378	0.649	0.000
<i>Jun</i>	-0.249	0.297	—	-0.312	0.271	0.000
<i>Tmem205</i>	-0.284	0.332	0.000	-0.292	0.288	0.000
<i>Igfbp4</i>	-0.256	0.443	0.000	-0.320	0.361	0.000
<i>Cela1</i>	-0.215	0.560	—	-0.370	0.404	0.000
<i>Ethe1</i>	-0.331	0.387	0.000	-0.266	0.377	0.000
<i>Gsta4</i>	-0.193	0.254	—	-0.408	0.116	0.000
<i>Rarres2</i>	-0.388	0.064	0.000	-0.214	0.131	—
<i>Slc34a1</i>	-0.276	0.835	0.000	-0.328	0.779	0.000
<i>Rsrp1</i>	-0.295	0.382	0.000	-0.316	0.345	0.000
<i>Spp1</i>	-0.225	0.439	—	-0.387	0.313	0.000
<i>Asl</i>	-0.255	0.372	0.000	-0.358	0.270	0.000
<i>Aldh7a1</i>	-0.262	0.394	0.000	-0.362	0.289	0.000
<i>Akr1c21</i>	-0.321	0.826	0.000	-0.306	0.786	0.000
<i>Malat1</i>	-0.291	0.905	0.000	-0.343	0.847	0.000
<i>D630023F18Rik</i>	-0.290	0.073	0.000	-0.345	0.037	0.000
<i>Mgst1</i>	-0.274	0.140	0.000	-0.361	0.067	0.000
<i>Cyp4a14</i>	-0.338	0.051	0.000	-0.310	0.051	0.000
<i>Maf</i>	-0.193	0.659	—	-0.470	0.470	0.000
<i>Gatm</i>	-0.222	0.651	—	-0.450	0.462	0.000
<i>Acsf1</i>	-0.261	0.293	0.000	-0.414	0.163	0.000
<i>Neat1</i>	-0.273	0.256	0.000	-0.413	0.202	0.000
<i>Gabarapl1</i>	-0.247	0.564	—	-0.476	0.389	0.000
<i>mt-Nd5</i>	-0.308	0.870	0.000	-0.415	0.808	0.000
<i>G6pc</i>	-0.281	0.365	0.000	-0.455	0.241	0.000
<i>Gpx3</i>	-0.349	0.945	0.000	-0.418	0.913	0.000
<i>Slc5a2</i>	-0.301	0.209	0.000	-0.481	0.108	0.000
<i>ldh1</i>	-0.312	0.568	0.000	-0.472	0.442	0.000
<i>Slc6a19</i>	-0.355	0.125	0.000	-0.440	0.066	0.000
<i>Slc5a12</i>	-0.349	0.161	0.000	-0.454	0.103	0.000
<i>Clec2h</i>	-0.394	0.088	0.000	-0.414	0.064	0.000
<i>Acaa2</i>	-0.368	0.362	0.000	-0.476	0.237	0.000
<i>mt-Atp8</i>	-0.411	0.537	0.000	-0.438	0.479	0.000
<i>mt-Nd4l</i>	-0.357	0.751	0.000	-0.515	0.658	0.000
<i>Apoe</i>	-0.221	0.326	—	-0.653	0.100	0.000
<i>Slc13a1</i>	-0.526	0.492	0.000	-0.397	0.502	0.000
<i>Spp2</i>	-0.463	0.309	0.000	-0.505	0.242	0.000
<i>Slc22a8</i>	-0.411	0.328	0.000	-0.591	0.176	0.000
<i>Gsta3</i>	-0.472	0.228	0.000	-0.583	0.138	0.000
<i>Dnase1</i>	-0.323	0.415	0.000	-0.782	0.168	0.000
<i>Ass1</i>	-0.434	0.792	0.000	-0.720	0.622	0.000
<i>Cyp2d12</i>	-0.517	0.081	0.000	-0.678	0.011	0.000
<i>Gsta2</i>	-0.660	0.498	0.000	-0.575	0.514	0.000
<i>Apob</i>	-0.671	0.082	0.000	-0.749	0.041	0.000

Appendix 9: Information and quality control metrics of scRNA-seq samples

Mouse	tdTomato	Sex	Condition	Time (days)	Time point	Cells	UMIs per cell	Genes per cell
OY1004	Positive	Male	Control	7	Early	13905	3051	1298
OY725	Positive	Female	Control	8	Early	18269	3271	1320
OY955	Positive	Female	Control	15	Early	11717	3616	1446
OY954	Positive	Female	Control	15	Early	12246	2838.5	1258
OY1021	Positive	Male	Vhl KO	7	Early	7169	8249	2457
OY1166	Positive	Male	Vhl KO	8	Early	29242	2927	1242
OY733	Positive	Female	Vhl KO	8	Early	22770	3586.5	1441
OY978	Positive	Female	Vhl KO	15	Early	13913	3737	1501
OY1004	Negative	Male	Control	7	Early	15128	2700	1107
OY725	Negative	Female	Control	8	Early	17560	2660.5	1041
OY955	Negative	Female	Control	15	Early	7288	2317	972
OY954	Negative	Female	Control	15	Early	11446	1492	760
OY1166	Negative	Male	Vhl KO	8	Early	17101	3082	1175
OY733	Negative	Female	Vhl KO	8	Early	19877	2921	1120
OY978	Negative	Female	Vhl KO	15	Early	14470	2520	1078
OY545	Positive	Male	Control	315	Late	19363	1985	888
OY622	Positive	Male	Control	427	Late	18383	3504	1256
OY552	Positive	Female	Control	128	Late	5104	4284	1527
OY611	Positive	Female	Control	132	Late	22925	2720	1120
OY1023	Positive	Male	Vhl KO	127	Late	16577	4101	1432
OY615	Positive	Male	Vhl KO	275	Late	15970	4251.5	1528
OY606	Positive	Female	Vhl KO	132	Late	16137	3905	1509
OY626	Positive	Female	Vhl KO	171	Late	10838	3540	1312
OY545	Negative	Female	Control	315	Late	14296	2804	1221
OY622	Negative	Male	Control	427	Late	24412	3087	1121
OY551	Negative	Female	Control	128	Late	6525	3328	1325
OY611	Negative	Female	Control	132	Late	17484	2722	1051
OY1023	Negative	Male	Vhl KO	127	Late	17056	2425	1019
OY615	Negative	Male	Vhl KO	275	Late	15650	3144	1378.5
OY606	Negative	Female	Vhl KO	132	Late	13587	2868	1144
OY626	Negative	Female	Vhl KO	171	Late	8254	3040	1237
VB177	Positive	Female	Vhl Pbrm1 KO	108	Late	6547	2379	949
VB229	Positive	Female	Vhl Pbrm1 KO	248	Late	24052	1822.5	854
VB174	Positive	Male	Vhl Pbrm1 KO	521	Late	18017	3745	1273

Appendix 10: Cell-type specific marker genes

PEC					
<i>Ackr3</i>	<i>Clu</i>	<i>Fhl2</i>	<i>Lbp</i>	<i>Rasl11a</i>	<i>Timp2</i>
<i>Aebp1</i>	<i>Col4a1</i>	<i>Fxyd1</i>	<i>Ltbp3</i>	<i>Rbp1</i>	<i>Tm4sf1</i>
<i>Akap12</i>	<i>Col4a2</i>	<i>Gpc3</i>	<i>Mcc</i>	<i>Slc6a6</i>	<i>Tshz2</i>
<i>Anxa3</i>	<i>Cp</i>	<i>Gsn</i>	<i>Mical3</i>	<i>Sparc</i>	<i>Tuba1a</i>
<i>C3</i>	<i>Cpe</i>	<i>Jun</i>	<i>Ncam1</i>	<i>Stc2</i>	
<i>Cald1</i>	<i>Cyr61</i>	<i>Kcnma1</i>	<i>Ptpn13</i>	<i>Tbc1d4</i>	
PT S1					
<i>Acsm2</i>	<i>Cryl1</i>	<i>Glyat</i>	<i>Nox4</i>	<i>Ptprd</i>	<i>Sord</i>
<i>Akr1c21</i>	<i>Dab2</i>	<i>Idh1</i>	<i>Osbpl6</i>	<i>Rab11fip3</i>	<i>Spp2</i>
<i>Ass1</i>	<i>Dcxr</i>	<i>Igfbp4</i>	<i>Pah</i>	<i>Slc34a1</i>	<i>Tmem27</i>
<i>Auts2</i>	<i>Dnase1</i>	<i>Keg1</i>	<i>Pakap</i>	<i>Slc4a4</i>	<i>Tnfrsf8</i>
<i>Bnc2</i>	<i>Enpp2</i>	<i>Maf</i>	<i>Pck1</i>	<i>Slc5a12</i>	

