TARGETING GLUTAMINE METABOLISM IN KIDNEY DEVELOPMENT
AND POLYCYSTIC KIDNEY DISEASE

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Dedication

In memory of my father, Michael A. Flowers.

ACKNOWLEDGEMENTS

Foremost, I would like to express my sincere gratitude to my advisor, Dr. Thomas Carroll, for the continuous support of my Ph.D. study and research, for his patience, motivation, enthusiasm, and immense knowledge. The door to Dr. Carroll's office was always open whenever I ran into difficulty concerning my research. He consistently allowed my work to be my own, but steered me in the right the direction whenever he thought I needed it.

Besides my advisor, I would like to thank the rest of my thesis committee: Dr. John Abrams, Dr. Melanie Cobb, Dr. Ralph DeBerardinis, and Dr. Hao Zhu for not only offering their expertise and insightful comments, but also for the hard questions which incentivized me to widen my research from various perspectives. I would like to offer a special mention to Dr. DeBerardinis and his laboratory for providing assistance with the design and execution of our metabolism-related experiments.

I would like to thank the members of the Carroll Lab for their interesting discussions, especially Genelyn Vadney, for technical assistance. I would also like to thank former Carroll Lab members, Dr. Amrita Das and Dr. Dibyendu Dutta, for their guidance and encouragement throughout their tenure in the lab.

Last but not the least, I would like to thank my family: my parents, Michael and Rosalyn, my brother, Marcus, and my best friend, Monique for supporting me throughout the duration of my graduate studies.

TARGETING GLUTAMINE METABOLISM IN KIDNEY DEVELOPMENT AND POLYCYSTIC KIDNEY DISEASE

By

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DISSERTATION

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

Doctor of Philosophy

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

August 2018

Abstract

Polycystic kidney disease is a hereditary disorder characterized by the progressive manifestation of numerous fluid-filled sacs, known as cysts, within the renal epithelia. The enlargement of the cysts causes the gradual replacement of normal kidney parenchyma which leads to impairment of renal function, and ultimately, renal failure. While the primary causes of PKD are genetic mutations in one of the polycystins that encode the PKD1 and PKD2 proteins, the age of onset and severity of PKD cases greatly varies, suggesting other genes/processes are involved.

Lkb1 is a serine-threonine kinase involved in the regulation of several molecular processes including cellular polarity, autophagy, mTOR signaling, and energetic stress response, all of which are dysregulated in PKD. We generated mice lacking Lkb1 in developing kidney epithelia to establish which of these processes contributed to cyst formation and progression in the absence of Lkb1. Surprisingly, Lkb1 mutant mice showed no defects in renal tubule development or maintenance. However, later studies revealed the co-ablation Lkb1 along with Tsc1, a gene known to play a role in human PKD, within the developing renal epithelia prompted a drastic acceleration in the timing, number, and size of cyst formation. We utilized in vitro cell culture coupled with ex vivo culture of embryonic kidneys with defined media allowed us to determine which metabolic pathways were affected by the deficit of Lkb1.

Our results revealed that Lkb1 mutant cells and embryonic kidneys require glutamine for growth while wild-type cells and kidneys did not. Subsequent studies

demonstrated that Pkd1 embryonic kidneys phenocopied Lkb1 mutant kidneys in respect to their reliance on glutamine for growth. Further investigation into defining which metabolic enzymes/pathways are regulated in normal kidney development and how the absence of Lkb1 or Pkd1 alters these metabolic processes will allow us to gain a greater understanding of the role of metabolism in PKD and potentially lead to t the development of therapeutics to reduce cyst number and/or size.