<i>Calm14</i>	<i>Fbp1</i>	<i>Malat1</i>	<i>Pdzk1</i>	<i>Slc5a2</i>	
<i>Car12</i>	<i>Fut9</i>	<i>Miox</i>	<i>Pik3c2g</i>	<i>Slc6a19</i>	
<i>Cela1</i>	<i>Gatm</i>	<i>Nhs</i>	<i>Plcb1</i>	<i>Snhg11</i>	
PT S2 Male					
<i>Acsm3</i>	<i>Ces1f</i>	<i>Cyp4a12a</i>	<i>Nat8</i>	<i>Slc7a13</i>	
<i>Adh1</i>	<i>Cyp2e1</i>	<i>Gm11128</i>	<i>RP23-306P12.3</i>	<i>Slco1a1</i>	
<i>Azgp1</i>	<i>Cyp2j13</i>	<i>Inmt</i>	<i>Slc22a6</i>		
PT S2 Female					
<i>BC089597</i>	<i>Cyp4a14</i>	<i>Papss2</i>	<i>Slc13a3</i>		
<i>Cyp4a10</i>	<i>Hpd</i>	<i>Prlr</i>	<i>Slc22a8</i>		
PT S3 Male					
<i>Acsm3</i>	<i>Cyp2d9</i>	<i>Gm853</i>	<i>Ppic</i>	<i>Slc22a13</i>	<i>Slc6a13</i>
<i>Acy1</i>	<i>Cyp51</i>	<i>Gpm6a</i>	<i>Rnf24</i>	<i>Slc22a19</i>	<i>Slc7a13</i>
<i>BC035947</i>	<i>Cyp7b1</i>	<i>Mep1b</i>	<i>Serpina1d</i>	<i>Slc22a2</i>	<i>Slc9a8</i>
<i>Cd36</i>	<i>Fgf1</i>	<i>Mettl7a2</i>	<i>Serpina1f</i>	<i>Slc22a7</i>	<i>Tet2</i>
<i>Cyp2a4</i>	<i>Fmo5</i>	<i>Mogat1</i>	<i>Slc10a2</i>	<i>Slc5a8</i>	<i>Tmigd1</i>
PT S3 Female					
<i>Aadat</i>	<i>BC089597</i>	<i>Gm4450</i>	<i>Ppic</i>	<i>Slc22a13</i>	<i>Slc9a8</i>
<i>Abcc3</i>	<i>Bdh1</i>	<i>Gm853</i>	<i>Prlr</i>	<i>Slc22a19</i>	<i>Slco1a6</i>
<i>Ace</i>	<i>Ccbl2</i>	<i>Gpm6a</i>	<i>Rnf24</i>	<i>Slc22a7</i>	<i>Socs2</i>
<i>Acot7</i>	<i>Cyp2a4</i>	<i>Hmgcr</i>	<i>Scd1</i>	<i>Slc5a10</i>	
<i>Acy1</i>	<i>Cyp4a31</i>	<i>Mep1b</i>	<i>Serpina1f</i>	<i>Slc6a13</i>	
<i>Agt</i>	<i>Cyp51</i>	<i>Mettl7a2</i>	<i>Slc10a2</i>	<i>Slc6a18</i>	
<i>Akr1c18</i>	<i>Fam25c</i>	<i>Myo5a</i>	<i>Slc16a4</i>	<i>Slc7a12</i>	
Loop of Henle					
<i>1700016C15Rik</i>	<i>Cldn8</i>	<i>Hacd4</i>	<i>Pcolce</i>	<i>Slc16a7</i>	<i>Vldlr</i>
<i>Abcc4</i>	<i>Cobll1</i>	<i>Hnmt</i>	<i>Phactr1</i>	<i>Slc39a8</i>	<i>Wfdc15b</i>
<i>Abi3bp</i>	<i>Col4a4</i>	<i>Hnmp1</i>	<i>Phgdh</i>	<i>Slc4a11</i>	<i>Wnk4</i>
<i>AC144408.3</i>	<i>Col4a5</i>	<i>Hspa1b</i>	<i>Pitx2</i>	<i>Slc5a3</i>	<i>Wnt7b</i>
<i>Ackr3</i>	<i>Cox7a1</i>	<i>Hspb1</i>	<i>Plat</i>	<i>Slc6a6</i>	<i>Wwc1</i>
<i>Aif1l</i>	<i>Cpm</i>	<i>Ifit3</i>	<i>Plet1</i>	<i>Slc7a6</i>	<i>Zfyve21</i>
<i>Akr1b3</i>	<i>Cryab</i>	<i>Ifit3b</i>	<i>Ppl</i>	<i>Slco4a1</i>	<i>Sostdc1</i>
<i>Aktip</i>	<i>Csrp2</i>	<i>Igfbp2</i>	<i>Ppp1r1a</i>	<i>Slit2</i>	<i>Sptssb</i>
<i>Alcam</i>	<i>Ctsh</i>	<i>Jund</i>	<i>Ppp1r1b</i>	<i>Sorcs3</i>	<i>Tacstd2</i>
<i>Angpt2</i>	<i>Dnm3</i>	<i>Kcnj1</i>	<i>Prom2</i>	<i>Sostdc1</i>	<i>Tfap2b</i>
<i>Ankrd1</i>	<i>Efemp1</i>	<i>Kcnk1</i>	<i>Proser2</i>	<i>Spint2</i>	<i>Thbs1</i>
<i>Anxa1</i>	<i>Efna5</i>	<i>Klf13</i>	<i>Prox1</i>	<i>Spon1</i>	<i>Them6</i>
<i>Apela</i>	<i>Egf</i>	<i>Kng2</i>	<i>Prrg1</i>	<i>Sptssb</i>	<i>Tiam1</i>
<i>Aqp1</i>	<i>Elovl7</i>	<i>Krt18</i>	<i>Pscs</i>	<i>Srgap3</i>	<i>Timp2</i>
<i>Arhgap6</i>	<i>Epcam</i>	<i>Lamb1</i>	<i>Ptgs</i>	<i>Stard13</i>	<i>Tmem171</i>
<i>Atp10b</i>	<i>Epha7</i>	<i>Lamb3</i>	<i>Ptger3</i>	<i>Tacstd2</i>	<i>Tmem207</i>
<i>Bckdhhb</i>	<i>Epn3</i>	<i>Lrba</i>	<i>Ptpn14</i>	<i>Tbc1d4</i>	<i>Tmem30b</i>
<i>Bcl6</i>	<i>Erbp4</i>	<i>Lynx1</i>	<i>Ptrf</i>	<i>Tfap2b</i>	<i>Tshz2</i>
<i>Bdh2</i>	<i>Esam</i>	<i>Lypd2</i>	<i>Rab3ip</i>	<i>Tfdp2</i>	<i>Tspan13</i>
<i>Bok</i>	<i>Esrrb</i>	<i>Mboat2</i>	<i>Ranbp3l</i>	<i>Thbs1</i>	<i>Tspan6</i>
<i>Bsnd</i>	<i>F2rl1</i>	<i>Mfsd4</i>	<i>Rap1gap</i>	<i>Them6</i>	<i>Ubr4</i>
<i>Bst1</i>	<i>Fabp5</i>	<i>Misp</i>	<i>Rasl11b</i>	<i>Tiam1</i>	<i>Ugt8a</i>
<i>Cacnb4</i>	<i>Fam101b</i>	<i>Mrps6</i>	<i>Rbms3</i>	<i>Timp2</i>	<i>Upk3b</i>
<i>Cd59b</i>	<i>Fcgbp</i>	<i>Mt2</i>	<i>Ryk</i>	<i>Tmem171</i>	<i>Vldlr</i>
<i>Cd82</i>	<i>Fry</i>	<i>Muc1</i>	<i>S100a6</i>	<i>Tmem207</i>	<i>Wnt7b</i>
<i>Cdh13</i>	<i>Frzb</i>	<i>Nbl1</i>	<i>Scel</i>	<i>Tmem30b</i>	<i>Zfyve21</i>
<i>Cdh16</i>	<i>Gal3st1</i>	<i>Nccrp1</i>	<i>Sdc4</i>	<i>Tmem52b</i>	
<i>Cfap52</i>	<i>Gls</i>	<i>Ndr4</i>	<i>Sema5a</i>	<i>Tns3</i>	
<i>Cgnl1</i>	<i>Gm14410</i>	<i>Npnt</i>	<i>Sep-04</i>	<i>Tshz2</i>	
<i>Chn2</i>	<i>Gm14546</i>	<i>Nrip3</i>	<i>Sgcz</i>	<i>Tspan13</i>	
<i>Ckb</i>	<i>Gm26561</i>	<i>Nupr1</i>	<i>Shank2</i>	<i>Tspan6</i>	
<i>Clcnka</i>	<i>Gm42697</i>	<i>Osr2</i>	<i>Sim1</i>	<i>Ubr4</i>	
<i>Clcnkb</i>	<i>Gm6878</i>	<i>Paqr5</i>	<i>Slc12a1</i>	<i>Ugt8a</i>	
<i>Cldn19</i>	<i>Gmpr</i>	<i>Pax2</i>	<i>Slc12a3</i>	<i>Upk3b</i>	
<i>Cldn4</i>	<i>Gpx6</i>	<i>Pax8</i>	<i>Slc16a5</i>	<i>Utrn</i>	
DCT					
<i>1700016C15Rik</i>	<i>Cldn19</i>	<i>Hnmt</i>	<i>Mt2</i>	<i>Rap1gap2</i>	<i>Tmem52b</i>
<i>Abca13</i>	<i>Cldn4</i>	<i>Hoxb5os</i>	<i>Nbl1</i>	<i>Rasl11b</i>	<i>Tmem72</i>
<i>Ackr3</i>	<i>Clu</i>	<i>Hoxd8</i>	<i>Nccrp1</i>	<i>Rhbg</i>	<i>Trpm6</i>

<i>Angpt2</i>	<i>Cobl1</i>	<i>Ier3</i>	<i>Ndr4</i>	<i>Rhcg</i>	<i>Tsc22d1</i>
<i>Bdh2</i>	<i>Cox7a1</i>	<i>Ifit3b</i>	<i>Osr2</i>	<i>Scnn1b</i>	<i>Tshz2</i>
<i>Bok</i>	<i>Cwh43</i>	<i>Kcnj1</i>	<i>Pcolce</i>	<i>Sfrp1</i>	<i>Tspan1</i>
<i>Bst1</i>	<i>Cyfp2</i>	<i>Kcnk1</i>	<i>Pgam2</i>	<i>Sgms2</i>	<i>Ubr4</i>
<i>Cables1</i>	<i>Dst</i>	<i>Kl</i>	<i>Phgdh</i>	<i>Slc12a1</i>	<i>Uroc1</i>
<i>Cacnb4</i>	<i>Egf</i>	<i>Khl3</i>	<i>Pitx2</i>	<i>Slc12a3</i>	<i>Wnk1</i>
<i>Cadps2</i>	<i>Emx1</i>	<i>Kng2</i>	<i>Ppp1r1a</i>	<i>Slc16a7</i>	<i>Wnk4</i>
<i>Calb1</i>	<i>Epha7</i>	<i>Lhx1</i>	<i>Ppp1r1b</i>	<i>Slc39a8</i>	<i>Zfyve21</i>
<i>Cav2</i>	<i>F2rl1</i>	<i>Lhx1os</i>	<i>Prkd1</i>	<i>Slc4a11</i>	
<i>Cfap52</i>	<i>Fabp3</i>	<i>Lpl</i>	<i>Proser2</i>	<i>Slc5a3</i>	
<i>Clcnka</i>	<i>Frzb</i>	<i>Ltc4s</i>	<i>Ptger3</i>	<i>Slc8a1</i>	
<i>Clcnkb</i>	<i>Gmpr</i>	<i>Mfsd4</i>	<i>Ptgfr</i>	<i>Sostdc1</i>	
<i>Cldn10</i>	<i>Gpx6</i>	<i>Misp</i>	<i>Pvalb</i>	<i>Tmem30b</i>	
CDPC					
<i>Adgrg1</i>	<i>Clu</i>	<i>Gstt3</i>	<i>Mcoln3</i>	<i>Ppic</i>	<i>Tacstd2</i>
<i>Adh1</i>	<i>Col26a1</i>	<i>Hes1</i>	<i>Me1</i>	<i>Prss23</i>	<i>Tbck</i>
<i>Aif1l</i>	<i>Cpe</i>	<i>Hnmpr</i>	<i>Mgat4c</i>	<i>Ptger1</i>	<i>Tfap2a</i>
<i>Aldh1a3</i>	<i>Crybg1</i>	<i>Hoxd8</i>	<i>Msln</i>	<i>Ptprj</i>	<i>Tfcp2l1</i>
<i>Apela</i>	<i>Defb29</i>	<i>Hsd11b2</i>	<i>Muc1</i>	<i>Ranbp3l</i>	<i>Tgm2</i>
<i>Aqp2</i>	<i>Dip2c</i>	<i>Hspa1b</i>	<i>Muc20</i>	<i>Rhbg</i>	<i>Tmem117</i>
<i>Aqp3</i>	<i>Dlg1</i>	<i>Kcnc2</i>	<i>Nckap5</i>	<i>Rhcg</i>	<i>Tmem178</i>
<i>Aqp4</i>	<i>Dnm3</i>	<i>Kcne1</i>	<i>Npnt</i>	<i>Rnf183</i>	<i>Tmem213</i>
<i>Atp4a</i>	<i>Efemp1</i>	<i>Kcnj1</i>	<i>Nr3c2</i>	<i>Rnf186</i>	<i>Tmem229a</i>
<i>Avpr2</i>	<i>Elf5</i>	<i>Kcnj10</i>	<i>Pappa</i>	<i>Samd12</i>	<i>Tmem30b</i>
<i>Bmpr1b</i>	<i>F3</i>	<i>Kcnq1</i>	<i>Pax2</i>	<i>Sbspon</i>	<i>Tmem45b</i>
<i>Btc</i>	<i>Fam129a</i>	<i>Kif26b</i>	<i>Pax8</i>	<i>Scd2</i>	<i>Tmem52b</i>
<i>Cacnb2</i>	<i>Fermt1</i>	<i>Kl</i>	<i>Pde3b</i>	<i>Scnn1a</i>	<i>Tmprss2</i>
<i>Calb1</i>	<i>Fmpd4</i>	<i>Krt18</i>	<i>Pde8b</i>	<i>Scnn1b</i>	<i>Tpd52l1</i>
<i>Calml3</i>	<i>Fxyd3</i>	<i>Krt19</i>	<i>Perp</i>	<i>Scnn1g</i>	<i>Tspan1</i>
<i>Cav1</i>	<i>Fxyd4</i>	<i>Krt23</i>	<i>Pgam2</i>	<i>Sdc1</i>	<i>Tspan6</i>
<i>Cav2</i>	<i>Gata3</i>	<i>Krt7</i>	<i>Phactr1</i>	<i>Serinc2</i>	<i>Tspan8</i>
<i>Cdh1</i>	<i>Gcom1</i>	<i>Krt8</i>	<i>Phactr2</i>	<i>Slc12a2</i>	<i>Ube2h</i>
<i>Cdo1</i>	<i>Gsdmc2</i>	<i>Lamb3</i>	<i>Phlda3</i>	<i>Smim5</i>	
<i>Clcnkb</i>	<i>Gsta3</i>	<i>Lcn2</i>	<i>Plcl1</i>	<i>Spink8</i>	
<i>Cldn7</i>	<i>Gsto1</i>	<i>Mal2</i>	<i>Plet1</i>	<i>Sptbn2</i>	
CDIC					
<i>2610016A17Rik</i>	<i>Car12</i>	<i>Grb10</i>	<i>Mme</i>	<i>Psd3</i>	<i>Slc4a1</i>
<i>Adgrf5</i>	<i>Ccbe1</i>	<i>Guca2a</i>	<i>Naaladl2</i>	<i>Ralgapa2</i>	<i>Slc4a9</i>
<i>Aif1l</i>	<i>Ckmt1</i>	<i>Hepacam2</i>	<i>Nbea</i>	<i>Rcan2</i>	<i>Spink8</i>
<i>Aldh1l1</i>	<i>Clcnkb</i>	<i>Hmx2</i>	<i>Ncoa7</i>	<i>Rhbg</i>	<i>Syn2</i>
<i>Aqp6</i>	<i>Clnk</i>	<i>Ica1</i>	<i>Nudt4</i>	<i>Rhcg</i>	<i>Tfcp2l1</i>
<i>Arhgap18</i>	<i>Col18a1</i>	<i>Igfbp5</i>	<i>Ociad2</i>	<i>Rnf152</i>	<i>Tmcc3</i>
<i>Atp6v0a4</i>	<i>Cox7a1</i>	<i>Insrr</i>	<i>Oxgr1</i>	<i>Rp1</i>	<i>Tmem117</i>
<i>Atp6v0d2</i>	<i>Dmrt2</i>	<i>Irs1</i>	<i>Pakap</i>	<i>Rtl4</i>	<i>Tmem163</i>
<i>Atp6v1b1</i>	<i>Dmx1</i>	<i>Itpr2</i>	<i>Pam</i>	<i>Serpinb9</i>	<i>Tmem61</i>
<i>Atp6v1c2</i>	<i>Efh1</i>	<i>Kcnq1</i>	<i>Pde1a</i>	<i>Sgms1</i>	<i>Wdr72</i>
<i>Atp6v1g3</i>	<i>Epb41l2</i>	<i>Kit</i>	<i>Pde4b</i>	<i>Slc16a5</i>	
<i>Atp6v1h</i>	<i>Eps8</i>	<i>Krt7</i>	<i>Plcg2</i>	<i>Slc26a4</i>	
<i>Bmpr1b</i>	<i>Fam13a</i>	<i>Lsmp</i>	<i>Prkca</i>	<i>Slc35f1</i>	
<i>Cacnb2</i>	<i>Foxi1</i>	<i>Malrd1</i>	<i>Prkg1</i>	<i>Slc43a2</i>	
Podocyte					
<i>2010111101Rik</i>	<i>Colec12</i>	<i>Gpsm3</i>	<i>Mmp23</i>	<i>Pls3</i>	<i>St6galnac6</i>
<i>43719</i>	<i>Cpq</i>	<i>Gpx8</i>	<i>Mpp5</i>	<i>Pmepa1</i>	<i>Synpo</i>
<i>9530026P05Rik</i>	<i>Cryab</i>	<i>Gsn</i>	<i>Mtss1</i>	<i>Podxl</i>	<i>Tcf21</i>
<i>Aebp1</i>	<i>Ctgf</i>	<i>H2-Q6</i>	<i>Myo1d</i>	<i>Prkg1</i>	<i>Thsd7a</i>
<i>Agpat4</i>	<i>Dach1</i>	<i>H2-Q7</i>	<i>Myo1e</i>	<i>Ptpro</i>	<i>Timp2</i>
<i>Agpr</i>	<i>Dag1</i>	<i>Hs3st6</i>	<i>Myom2</i>	<i>Qk</i>	<i>Tjp1</i>
<i>Alcam</i>	<i>Ddn</i>	<i>Hspa12a</i>	<i>Nars2</i>	<i>Rab3b</i>	<i>Tm4sf1</i>
<i>Angptl2</i>	<i>Dock4</i>	<i>Hspb8</i>	<i>Nebi</i>	<i>Rarres1</i>	<i>Tmed9</i>
<i>Anxa1</i>	<i>Dock5</i>	<i>Htra1</i>	<i>Nes</i>	<i>Rasl11a</i>	<i>Tmem108</i>
<i>Anxa3</i>	<i>Dpp4</i>	<i>Ifngr1</i>	<i>Nphs1</i>	<i>Rhpn1</i>	<i>Tmem109</i>
<i>Aplp1</i>	<i>Dpysl2</i>	<i>Illdr2</i>	<i>Nphs2</i>	<i>Robo2</i>	<i>Tmem150c</i>
<i>Arhgap28</i>	<i>Efnb1</i>	<i>Iqgap2</i>	<i>Npnt</i>	<i>Scd2</i>	<i>Tmem54</i>
<i>Arvcf</i>	<i>Ehd2</i>	<i>Itga3</i>	<i>Npr1</i>	<i>Sema3e</i>	<i>Tmod3</i>
<i>Astn2</i>	<i>Eml2</i>	<i>Itgb1</i>	<i>Npr3</i>	<i>Sema3g</i>	<i>Trib2</i>