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List of Abbreviations

4E-BP1 - 4E-binding protein 1

AMP - adenosine monophosphate

AMPK - AMP-activated protein kinase

AKT - Protein kinase B

ADPKD - autosomal dominant polycystic kidney disease

ARE – androgen receptor element

ARPKD - autosomal recessive polycystic kidney disease

ATM – ataxia-telegiectasia mutated

ATP - adenosine triphosphate

BCH - 2-amino-2-norbornanecarboxylic acid

BPTES - bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulfide

CD - collecting duct

CDC- cell division cycle

COX – cyclooxygenases

DEPTOR - DEP domain-containing mTOR-interacting protein

DCT - distal convoluted tubule

DMEM - Dulbecco's Modified Eagle Medium

DMSO – dimethyl sulfoxide

E – embryonic day

EAA – essential amino acid

ECM – extracellular matrix

EMT - epithelial-to-mesenchymal transition

ESRD - end-stage renal disease

ERα – Estrogen receptor alpha

GCL – glutamate-cysteine ligase

GDNF - glial-derived neurotropic factor

GPNA - l-γ-glutamyl-p-nitroanilide

GPX - glutathione peroxidase

GSH - reduced glutathione

GSSG- oxidized glutathione

GST - glutathione-s-transferase

IMCD – inner medullary collecting duct

INR - Insulin receptor

LKB1 - Liver Kinase B1

MARK – microtubule affinity-regulating kinase

MEF – mouse embryonic fibroblast

MM - metanephric mesenchyme

MMP – metalloproteinase

MO25 - mouse protein 25

mLST8/GβL - mammalian lethal with SEC13 protein 8

MSEA - metabolite set enrichment analysis

mTOR - mammalian target of rapamycin

mTORC1 - mammalian target of rapamycin complex 1

mTORC2 - mammalian target of rapamycin complex 2

NEAA - non-essential amino acid

NLS – nuclear localization signal

NRF2 – nuclear factor erythroid 2-related factor 2

NOX - NADPH oxidase

P – postnatal day

PC1 - Polycystin-1

PC2 - Polycystin-2

PCA - Principal Component Analysis

PCNA - proliferating cell nuclear antigen

PJS - Peutz-Jeghers syndrome

PKD - Polycystic kidney disease

PKHD1 - fibrocystin/polyductin

PIKK - phosphoinositide kinase-related kinase

PRAS40 - proline- rich Akt substrate 40

RAPTOR - Regulatory-associated protein of mTOR

RHEB – Ras homolog enriched in brain

RICTOR - Rapamycin-insensitive companion of mammalian target of rapamycin

ROS - reactive oxygen species

S6K - ribosomal protein S6 kinase

SEM - standard error of the means

SIK – salt-inducible kinase

SIN1 - stress-activated protein kinase interacting protein 1

SLC - solute-linked carrier

STRAD - STE20-related adaptor

TCA - tricarboxylic acid

TEC – tubular epithelial cell

TSC - Tuberous Sclerosis Complex

UB - ureteric Bud

VIP – variable importance

CHAPTER ONE

INTRODUCTION

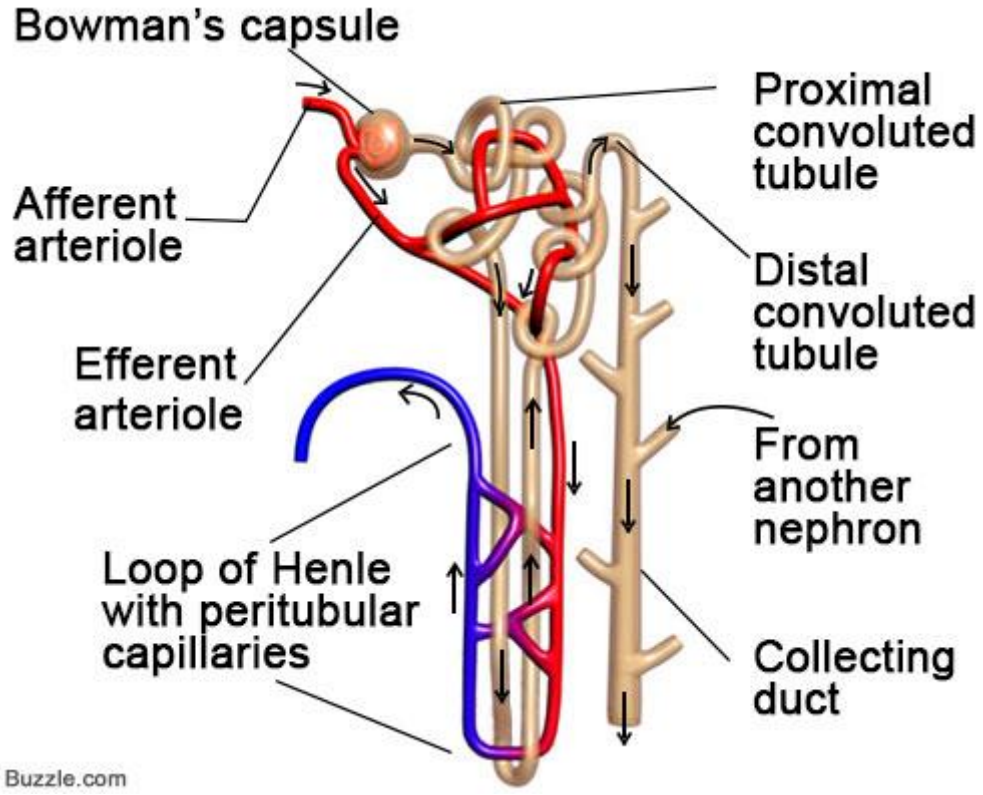

Figure 1-1: Schematic of mammalian nephron, the functional unit of the kidney. As blood enters the glomerulus, plasma is filtered into the tubule lumen. Obligatory electrolytes and water are absorbed while unnecessary electrolytes are excreted. Final urine concentration is determined within the collecting duct segments of the nephron which is then transported from the renal pelvis to the bladder.

Ureteric Bud Development

In vertebrates, all renal structures are derived from the intermediate mesoderm. In organisms with metanephric kidney, the majority of the mature cell types arise from 4 distinct progenitors: a vascular progenitor, a nephron progenitor, a stromal progenitor and the ureteric bud (UB). The UB begins as a simple epithelial structure that arises from the primary nephric duct[1-3]. The primary nephric duct branches laterally, forming the ureteric bud and invades the surrounding metanephric mesenchyme (consisting of the vascular, stromal and nephron progenitor populations) at approximately 4-5 weeks of

gestation in humans (embryonic day 10.5 in mouse)[3]. After migrating into the metanephric mesenchyme, the UB proliferates and arborizes in response to secreted growth factors from the metanephric mesenchyme. Reciprocal induction from the ureteric bud and the metanephric mesenchyme is a crucial juncture in the initiation of mammalian renal development. After sequential branching and elongation events, the morphogenesis of the UB concludes with the maturation, remodeling, and differentiation of the UB epithelia to ultimately give rise to the urinary collecting system (collecting ducts and ureter). The initial branching of the UB is regulated through the activation of UB-specific Ret receptor by the glial-derived neurotropic factor (*Gdnf*) ligand secreted from the metanephric mesenchyme at the tips of the ureteric bud. The Gdnf signals act in a permissive manner, driving the majority of cellular proliferation in the UB tips through Ret-activity[1,4-6]. After the initial invasion of the bud, the early branching events result in the formation of the renal pelvises and calyces within the papilla and inner medullary regions. Subsequent branching events gives rise to the mature collecting ducts in the outer medulla and cortex. As the ureteric bud undergoes reiterative branching events, the metanephric mesenchyme continues to proliferate, then aggregates at the bifurcations of the UB, condenses, and undergoes mesenchymal-to-epithelial transition forming an epithelial structure known as the renal vesicle that differentiates into various segments of the nephron[1,4,6]. The medullary region of the UB does not undergo further branching, due to lack of signals from the MM as well as the presence of inhibitory signals from the medullary stroma, although it continues to proliferate and elongate. Ultimately, the UB derived ureter will connect the kidney to the bladder.

Collecting Duct Differentiation

The collecting ducts are the terminal site of electrolyte and water reabsorption determining the final urine excretion of the kidney. Proper formation of the cortical and medullary collecting ducts is a major contributing factor in how the medulla of the kidney adapts to fluctuations in blood volume and other physiological conditions.

Collecting duct differentiation originates in the inner medullary region of the kidney then radially extends into the outer medullary and cortical regions throughout gestation, making the cortical-most cells of the collecting ducts the most immature/undifferentiated compared to the medullary CD cells[1,4,6]. As the CDs branch and elongate, synchronized maturation of the adjacent MM-derived epithelia also occurs to give rise to the functional unit of the kidney, the nephron. The collecting duct unites to the rest of the nephron via the transition into the connecting segment via the distal convoluted tubule (DCT)[1,4,6].