<i>Bcam</i>	<i>Emp3</i>	<i>Itgb5</i>	<i>Nsf</i>	<i>Sema5a</i>	<i>Tspan13</i>
<i>C1qtnf7</i>	<i>Enpep</i>	<i>Kank1</i>	<i>Ntrk3</i>	<i>Sgip1</i>	<i>Tspan15</i>
<i>Cald1</i>	<i>Fam65a</i>	<i>Kcnq1ot1</i>	<i>Nupr1</i>	<i>Shisa3</i>	<i>Tspan2</i>
<i>Cd151</i>	<i>Fat1</i>	<i>Lamb2</i>	<i>Optn</i>	<i>Sirpa</i>	<i>Tspan4</i>
<i>Cd55</i>	<i>Fgfbp1</i>	<i>Lrch1</i>	<i>Ormdl3</i>	<i>Siva1</i>	<i>Tuba1a</i>
<i>Cd59a</i>	<i>Fgfr1</i>	<i>Lrrfp1</i>	<i>P3h2</i>	<i>Slc31a2</i>	<i>Vamp5</i>
<i>Cdc14a</i>	<i>Fmnl2</i>	<i>Mafb</i>	<i>Pak1</i>	<i>Slc48a1</i>	<i>Vegfa</i>
<i>Cdkn1c</i>	<i>Fry</i>	<i>Magi2</i>	<i>Pard3b</i>	<i>Slc9a3r2</i>	<i>Vim</i>
<i>Chst1</i>	<i>Fryl</i>	<i>Maml3</i>	<i>Pcolce</i>	<i>Snaip</i>	<i>Wif1</i>
<i>Cldn5</i>	<i>Fzd2</i>	<i>Man1a2</i>	<i>Pdlim2</i>	<i>Snx31</i>	<i>Wt1</i>
<i>Clic3</i>	<i>Gadd45a</i>	<i>Mapt</i>	<i>Pdpn</i>	<i>Sox5</i>	<i>Zbtb7c</i>
<i>Clic5</i>	<i>Galnt10</i>	<i>Mcc</i>	<i>Plat</i>	<i>Sparc</i>	<i>Zbtb8a</i>
<i>Cmtm7</i>	<i>Ggt5</i>	<i>Mertk</i>	<i>Plce1</i>	<i>Srgap1</i>	<i>Zfp385b</i>
<i>Col1a2</i>	<i>Gm29266</i>	<i>Metnl</i>	<i>Plekkg1</i>	<i>St3gal1</i>	
<i>Col4a3</i>	<i>Gm45062</i>	<i>Mgat5</i>	<i>Plekho1</i>	<i>St3gal6</i>	
<i>Col4a4</i>	<i>Golim4</i>	<i>Mme</i>	<i>Plod2</i>	<i>St6galnac3</i>	
Fibroblast					
<i>4930578G10Rik</i>	<i>Dkk2</i>	<i>Ifitm3</i>	<i>Mpeg1</i>	<i>Prkg1</i>	<i>Sep-04</i>
<i>Adamts5</i>	<i>Dlc1</i>	<i>Itga8</i>	<i>Ms4a6c</i>	<i>Rap1a</i>	<i>Serpine2</i>
<i>Aebp1</i>	<i>Ecm1</i>	<i>Lbh</i>	<i>Msn</i>	<i>Rarres2</i>	<i>Serping1</i>
<i>Agtr1a</i>	<i>Emilin1</i>	<i>Ldb2</i>	<i>Mxra8</i>	<i>Rasgrp2</i>	<i>Tshz2</i>
<i>Bgn</i>	<i>Fam49b</i>	<i>Lgals3</i>	<i>Myl9</i>	<i>Rasl11a</i>	<i>Ttc28</i>
<i>Cfh</i>	<i>Fhl2</i>	<i>Lhfp</i>	<i>Pcolce</i>	<i>Rbms3</i>	<i>Ucp2</i>
<i>Clec4a3</i>	<i>Gngt2</i>	<i>Lrp1</i>	<i>Pdgfra</i>	<i>Rcn3</i>	
<i>Col1a2</i>	<i>Gucy1a2</i>	<i>Lst1</i>	<i>Pdgfrb</i>	<i>Robo2</i>	
<i>Ctgf</i>	<i>Gucy1a3</i>	<i>Lyz2</i>	<i>Pdlim2</i>	<i>S100a4</i>	
<i>Des</i>	<i>Ifi272a</i>	<i>Mgp</i>	<i>Plac8</i>	<i>Samhd1</i>	
Macrophage					
<i>Adgre1</i>	<i>Cd300c2</i>	<i>Cx3cr1</i>	<i>H2-DMa</i>	<i>Ms4a6c</i>	<i>Tgfb1</i>
<i>Aif1</i>	<i>Cd68</i>	<i>Cxcl16</i>	<i>H2-DMb1</i>	<i>Ms4a6d</i>	<i>Tgfb1</i>
<i>Apoe</i>	<i>Cd72</i>	<i>Cybb</i>	<i>H2-Eb1</i>	<i>Ms4a7</i>	<i>Tm6sf1</i>
<i>C1qa</i>	<i>Cd74</i>	<i>Fcgr1</i>	<i>Hexa</i>	<i>P2ry6</i>	<i>Trf</i>
<i>C1qb</i>	<i>Clec4a2</i>	<i>Fcgr2b</i>	<i>Hexb</i>	<i>Pid1</i>	<i>Unc93b1</i>
<i>C1qc</i>	<i>Csf1r</i>	<i>Fcgr3</i>	<i>Inpp5d</i>	<i>Pld4</i>	<i>Zeb2</i>
<i>C3ar1</i>	<i>Ctsc</i>	<i>Fyb</i>	<i>Ly86</i>	<i>Pou2f2</i>	
<i>Ccnd3</i>	<i>Ctsh</i>	<i>H2-Aa</i>	<i>Mafb</i>	<i>Rgs10</i>	
<i>Ccr5</i>	<i>Ctss</i>	<i>H2-Ab1</i>	<i>Ms4a6b</i>	<i>Snx5</i>	
NK Cell					
<i>Ace</i>	<i>Cd300a</i>	<i>Gzma</i>	<i>Jak1</i>	<i>Nkg7</i>	<i>S100a4</i>
<i>Adgre4</i>	<i>Clec10a</i>	<i>Gzmb</i>	<i>Klrb1c</i>	<i>Nr4a1</i>	<i>Samsn1</i>
<i>Alox5ap</i>	<i>Clec12a</i>	<i>H2-DMb2</i>	<i>Klrd1</i>	<i>Pglyrp1</i>	<i>Selp1g</i>
<i>Bin2</i>	<i>Clec4a1</i>	<i>H2-Oa</i>	<i>Klre1</i>	<i>Pid1</i>	<i>Sirpb1c</i>
<i>Ccl5</i>	<i>Clec4a3</i>	<i>Hp</i>	<i>Klrk1</i>	<i>Plbd1</i>	<i>Slamf7</i>
<i>Ccl6</i>	<i>Clec4b1</i>	<i>Ifi30</i>	<i>Lsp1</i>	<i>Pld4</i>	<i>Spi1</i>
<i>Ccl9</i>	<i>Ctsw</i>	<i>Ifitm6</i>	<i>Lst1</i>	<i>Pou2f2</i>	<i>Txk</i>
<i>Ccnd2</i>	<i>Cybb</i>	<i>Itgal</i>	<i>Ltb4r1</i>	<i>Ptpn6</i>	<i>Wfdc17</i>
<i>Ccr2</i>	<i>Dusp2</i>	<i>Itgb2</i>	<i>Ms4a4c</i>	<i>Ptprc</i>	
<i>Cd209a</i>	<i>Ear2</i>	<i>Itgb7</i>	<i>Ms4a6c</i>	<i>Rnase6</i>	
Pericyte					
<i>9530026P05Rik</i>	<i>Dgkb</i>	<i>Gpc6</i>	<i>Pde3a</i>	<i>Sox17</i>	
<i>Adgrl3</i>	<i>Dlc1</i>	<i>Hip1</i>	<i>Prkg1</i>	<i>Sox5</i>	
<i>Cald1</i>	<i>Ebf1</i>	<i>Lhfp</i>	<i>Rbpms</i>	<i>Vim</i>	
<i>Cd93</i>	<i>Fry</i>	<i>Maml3</i>	<i>Slit3</i>	<i>Zfhx3</i>	
Endothelial					
<i>Adgrf5</i>	<i>Ctla2a</i>	<i>Fgd5</i>	<i>Kdr</i>	<i>Pi16</i>	<i>Samd12</i>
<i>Adgrl4</i>	<i>Cyrr1</i>	<i>Flt1</i>	<i>Klf13</i>	<i>Plat</i>	<i>Serinc3</i>
<i>Akt3</i>	<i>Dlc1</i>	<i>Fmnl2</i>	<i>Klf3</i>	<i>Plcb4</i>	<i>Serpinh1</i>
<i>Anxa3</i>	<i>Dnm3</i>	<i>Fry</i>	<i>Klf7</i>	<i>Plekkg1</i>	<i>Sgk1</i>
<i>Arhgap31</i>	<i>Dock4</i>	<i>Gimap5</i>	<i>Ldb2</i>	<i>Plpp1</i>	<i>Shank3</i>
<i>Bst2</i>	<i>Ebf1</i>	<i>Gimap6</i>	<i>Lima1</i>	<i>Plpp3</i>	<i>Slc9a3r2</i>
<i>Cavin3</i>	<i>Ecscr</i>	<i>Gm20388</i>	<i>Lims2</i>	<i>Plvap</i>	<i>Smad6</i>
<i>Cd151</i>	<i>Efna5</i>	<i>Gm4070</i>	<i>Lrrfp1</i>	<i>Plxna4</i>	<i>Snaip</i>
<i>Cd200</i>	<i>Egfl7</i>	<i>Gnaq</i>	<i>Ly6c1</i>	<i>Podxl</i>	<i>Sparc</i>
<i>Cd24a</i>	<i>Ehd3</i>	<i>Gng11</i>	<i>Maml2</i>	<i>Prex2</i>	<i>Synpo</i>

<i>Cd300lg</i>	<i>Ehd4</i>	<i>Gpihbp1</i>	<i>Mef2c</i>	<i>Ptk2</i>	<i>Tcf4</i>
<i>Cd34</i>	<i>Elmo1</i>	<i>Heg1</i>	<i>Meis2</i>	<i>Ptprb</i>	<i>Tcf7l1</i>
<i>Cd93</i>	<i>Eln</i>	<i>Hip1</i>	<i>Mill2</i>	<i>Ptprj</i>	<i>Tcim</i>
<i>Cdh13</i>	<i>Emcn</i>	<i>Hspb1</i>	<i>Mmrn2</i>	<i>Ptpm</i>	<i>Tek</i>
<i>Cdh5</i>	<i>Eng</i>	<i>Icam2</i>	<i>Mxd4</i>	<i>Ramp2</i>	<i>Tinagl1</i>
<i>Chn2</i>	<i>Entpd1</i>	<i>Id1</i>	<i>Nfib</i>	<i>Rapgef1</i>	<i>Tm4sf1</i>
<i>Chrm3</i>	<i>Epas1</i>	<i>Id3</i>	<i>Nrp1</i>	<i>Rapgef4</i>	<i>Tmtc1</i>
<i>Clic4</i>	<i>Esam</i>	<i>Igfbp3</i>	<i>Pbx1</i>	<i>Rapgef5</i>	<i>Vamp5</i>
<i>Col4a1</i>	<i>Fabp4</i>	<i>Igfbp5</i>	<i>Pcdh9</i>	<i>Rgcc</i>	<i>Zeb1</i>
<i>Col4a2</i>	<i>Fam167b</i>	<i>Inpp4b</i>	<i>Pde3a</i>	<i>Rhoc</i>	
<i>Crip2</i>	<i>Fbxl7</i>	<i>Itga9</i>	<i>Pecam1</i>	<i>S100a16</i>	
VSMC					
<i>Acta2</i>	<i>Ctgf</i>	<i>Gucy1b3</i>	<i>Mxra8</i>	<i>Pdgfrb</i>	<i>Sep-04</i>
<i>Actn1</i>	<i>Des</i>	<i>Itga8</i>	<i>Myh11</i>	<i>Pdlim2</i>	<i>Serpine2</i>
<i>Adamts5</i>	<i>Dkk2</i>	<i>Kcna5</i>	<i>Myh9</i>	<i>Pln</i>	<i>Serping1</i>
<i>Aebp1</i>	<i>Ecm1</i>	<i>Lbh</i>	<i>Ndufa4l2</i>	<i>Ppp1r14a</i>	<i>Serpini1</i>
<i>Agtr1a</i>	<i>Emilin1</i>	<i>Lhfp</i>	<i>Ngf</i>	<i>Ptrf</i>	<i>Sncg</i>
<i>Akr1b7</i>	<i>Fhl2</i>	<i>Lmod1</i>	<i>Notch3</i>	<i>Rarres2</i>	<i>Sparcl1</i>
<i>Atp1b2</i>	<i>Flna</i>	<i>Lrp1</i>	<i>Nrip2</i>	<i>Rasgrp2</i>	<i>Tagln</i>
<i>Bgn</i>	<i>Fxyd1</i>	<i>Map3k7cl</i>	<i>Olfr558</i>	<i>Rasl11a</i>	<i>Tpm2</i>
<i>Cfh</i>	<i>Gm13861</i>	<i>Mgp</i>	<i>Pcolce</i>	<i>Rcn3</i>	
<i>Col1a2</i>	<i>Gpc6</i>	<i>Mir143hg</i>	<i>Pcp4l1</i>	<i>Rergl</i>	
<i>Cox4i2</i>	<i>Gucy1a3</i>	<i>Mustn1</i>	<i>Pdgfra</i>	<i>Rgs5</i>	
Neutrophil					
<i>Alox5ap</i>	<i>Fxyd5</i>	<i>Hp</i>	<i>Junb</i>	<i>Retnlg</i>	<i>S100a9</i>
<i>Ccl6</i>	<i>Gmfg</i>	<i>Ifitm1</i>	<i>Mxd1</i>	<i>S100a6</i>	<i>Srgn</i>
<i>Dusp1</i>	<i>Hdc</i>	<i>Il1b</i>	<i>Pglyrp1</i>	<i>S100a8</i>	<i>Tyrobp</i>
<i>Fos</i>					
Granulocyte					
<i>AA467197</i>	<i>Cd44</i>	<i>Ets2</i>	<i>Lilr4b</i>	<i>Nfkbiz</i>	<i>Samsn1</i>
<i>Basp1</i>	<i>Clec4d</i>	<i>Fbxl5</i>	<i>Lsp1</i>	<i>Nlrp3</i>	<i>Selplg</i>
<i>Bcl2a1b</i>	<i>Clec4e</i>	<i>Fosl2</i>	<i>Lst1</i>	<i>Pirb</i>	<i>Slc7a11</i>
<i>C5ar1</i>	<i>Clec4n</i>	<i>Gcnt2</i>	<i>Marcks</i>	<i>Pla2g7</i>	<i>Slfn1</i>
<i>Ccl3</i>	<i>Csf3r</i>	<i>Gda</i>	<i>Marcksl1</i>	<i>Plaur</i>	<i>Slpi</i>
<i>Ccr1</i>	<i>Csrnp1</i>	<i>Gpsm3</i>	<i>Mcemp1</i>	<i>Plek</i>	<i>Spi1</i>
<i>Ccr12</i>	<i>Cxcr4</i>	<i>Hcar2</i>	<i>Mxd1</i>	<i>Ptafr</i>	<i>Tnfaip2</i>
<i>Cd300c2</i>	<i>Cyp4f18</i>	<i>Il1r2</i>	<i>Ncf2</i>	<i>Ptgs2</i>	<i>Trib1</i>
<i>Cd300lf</i>	<i>Dennd4a</i>	<i>Il1rn</i>	<i>Ndel1</i>	<i>Pygl</i>	<i>Zyx</i>
Monocyte					
<i>AA467197</i>	<i>Cd300a</i>	<i>Emp3</i>	<i>Ifi207</i>	<i>Ms4a4c</i>	<i>Pou2f2</i>
<i>Adam8</i>	<i>Cd68</i>	<i>F13a1</i>	<i>Ifi27l2a</i>	<i>Ms4a6b</i>	<i>S100a4</i>
<i>Ap1s2</i>	<i>Cfp</i>	<i>Fcgr1</i>	<i>Ifitm6</i>	<i>Ms4a6c</i>	<i>Samhd1</i>
<i>Arg1</i>	<i>Chil3</i>	<i>Fcgr2b</i>	<i>Itgal</i>	<i>Ms4a6d</i>	<i>Sirpb1c</i>
<i>Basp1</i>	<i>Clec4a1</i>	<i>Fcgr4</i>	<i>Itgb2</i>	<i>Msr1</i>	<i>Tgfb1</i>
<i>C3ar1</i>	<i>Clec4a2</i>	<i>Fn1</i>	<i>Lrp1</i>	<i>Nr4a1</i>	<i>Thbs1</i>
<i>Ccl2</i>	<i>Clec4a3</i>	<i>Fyb</i>	<i>Lst1</i>	<i>Pid1</i>	<i>Zeb2</i>
<i>Ccl6</i>	<i>Csf1r</i>	<i>Gda</i>	<i>Lyn</i>	<i>Pla2g7</i>	
<i>Ccl9</i>	<i>Cybb</i>	<i>Gngt2</i>	<i>Mafb</i>	<i>Plac8</i>	
<i>Ccr2</i>	<i>Ecm1</i>	<i>Hmox1</i>	<i>Mpeg1</i>	<i>Pld4</i>	
B Cell					
<i>Apobec3</i>	<i>Cnp</i>	<i>Gm8369</i>	<i>Ighd</i>	<i>Mef2c</i>	<i>Sell</i>
<i>Bank1</i>	<i>Ebf1</i>	<i>H2-Aa</i>	<i>Ighm</i>	<i>Ms4a1</i>	<i>Serpinb1a</i>
<i>Cd19</i>	<i>Ets1</i>	<i>H2-Ab1</i>	<i>Igkc</i>	<i>Ms4a4c</i>	<i>Siglecg</i>
<i>Cd2</i>	<i>Fcer2a</i>	<i>H2-DMb2</i>	<i>Iglc2</i>	<i>Mzb1</i>	<i>Tnfrsf13c</i>
<i>Cd37</i>	<i>Fcmr</i>	<i>H2-Eb1</i>	<i>Iglc3</i>	<i>Ptpn6</i>	<i>Vpreb3</i>
<i>Cd53</i>	<i>Fcrla</i>	<i>H2-Oa</i>	<i>Limd2</i>	<i>Ptprcap</i>	
<i>Cd55</i>	<i>Foxp1</i>	<i>H2-Ob</i>	<i>Lsp1</i>	<i>Rac2</i>	
<i>Cd79a</i>	<i>Gimap6</i>	<i>Hmha1</i>	<i>Ltb</i>	<i>Ralgps2</i>	
<i>Cd79b</i>	<i>Gm43603</i>	<i>Hvcn1</i>	<i>Ly6d</i>	<i>Rho</i>	
T Cell					
<i>Arhgap15</i>	<i>Cd3g</i>	<i>Fgl2</i>	<i>Hmha1</i>	<i>Ltb</i>	<i>Selplg</i>
<i>Arhgap45</i>	<i>Cd48</i>	<i>Gimap1</i>	<i>Il7r</i>	<i>Ly6c2</i>	<i>Sep-01</i>
<i>B4galnt1</i>	<i>Ctsw</i>	<i>Gimap3</i>	<i>Itgb2</i>	<i>Ms4a4b</i>	<i>Skap1</i>
<i>Ccl5</i>	<i>Cxcr3</i>	<i>Gimap4</i>	<i>Itgb7</i>	<i>Ms4a6b</i>	<i>Slamf6</i>

<i>Ccnd3</i>	<i>Cxcr6</i>	<i>Gimap6</i>	<i>Lat</i>	<i>Nkg7</i>	<i>Tcf7</i>
<i>Ccr7</i>	<i>Dapl1</i>	<i>Gimap7</i>	<i>Lck</i>	<i>Ptpn22</i>	<i>Thy1</i>
<i>Cd2</i>	<i>Dusp2</i>	<i>Gm2682</i>	<i>Lef1</i>	<i>Ptpnc</i>	<i>Trac</i>
<i>Cd28</i>	<i>Dusp5</i>	<i>Gm8369</i>	<i>Lgals1</i>	<i>Ptpncap</i>	<i>Trbc1</i>
<i>Cd3d</i>	<i>Epsti1</i>	<i>H2-K1</i>	<i>Limd2</i>	<i>Rac2</i>	<i>Vps37b</i>
<i>Cd3e</i>	<i>Ets1</i>	<i>Hcst</i>	<i>Lsp1</i>	<i>S100a4</i>	<i>Xcl1</i>