Though branching plays a significant role in the formation of the collecting ducts, cellular proliferation and oriented cell division are also essential to terminal collecting duct morphology. As the collecting ducts elongate, there is a substantial dependence on convergent extension, which involves both non-oriented and polarized cell divisions, to define the proper epithelial tubule diameter[4,6].

The mature collecting ducts consist of two cell types, principal cells and intercalated cells. Principal cells, the most abundant cell type, function as the primary site

of Na^+ reabsorption, K^+ excretion, and water transport via hormonal signals from aldosterone and vasopressin. Intercalated cells are the primary site for acid/base homeostasis via signals from pendrin and H^+-ATPase[4,6]. In the developing mammalian kidney, intercalated cells are the most abundant within the medulla while very few are present in the cortical region of the kidney. Postnatally, there is a marked increase in cortical intercalated cells which indicates that the collecting ducts continue to reorganize after birth[4,6].

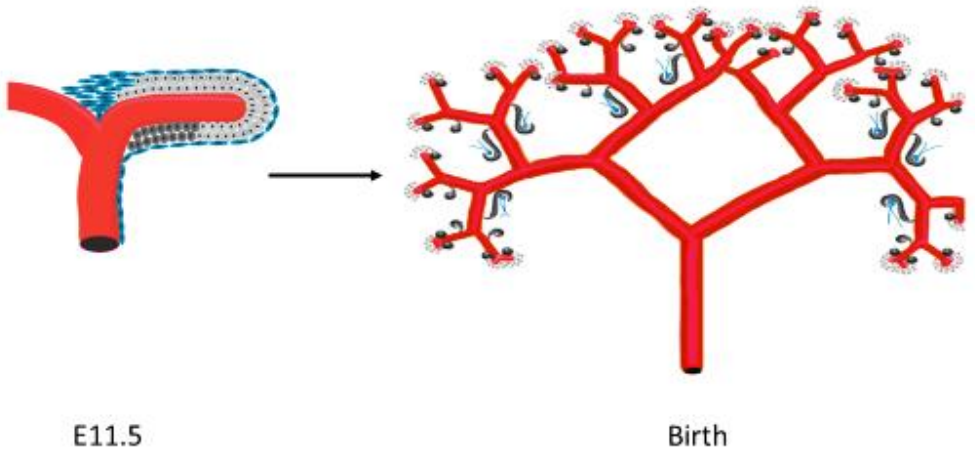

Figure 1-2: Development of the collecting duct system from the ureteric bud. Formation of the collecting ducts begins as the ureteric bud (shown in red). Reiterative branching events of the ureteric bud followed by a maturation/differentiation period gives rise to the collecting ducts.

LKB1/STK11

Liver Kinase B1 (LKB1) is a serine/threonine kinase also known as STK11. LKB1 exists in mammalian cells in a complex with STE20-related adaptor (STRAD) and Calcium-binding protein 39/mouse protein 25 (CAB39/MO25), which augment the proper localization and activity of LKB1 within a cell[7,8]. LKB1 is bound to STRAD as a pseudosubstrate which mediates a conformational change, and MO25 stabilizes this STRAD-LKB1 active conformation, enhancing LKB1 activity. When STRAD and MO25 are present, LKB1 is localized to the cytoplasm where the catalytic activity and affinity of LKB1 to its substrates is 10-fold higher compared to the nucleus. When not in a complex, LKB1 is predominantly localized to the nucleus, facilitated by the presence of a nuclear localization signal (NLS) within the N-terminal domain of LKB1[7-9].

Lkb1 was first identified as the gene that caused Peutz-Jeghers syndrome (PJS), a rare autosomal dominantly inherited disorder characterized by the formation of benign tumor-like polyps within the gastrointestinal tract and discoloration of the mucous membrane of several organs. Peutz-Jeghers syndrome was first described by Dr. Joannes Peutz in 1922 and further characterized by Dr. Harold Jeghers in the 1940s[10-12]. Later studies discovered that germline mutations of LKB1 are strongly correlated with an increased risk for predisposition to several cancers and patients with PJS are at an eight-fold higher risk for cancer than the general population[13,14]. In PJS patients with mutations in SL26 of LKB1, in which the three amino acids involved in STRAD binding are mutated,

LKB1 accumulates in the nucleus but retains catalytic activity which is believed to contribute to PJS pathogenesis[15].