Appendix 11: HIF Target Genes

Mouse Orthologues of HIF Target Genes						
<i>A4galt</i>	<i>Cdcp1</i>	<i>Fbxl8</i>	<i>Kdm4b</i>	<i>P4hb</i>	<i>Rnf217</i>	<i>Tiparp</i>
<i>Abca3</i>	<i>Cdh10</i>	<i>Fbxo17</i>	<i>Kdm4c</i>	<i>Pabpc1</i>	<i>Rnf223</i>	<i>Tk1</i>
<i>Abca7</i>	<i>Cdk19</i>	<i>Fbxo21</i>	<i>Kdm5b</i>	<i>Pacsin2</i>	<i>Rnf24</i>	<i>Tle1</i>
<i>Abcb6</i>	<i>Cdk2ap2</i>	<i>Fbxo42</i>	<i>Kdm5c</i>	<i>Padi1</i>	<i>Rnf4</i>	<i>Tll1</i>
<i>Abhd12</i>	<i>Cdkn1b</i>	<i>Fchsd2</i>	<i>Kdm6b</i>	<i>Pafah1b1</i>	<i>Rnmt</i>	<i>Tm4sf1</i>
<i>Abhd6</i>	<i>Cdkn1c</i>	<i>Fem1c</i>	<i>Keap1</i>	<i>Pag1</i>	<i>Rnpep</i>	<i>Tm4sf5</i>
<i>Abr</i>	<i>Ceacam1</i>	<i>Fgd4</i>	<i>Khdrbs1</i>	<i>Paics</i>	<i>Rnpepl1</i>	<i>Tmem102</i>
<i>Acadvl</i>	<i>Cebpd</i>	<i>Fgd5</i>	<i>Kif12</i>	<i>Palm</i>	<i>Rock2</i>	<i>Tmem123</i>
<i>Acap1</i>	<i>Celsr2</i>	<i>Fgf11</i>	<i>Kif21a</i>	<i>Pam</i>	<i>Ror2</i>	<i>Tmem129</i>
<i>Acsf3</i>	<i>Celsr3</i>	<i>Fgfr1</i>	<i>Klf10</i>	<i>Paqr5</i>	<i>Rpap1</i>	<i>Tmem139</i>
<i>Actb</i>	<i>Cep250</i>	<i>Fggy</i>	<i>Klf5</i>	<i>Paqr7</i>	<i>Rph3al</i>	<i>Tmem140</i>
<i>Actg1</i>	<i>Cep350</i>	<i>Fhl2</i>	<i>Klf6</i>	<i>Paqr8</i>	<i>Rpl3</i>	<i>Tmem141</i>
<i>Adam8</i>	<i>Cerk</i>	<i>Fkbp8</i>	<i>Klhl38</i>	<i>Parp16</i>	<i>Rps6ka4</i>	<i>Tmem143</i>
<i>Adamts13</i>	<i>Chd7</i>	<i>Fkbp9</i>	<i>Krcc1</i>	<i>Patl1</i>	<i>Rraga</i>	<i>Tmem170b</i>
<i>Adap1</i>	<i>Cherp</i>	<i>Flii</i>	<i>Krt15</i>	<i>Pbrm1</i>	<i>Rrp1</i>	<i>Tmem181a</i>
<i>Adar</i>	<i>Chml</i>	<i>Flnb</i>	<i>Krt18</i>	<i>Pcx</i>	<i>Rsb1</i>	<i>Tmem214</i>
<i>Add1</i>	<i>Chrac1</i>	<i>Flot2</i>	<i>Krt19</i>	<i>Pcbp1</i>	<i>Rundc3b</i>	<i>Tmem248</i>
<i>Adm</i>	<i>Chrn1b</i>	<i>Fn1</i>	<i>Krt8</i>	<i>Pcgf5</i>	<i>Runx1</i>	<i>Tmem37</i>
<i>Aff1</i>	<i>Chst15</i>	<i>Fndc3b</i>	<i>Ksr2</i>	<i>Pdcd6ip</i>	<i>Runx2</i>	<i>Tmem39b</i>
<i>Agap3</i>	<i>Chsy1</i>	<i>Fnip1</i>	<i>Kxd1</i>	<i>Pde10a</i>	<i>Runx3</i>	<i>Tmem44</i>
<i>Agbl5</i>	<i>Cic</i>	<i>Fntb</i>	<i>Lama5</i>	<i>Pde4c</i>	<i>Ruvbl2</i>	<i>Tmem45a</i>
<i>Agfg1</i>	<i>Cited2</i>	<i>Fos</i>	<i>Lamp1</i>	<i>Pde9a</i>	<i>Rxa</i>	<i>Tmem45b</i>
<i>Agfg2</i>	<i>Ckb</i>	<i>Fosl1</i>	<i>Lbh</i>	<i>Pdgfa</i>	<i>S100a10</i>	<i>Tmem51</i>
<i>Aggf1</i>	<i>Cldn3</i>	<i>Fosl2</i>	<i>Ldha</i>	<i>Pdgfb</i>	<i>S100a4</i>	<i>Tmem63b</i>
<i>Ago2</i>	<i>Clic6</i>	<i>Foxa2</i>	<i>Leo1</i>	<i>Pdgfc</i>	<i>Samd4</i>	<i>Tmod3</i>
<i>Agpat2</i>	<i>Clk3</i>	<i>Foxd1</i>	<i>Lgals1</i>	<i>Pdgfr1</i>	<i>Sap30</i>	<i>Tmsb10</i>
<i>Agpat5</i>	<i>Clpb</i>	<i>Foxl1</i>	<i>Lgals8</i>	<i>Pdia3</i>	<i>Sart1</i>	<i>Tmtc2</i>
<i>Ahd1c</i>	<i>Cmip</i>	<i>Foxo1</i>	<i>Lgmn</i>	<i>Pdia5</i>	<i>Sav1</i>	<i>Tmub1</i>
<i>Ahnak</i>	<i>Cmtm3</i>	<i>Foxo3</i>	<i>Lhpl2</i>	<i>Pdk1</i>	<i>Sbf2</i>	<i>Tnfaip3</i>
<i>Ahr</i>	<i>Cnih3</i>	<i>Fscn1</i>	<i>Lhpp</i>	<i>Pdk3</i>	<i>Sbk2</i>	<i>Tnfaip8l1</i>
<i>Ahsg</i>	<i>Cnnm1</i>	<i>Fscn2</i>	<i>Lhx2</i>	<i>Pdlim1</i>	<i>Scaf11</i>	<i>Tnfrsf10b</i>
<i>Ajuba</i>	<i>Cnot8</i>	<i>Fth1</i>	<i>Limch1</i>	<i>Pdlim2</i>	<i>Scamp2</i>	<i>Tnfrsf10b</i>
<i>Ak4</i>	<i>Col6a1</i>	<i>Fto</i>	<i>Lin54</i>	<i>Pdlim4</i>	<i>Scarb1</i>	<i>Tnfrsf10b</i>
<i>Alcam</i>	<i>Col6a2</i>	<i>Fut11</i>	<i>Llgl1</i>	<i>Pdlim7</i>	<i>Scd1</i>	<i>Tnfrsf19</i>
<i>Aldh16a1</i>	<i>Cotl1</i>	<i>Fxyd1</i>	<i>Lmcd1</i>	<i>Pdxk</i>	<i>Scd2</i>	<i>Tnip1</i>
<i>Aldh1a3</i>	<i>Cox20</i>	<i>Fzd7</i>	<i>Lmo2</i>	<i>Peg3</i>	<i>Scn1b</i>	<i>Tnnt1</i>
<i>Aldh3b1</i>	<i>Cp</i>	<i>Fzd8</i>	<i>Lonp1</i>	<i>Pelp1</i>	<i>Scn9a</i>	<i>Tns1</i>
<i>Aldoa</i>	<i>Cpox</i>	<i>Fzr1</i>	<i>Lox</i>	<i>Per2</i>	<i>Scnn1b</i>	<i>Tns3</i>
<i>Aldoc</i>	<i>Crebzf</i>	<i>G6pc3</i>	<i>Loxl2</i>	<i>Perp</i>	<i>Scyl1</i>	<i>Tns4</i>
<i>Alkbh5</i>	<i>Crkl</i>	<i>Gaa</i>	<i>Loxl4</i>	<i>Pfkfb4</i>	<i>Sdc1</i>	<i>Tollip</i>
<i>Alox12b</i>	<i>Crtac1</i>	<i>Gabre</i>	<i>Lpcat1</i>	<i>Pfkl</i>	<i>Sdc2</i>	<i>Tomm20</i>
<i>Alpk2</i>	<i>Cryab</i>	<i>Gadd45b</i>	<i>Lrfn4</i>	<i>Pfkb</i>	<i>Sec14l4</i>	<i>Tor4a</i>
<i>Alpl</i>	<i>Cryl1</i>	<i>Gal3st1</i>	<i>Lrp1</i>	<i>Pgam1</i>	<i>Sec24a</i>	<i>Trp53inp2</i>
<i>Amigo2</i>	<i>Csrnp1</i>	<i>Galnt18</i>	<i>Lrp2bp</i>	<i>Pgap3</i>	<i>Sec61g</i>	<i>Tpbp</i>
<i>Amotl2</i>	<i>Cstb</i>	<i>Gapdh</i>	<i>Lrp5</i>	<i>Pgk1</i>	<i>Sema4b</i>	<i>Tpd52</i>
<i>Anapc16</i>	<i>Ctbp2</i>	<i>Gas2l3</i>	<i>Lrrc25</i>	<i>Pgm1</i>	<i>Sema4f</i>	<i>Tpi1</i>
<i>Angptl4</i>	<i>Ctdp1</i>	<i>Gbe1</i>	<i>Lrrc26</i>	<i>Pgrmc2</i>	<i>Sema5b</i>	<i>Traf3ip1</i>
<i>Ankrd13a</i>	<i>Ctdsp1</i>	<i>Gbf1</i>	<i>Lrrc3</i>	<i>Phf3</i>	<i>Sephs2</i>	<i>Tram2</i>
<i>Ankrd33b</i>	<i>Ctif</i>	<i>Gcat</i>	<i>Lrrk1</i>	<i>Pi4k2a</i>	<i>Serbp1</i>	<i>Trappc9</i>
<i>Ankrd37</i>	<i>Ctsd</i>	<i>Gdf15</i>	<i>Lsp1</i>	<i>Pias2</i>	<i>Sergef</i>	<i>Trdmt1</i>
<i>Ankzf1</i>	<i>Cuedc1</i>	<i>Gfra2</i>	<i>Ltpb3</i>	<i>Picalm</i>	<i>Serinc2</i>	<i>Trib1</i>
<i>Ano6</i>	<i>Cux1</i>	<i>Gja3</i>	<i>Luzp1</i>	<i>Pigz</i>	<i>Serinc5</i>	<i>Trib2</i>