Since the initial characterization of LKB1 in PJS, numerous studies have indicated a conserved role for LKB1 in the regulation of cell polarity, epithelial-to-mesenchymal transition (EMT), cell growth and proliferation, and energetic stress[16-21]. Loss of LKB1 in tubular epithelial cells (TECs) is also associated with dedifferentiation, marked by increased expression of collagen genes Col1a1, Col3a1, and smooth muscle gene, Acta2[22]. In mouse models, total ablation of Lkb1 during development causes embryonic lethality[23]. Mice with heterozygous Lkb1 mutations have no explicit phenotype and are viable until late stages, approximately 45-50 weeks of age, when they typically develop hepatocellular or gastrointestinal polyps similar to what is observed in human PJS patients[12,24].

LKB1 phosphorylates 14 kinases, with the best characterized being AMP-activated protein kinase (AMPK)[25,26]. AMPK is an established metabolic regulator. LKB1/AMPK acts as a critical cell cycle and metabolic checkpoint in response to low ATP or nutrient levels[27]. When AMPK senses ATP depletion, it inhibits several anabolic pathways in order to reduce energy expenditure. LKB1/AMPK signaling is also responsible for the physiological feedback to different types of metabolic stress including hypoxia, nutrient deficit, and energy expenditure in a context-specific manner. This signaling is distinctive in its ability to integrate these different forms of energetic stress and reprogramming metabolism allowing cell-specific responses to changes in the surrounding microenvironment. Lkb1 has also been shown to have a long-term impact on cancer cell metabolism in mice

through the activation of several transcriptional regulators of energy metabolism including *Foxo3*, *Hnf4a*, and *Ppargc1a*[22], some of which are AMPK-independent, but are mediated by AMPK-related kinases such as microtubule affinity-regulating kinases (MARKs) and salt-inducible kinases (SIKs)[22].