Mouse Orthologues of HIF Target Genes						
Anxa2	Cxcl16	Gjb2	Lypla2	Pih1d1	Serpina1b	Trtb3
Anxa4	Cxcr4	Gjc2	Lzts2	Pik3r1	Serpina1e	Trim33
Anxa6	Cyb5d1	Glg1	Mab21l3	Pim1	Serpina1a	Trim56
Ap1m1	Cyb5r2	Glrx	Macrocl	Pitpnm1	Serpina6a	Trim8
Ap3d1	Cyb5r3	Gmcl1	Maea	Pitrm1	Serpina9	Trio
Apaf1	Cyp26b1	Gng4	Maf	Pkm	Serpine1	Triobp
Apoa4	Cyp2s1	Golga7b	Maf1	Pkp2	Sertad1	Trpm4
Apoc3	Cyth1	Gorasp2	Maff	Pkp3	Sertad2	Trpt1
Apol9a	Dab2ip	Gphn	Mafk	Pla2g4d	Setbp1	Trpv4
Apol8	Dag1	Gpi1	Mall	Plac8	Setd1a	Tsc2
Apold1	Dapk1	Gpnmb	Maml3	Plcb1	Sgcd	Tsc22d3
Aqr	Dapk2	Gpr157	Map1lc3a	Plcd1	Sh2d4a	Tsku
Arhgap18	Dbn1	Gprc5a	Map2k1	Plec	Sh3gl1	Tspan15
Arhgap35	Dclk2	Gprc5c	Map3k1	Plekha1	Sh3pxd2a	Tspan2
Arhgdia	Dcp1b	Gprin2	Map3k8	Plekha2	Shank2	Tspo
Arhgef12	Ddit4	Gpt2	Map3k9	Plekhd1	Siah2	Tspyl5
Arhgef16	Ddx41	Gpx3	Mapk13	Plin2	Sik1	Tsr1
Arhgef28	Dennd1a	Gramd4	Mapk14	Plod1	Sipa1	Tst
Arhgef37	Dennd2a	Grb10	Mapk7	Plod2	Slain2	Ttc3
Arhgef39	Dhcr7	Greb1	Marveld1	Plod3	Slamf7	Ttc30a1
Arid2	Dhrs13	Grem1	Mbd3	Plscr4	Slc11a1	Ttyh3
Arid3a	Dhrs3	Grid1	Mc1r	Plxnd1	Slc11a2	Tubb1
Arid3b	Dhx30	Grk6	Mctp2	Pmepa1	Slc16a3	Tubb3
Arid5a	Disp2	Gse1	Med23	Pnma2	Slc17a5	Tubb6
Arl10	Dixdc1	Gsk3a	Mef2d	Pnrc1	Slc1a1	Tubgcp2
Arl8a	Dnah11	Gsn	Megf6	Polr2e	Slc1a2	Tulp3
Artn	Dnah5	Gtf3c1	Met	Polrmt	Slc22a18	Tulp4
Asap2	Dnajb2	Gtpbp1	Mfsd10	Pom121	Slc25a36	Txnrd1
Asb2	Dnm2	Gtse1	Mfsd2a	Pou2f1	Slc2a1	Uaca
Ascl2	Dnttip1	Gys1	Mfsd3	Ppan	Slc2a3	Ubc
Asgr1	Dock6	Hagh	Mgat5b	Ppard	Slc30a10	Ube2o
Ash1l	Dot1l	Havcr1	Mib2	Ppfia3	Slc35e1	Ube2q1
Asph	Dpcd	Hcfc1r1	Midn	Ppfia4	Slc38a1	Ubqln1
Aspscr1	Dpp9	Hdac11	Mknk2	Ppfibp2	Slc38a2	Ucn2
Atf3	Dpysl2	Hdac3	Mltt6	Ppme1	Slc45a1	Ufsp2
Atg16l1	Dpysl3	Hectd1	Mmrn2	Ppp1r13l	Slc45a3	Ugp2
Atg4b	Dsp	Heg1	Mnt	Ppp1r3b	Slc4a11	Uhrf1
Atg4d	Dtna	Helz	Moap1	Ppp1r3c	Slc51a	Ulk1
Atg9a	Dusp1	Herc3	Mob3a	Ppp1r3e	Slc6a6	Unc93b1
Atl1	Dusp5	Hes1	Mospd3	Ppp1r3g	Slc6a8	Upk1a
Atoh8	Dync1h1	Hes2	Mpi	Ppp2r5b	Slc7a2	Upk1b
Atp10a	Dynlrb1	Hey1	Mpst	Preld2	Slc7a5	Uprt
Atp11a	Dyrk1a	Hfe	Mrgbp	Prex2	Slc9a3r1	Usp5
Atp13a2	Dyrk1b	Hgs	Mt1	Prickle3	Slc9a3r2	Usp7
Atp8b3	Ech1	Hilpda	Mtff1	Prkaa2	Slx4	Vdac1
Atp9a	Edem1	Hip1	Mthfd1l	Prkar1b	Smad3	Vegfa
Atrn	Edf1	Hivep2	Mtss1	Prkca	Smad7	Vegfc
Atxn1	Edn2	Hk1	Muc1	Proc	Smad2	Vgf
Avpi1	Eef1g	Hk2	Muc5b	Proser2	Smarca1	Vgll4
Axin1	Eefsec	H13	Mvd	Prox1	Smarcc1	Vill
Axin2	Efh2	Hmox1	Mxi1	Prr7	Smarcd2	Vkorc1
Axl	Efna1	Hnf1b	Myh10	Prrc2b	Smc2	Vldlr
B3gnt4	Efna3	Hnrnpk	Myh9	Prss53	Smcr8	Vps37a
B9d2	Efna5	Hnrnpl	Myllk4	Prtn3	Smim3	Vps37d
Bach1	Egfl7	Hnrnpu	Myo18a	Prx	Smpd13a	Vps53
Bag3	Egfr	Hoxb8	Myo1b	Psd	Smurf1	Vsig10l
Bbx	Egln1	Hoxb9	Myof	Psd4	Smyd2	Vwa1
Bckdha	Egln3	Hpcal1	Mysm1	Ptbp1	Smyd3	Wasf2
Bckdk	Eif2ak3	Hpn	Mzt2	Ptch1	Snape3	Wbp11
Bcl3	Eif3a	Hps3	N4bp2l1	Ptprm	Snx33	Wdr18
Bcl6	Eif3d	Hr	N4bp3	Ptprs	Sorl1	Wdr26
Begain	Eif4b	Hs3st3b1	Nags	Ptpru	Sos1	Wdr45b
Bhlhe40	Eif4g3	Hsp90ab1	Nalcn	Pttg1ip	Sowahd	Wdr54
Bhlhe41	Eif4h	Iba57	Narf	Ptx3	Sox12	Wdr72
Bicd1	Elavl1	Icam1	Nat10	Pwwp2b	Sp5	Wdr82
Bik	Elf3	Icam5	Nbas	Pxdn	Spag4	Wnt11

Mouse Orthologues of HIF Target Genes						
<i>Birc3</i>	<i>Elk3</i>	<i>Icosl</i>	<i>Ncbp2</i>	<i>Pygl</i>	<i>Spns2</i>	<i>Wnt6</i>
<i>Birc6</i>	<i>El1</i>	<i>Id1</i>	<i>Ncf2</i>	<i>Qsox1</i>	<i>Spock2</i>	<i>Wrip1</i>
<i>Blcap</i>	<i>El12</i>	<i>Id2</i>	<i>Nckap5</i>	<i>R3hdm4</i>	<i>Spred1</i>	<i>Wsb1</i>
<i>Bmp4</i>	<i>Emd</i>	<i>Idh2</i>	<i>Nckipsd</i>	<i>Rab11b</i>	<i>Spry1</i>	<i>Wsb2</i>
<i>Bmp6</i>	<i>Emp3</i>	<i>Ier2</i>	<i>Ncoa4</i>	<i>Rab15</i>	<i>Spryd3</i>	<i>Wt1</i>
<i>Bnc2</i>	<i>Eno1</i>	<i>Ilfo1</i>	<i>Ncor2</i>	<i>Rab20</i>	<i>Sptlc2</i>	<i>Wwc1</i>
<i>Bnip3</i>	<i>Eno2</i>	<i>Ift140</i>	<i>Ndel1</i>	<i>Rab30</i>	<i>Srebf1</i>	<i>Wwc2</i>
<i>Bnip3l</i>	<i>Enpp3</i>	<i>Igf1r</i>	<i>Ndr1</i>	<i>Rab37</i>	<i>Srsf9</i>	<i>Xylt1</i>
<i>Brsk2</i>	<i>Epas1</i>	<i>Igf2bp2</i>	<i>Nebi</i>	<i>Rab3a</i>	<i>Ssbp4</i>	<i>Yeats2</i>
<i>Bsg</i>	<i>Epb41l1</i>	<i>Igfbp4</i>	<i>Nedd4</i>	<i>Rab3d</i>	<i>Ssh1</i>	<i>Ypel2</i>
<i>Btbd16</i>	<i>Epo</i>	<i>Igsf11</i>	<i>Nedd9</i>	<i>Rab40b</i>	<i>Sspn</i>	<i>Ypel5</i>
<i>Btbd2</i>	<i>Eps8l2</i>	<i>Il1rap</i>	<i>Nek6</i>	<i>Rab42</i>	<i>Sstr3</i>	<i>Ywhag</i>
<i>Btg1</i>	<i>Erbb2</i>	<i>Ilvbl</i>	<i>Nelfb</i>	<i>Rabggta</i>	<i>St3gal1</i>	<i>Zbtb1</i>
<i>1810055G02Rik</i>	<i>Erbb3</i>	<i>Imp3</i>	<i>Nfe2l1</i>	<i>Rabl6</i>	<i>St6gal1</i>	<i>Zbtb25</i>
<i>2310030G06Rik</i>	<i>Ercc1</i>	<i>Incenp</i>	<i>Nfic</i>	<i>Rac2</i>	<i>St8sia6</i>	<i>Zc3hav11</i>
<i>Al837181</i>	<i>Erf</i>	<i>Inha</i>	<i>Nfil3</i>	<i>Rac3</i>	<i>Stbd1</i>	<i>Zcchc2</i>
<i>2010003K11Rik</i>	<i>Ergic1</i>	<i>Inhbb</i>	<i>Nfix</i>	<i>Rad23a</i>	<i>Stc2</i>	<i>Zcchc3</i>
<i>1700017B05Rik</i>	<i>Ermp1</i>	<i>Ino80d</i>	<i>Nfkb2</i>	<i>Rad51b</i>	<i>Steap3</i>	<i>Zdhhc7</i>
<i>AA986860</i>	<i>Errf1</i>	<i>Insig1</i>	<i>Nfrkb</i>	<i>Rad54b</i>	<i>Stk32c</i>	<i>Zfhx3</i>
<i>C2cd3</i>	<i>Espn</i>	<i>Insig2</i>	<i>Nfs1</i>	<i>Rai1</i>	<i>Stk39</i>	<i>Zfp36</i>
<i>1700003E16Rik</i>	<i>Esyt1</i>	<i>Insr</i>	<i>Ngly1</i>	<i>Ralgapa2</i>	<i>Stmnd1</i>	<i>Zfp64</i>
<i>C3</i>	<i>Esyt2</i>	<i>Ints1</i>	<i>Nkd1</i>	<i>Ralgds</i>	<i>Stxbp1</i>	<i>Zfpm1</i>
<i>1810037117Rik</i>	<i>Etfb</i>	<i>Ipo13</i>	<i>Nog</i>	<i>Rap1gap</i>	<i>Sult2b1</i>	<i>Zfyve28</i>
<i>9530068E07Rik</i>	<i>Ets2</i>	<i>Irf2bp2</i>	<i>Nol3</i>	<i>Rap2b</i>	<i>Sumf1</i>	<i>Zhx2</i>
<i>9930012K11Rik</i>	<i>Etv3</i>	<i>Itga5</i>	<i>Nop14</i>	<i>Rapgef6</i>	<i>Susd3</i>	<i>Zic2</i>
<i>Car12</i>	<i>Exoc3</i>	<i>Itga6</i>	<i>Notch1</i>	<i>Rara</i>	<i>Sybu</i>	<i>Zkscan2</i>
<i>Car9</i>	<i>Exoc3l4</i>	<i>Itgal</i>	<i>Npepps</i>	<i>Rasd1</i>	<i>Syde1</i>	<i>Zmiz2</i>
<i>Cabin1</i>	<i>Exosc5</i>	<i>Itgb2</i>	<i>Nptxr</i>	<i>Rassf2</i>	<i>Syncrip</i>	<i>Zmynd15</i>
<i>Cables1</i>	<i>Ext1</i>	<i>Itgb3</i>	<i>Npw</i>	<i>Rassf3</i>	<i>Syt17</i>	<i>Zmynd8</i>
<i>Cacng6</i>	<i>Eya2</i>	<i>Itgb4</i>	<i>Nr2f2</i>	<i>Rassf7</i>	<i>Syt7</i>	<i>Zfp148</i>
<i>Calb1</i>	<i>Ezr</i>	<i>Itgb5</i>	<i>Nr2f6</i>	<i>Raver1</i>	<i>Szrd1</i>	<i>Zfp160</i>
<i>Calcoco1</i>	<i>F10</i>	<i>Itpk1</i>	<i>Nr3c1</i>	<i>Rb1</i>	<i>Tacc1</i>	<i>Zfp248</i>
<i>Camk2n1</i>	<i>F2</i>	<i>Jag2</i>	<i>Nr4a1</i>	<i>Rb1cc1</i>	<i>Tada2b</i>	<i>Zfp292</i>
<i>Caprin1</i>	<i>Fa2h</i>	<i>Jam2</i>	<i>Nr4a3</i>	<i>Rbck1</i>	<i>Taf15</i>	<i>Zfp318</i>
<i>Card10</i>	<i>Fabp1</i>	<i>Jmjd6</i>	<i>Nrarp</i>	<i>Rbpj</i>	<i>Taf9b</i>	<i>Zfp385a</i>
<i>Casc3</i>	<i>Fads1</i>	<i>Josd1</i>	<i>Nrf1</i>	<i>Rcor1</i>	<i>Tagln2</i>	<i>Zfp395</i>
<i>Casp14</i>	<i>Fads2</i>	<i>Jun</i>	<i>Nrp1</i>	<i>Rcor2</i>	<i>Tanc2</i>	<i>Zfp469</i>
<i>Casp9</i>	<i>Fahd1</i>	<i>Junb</i>	<i>Nrsn2</i>	<i>Reep1</i>	<i>Tbc1d22a</i>	<i>Zfp532</i>
<i>Cav1</i>	<i>Fam107b</i>	<i>Kank3</i>	<i>Nsmf</i>	<i>Rela</i>	<i>Tbc1d4</i>	<i>Zfp160</i>
<i>Cblb</i>	<i>Fam110c</i>	<i>Kansl1</i>	<i>Nt5e</i>	<i>Rexo4</i>	<i>Tbc1d8</i>	<i>Zfp534</i>
<i>Cbx2</i>	<i>Fam13a</i>	<i>Kat6b</i>	<i>Nudt13</i>	<i>Rgs12</i>	<i>Tbl1xr1</i>	<i>Zfp677</i>
<i>Cbx4</i>	<i>Fam13c</i>	<i>Kcmf1</i>	<i>Nudt14</i>	<i>Rhobtb1</i>	<i>Tcf3</i>	<i>Zfp458</i>
<i>Cbx5</i>	<i>Fam162a</i>	<i>Kcnh3</i>	<i>Nudt18</i>	<i>Rhof</i>	<i>Tcf7l1</i>	<i>Zfp653</i>
<i>Cbx8</i>	<i>Fam168a</i>	<i>Kcnk1</i>	<i>Nudt22</i>	<i>Rimk1a</i>	<i>Tcfl5</i>	<i>Zfp654</i>
<i>Ccdc106</i>	<i>Fam210a</i>	<i>Kcnk15</i>	<i>Nup210</i>	<i>BC039771</i>	<i>Tcp11l2</i>	<i>Zfp707</i>
<i>Ccdc124</i>	<i>Fam219b</i>	<i>Kcnma1</i>	<i>Nxn</i>	<i>Rinl</i>	<i>Tes</i>	<i>Zfp740</i>
<i>Ccdc177</i>	<i>Fam3a</i>	<i>Kcnmb4</i>	<i>Obsl1</i>	<i>Riok3</i>	<i>Tesc</i>	<i>E430018J23Rik</i>
<i>Ccdc68</i>	<i>Fam3c</i>	<i>Kctd1</i>	<i>Ocel1</i>	<i>Rit1</i>	<i>Tet1</i>	<i>Zfp764</i>
<i>Ccl20</i>	<i>Fam50a</i>	<i>Kctd11</i>	<i>Opa3</i>	<i>Rlf</i>	<i>Tet2</i>	<i>Zfp768</i>
<i>Ccnd1</i>	<i>Fam83d</i>	<i>Kctd14</i>	<i>Opn3</i>	<i>Rmnd5a</i>	<i>Trf</i>	
<i>Ccng2</i>	<i>Fam83e</i>	<i>Kctd17</i>	<i>Orai3</i>	<i>Rnaset2a</i>	<i>Tfap4</i>	
<i>Ccny</i>	<i>Fam83h</i>	<i>Kctd18</i>	<i>Osblp10</i>	<i>Rnf103</i>	<i>Tfrc</i>	
<i>Ccr7</i>	<i>Fam89b</i>	<i>Kctd3</i>	<i>Osmr</i>	<i>Rnf122</i>	<i>Tgfa</i>	
<i>Cd4</i>	<i>Fam98b</i>	<i>Kctd9</i>	<i>Ovol1</i>	<i>Rnf128</i>	<i>Tgfb1</i>	
<i>Cd99l2</i>	<i>Farp1</i>	<i>Kdm1a</i>	<i>Oxsr1</i>	<i>Rnf144b</i>	<i>Tgfb2</i>	
<i>Cdc42bpb</i>	<i>Fbl</i>	<i>Kdm2a</i>	<i>P2ry2</i>	<i>Rnf145</i>	<i>Tgfb1</i>	
<i>Cdc42ep4</i>	<i>Fbrsl1</i>	<i>Kdm2b</i>	<i>P4ha1</i>	<i>Rnf165</i>	<i>Thoc6</i>	
<i>Cdca7l</i>	<i>Fbxl16</i>	<i>Kdm3a</i>	<i>P4ha2</i>	<i>Rnf187</i>	<i>Timp3</i>	

Appendix 12: Genes upregulated in ccRCC

Mouse Orthologues of Upregulated Genes in ccRCC					
<i>Abca1</i>	<i>Doc2a</i>	<i>Hk1</i>	<i>Neto2</i>	<i>Ppp1r13l</i>	<i>Tcaf2</i>
<i>Abcc1</i>	<i>Dpp6</i>	<i>Hk2</i>	<i>Nnmt</i>	<i>Prelid2</i>	<i>Tcerg1</i>
<i>Abcc3</i>	<i>Dscaml1</i>	<i>Hpcal1</i>	<i>Nol3</i>	<i>Prkag2</i>	<i>Tmtc2</i>
<i>Adcy5</i>	<i>Dusp1</i>	<i>Hsf4</i>	<i>Nr3c1</i>	<i>Psmg2</i>	<i>Tnfaip8</i>
<i>Alg13</i>	<i>Efcab3</i>	<i>Ifi205</i>	<i>Osmr</i>	<i>Rlf</i>	<i>Tnip1</i>
<i>Ampd3</i>	<i>Efna5</i>	<i>Immp2l</i>	<i>P4ha1</i>	<i>Rnf145</i>	<i>Tpm1</i>
<i>Angptl4</i>	<i>Egfr</i>	<i>Kcnp4</i>	<i>Pag1</i>	<i>Rnf24</i>	<i>Trim9</i>
<i>Asph</i>	<i>Egln1</i>	<i>Kcnma1</i>	<i>Pam</i>	<i>Scarb1</i>	<i>U2surp</i>
<i>Atxn1</i>	<i>Egln3</i>	<i>Kctd3</i>	<i>Pcsk6</i>	<i>Sema5b</i>	<i>Ube2d2a</i>
<i>Babam2</i>	<i>Eno1</i>	<i>Kdm3a</i>	<i>Pdia5</i>	<i>Sema6a</i>	<i>Ubr3</i>
<i>Barx2</i>	<i>Eno2</i>	<i>Kdm4c</i>	<i>Pdk1</i>	<i>Sgpp2</i>	<i>Utrn</i>
<i>Bicd1</i>	<i>Enpp3</i>	<i>Krba1</i>	<i>Per2</i>	<i>Slc15a4</i>	<i>Vegfa</i>
<i>Birc3</i>	<i>Esyt2</i>	<i>Ldha</i>	<i>Pfkfb3</i>	<i>Slc16a3</i>	<i>Vps13b</i>
<i>Car9</i>	<i>Eva1c</i>	<i>Lpcat1</i>	<i>Pfkfb4</i>	<i>Slc38a1</i>	<i>Wsb1</i>
<i>Cast</i>	<i>Fam13a</i>	<i>Map3k20</i>	<i>Pfkip</i>	<i>Slc41a2</i>	<i>Yeats2</i>
<i>Ccnd1</i>	<i>Fam149a</i>	<i>Mctp2</i>	<i>Pgk1</i>	<i>Slc6a3</i>	<i>Zfp248</i>
<i>Ccnd3</i>	<i>Fndc3b</i>	<i>Met</i>	<i>Phka2</i>	<i>Snd1</i>	<i>Zfp395</i>
<i>Cdk18</i>	<i>Fto</i>	<i>Mgll</i>	<i>Pkm</i>	<i>Sorbs1</i>	<i>Zfp532</i>
<i>Cdkal1</i>	<i>Gas2l3</i>	<i>Mtcp1</i>	<i>Plcb1</i>	<i>Spire1</i>	<i>Zfp608</i>
<i>Col23a1</i>	<i>Gatm</i>	<i>Myo3a</i>	<i>Plod2</i>	<i>Stk39</i>	
<i>Cp</i>	<i>Git2</i>	<i>Myof</i>	<i>Plscr1</i>	<i>Tanc2</i>	
<i>Ddb2</i>	<i>Hilpda</i>	<i>Ncor2</i>	<i>Pou5f1</i>	<i>Tbc1d8</i>	
<i>Dnah11</i>	<i>Hipk2</i>	<i>Ndrp1</i>	<i>Ppfia4</i>	<i>Tbxas1</i>	

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