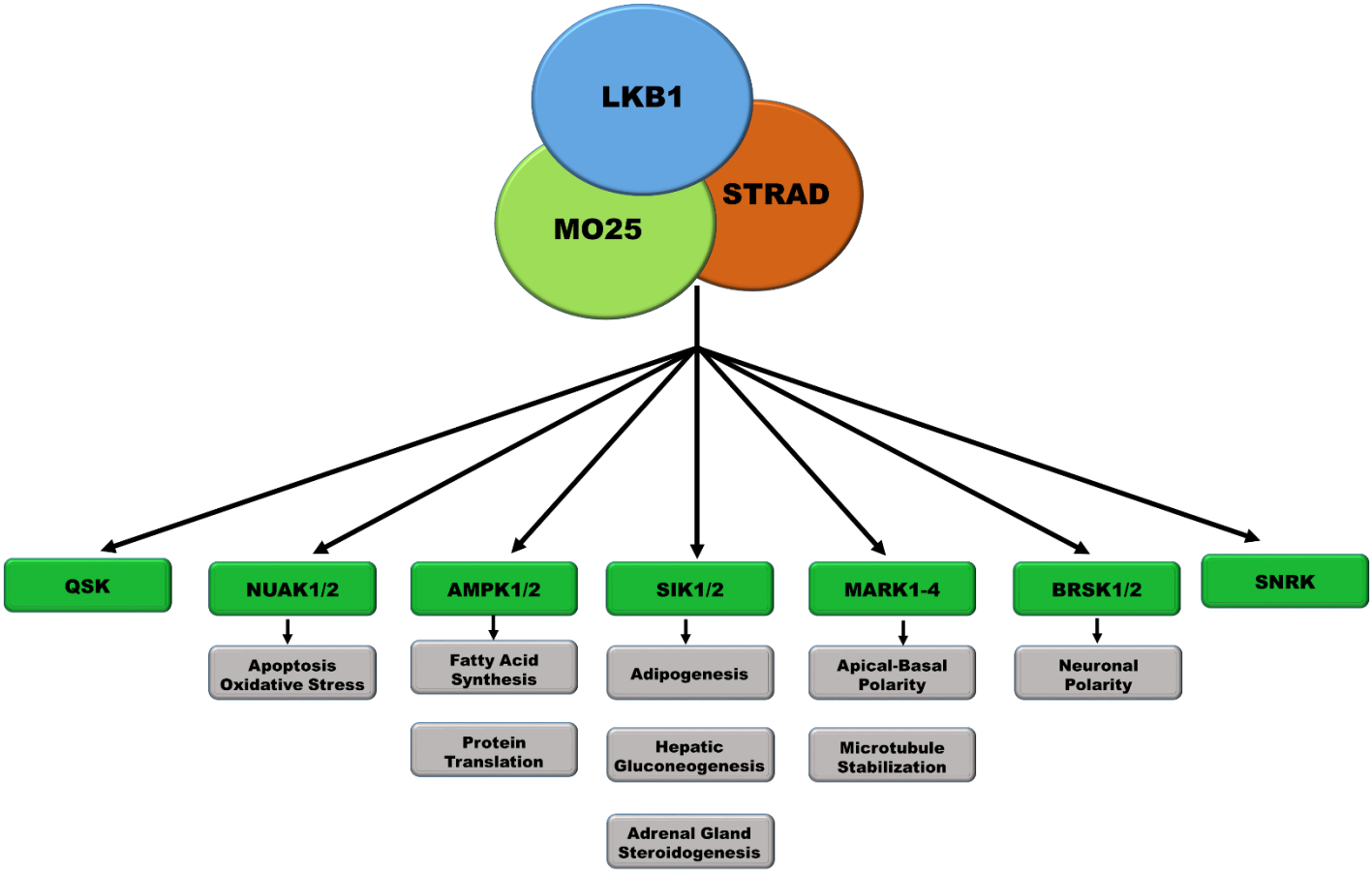

Figure 1-3: Lkb1 downstream targets. Kinases phosphorylated and activated by the LKB1 complex and their associated functional pathways.

LKB1 is regulated by several upstream mechanisms. LKB1 gene expression can be transcriptionally regulated by the sex hormones estrogen and androgen. Estrogen-mediated regulation of LKB1 through the binding of estrogen receptor alpha (ERα) binding of the LKB1 promoter to either increase or decrease LKB1 mRNA and protein expression levels in a cell-type dependent manner[28,29]. Androgen can also regulate LKB1 mRNA expression and can be modulated by changes in testosterone, but the absence of an androgen receptor element (ARE) in the LKB1 promoter suggests that this regulation is indirect[30,31].

Epigenetic studies have shown that several primary tumors and cell cancer lines have hypermethylation of the CpG island of LKB1 promoter region, causing the LKB1 transcript to be rendered undetectable[32-34]. This corresponded with the observation that regions of benign polyps in PJS patients but not analogous normal tissue displayed hypermethylation of the LKB1 promoter[32]. LKB1 can also undergo posttranslational modifications including phosphorylation, prenylation, ubiquitination, acetylation, and farnesylation, and SUMOylation which all regulate the localization and function of LKB1 under different conditions. The serine-threonine kinase Protein Kinase B, also known as AKT, directly phosphorylates LKB1, preventing its interaction with STRAD, sequestering it to the nucleus and inhibiting its function[35,36]. Conversely, polyubiquitination of LKB1 is essential to the maintenance of the LKB1-STRAD-MO25 complex. LKB1 can also be phosphorylated by the DNA response factor, ataxia-telegiectasia mutated (ATM), when reactive oxygen species are present to induce autophagy through the mTOR pathway[37,38].

In addition to phosphorylation, SUMOylation of LKB1 during times of energetic stress promotes the LKB1-AMPK interaction and subsequent AMPK activation[39,40].

mTOR pathway

Mammalian cells require a constant supply of nutrients to provide the cellular material necessary for growth and proliferation. Cancerous or other rapidly proliferating cells require a higher influx of nutrients to provide the energy required for the accumulation of carbohydrates, lipids, and proteins needed for amplified cell division.

Mammalian target of rapamycin (mTOR), a member of the phosphoinositide kinase-related kinase (PIKK) family, is an evolutionary conserved serine/threonine kinase complex that integrates and coordinates external signals such as nutrient availability, energetic stresses, and growth signals to stimulate cell survival, growth, and division[35,41]. The mTOR complex has also been shown to manage autophagy and protein synthesis when catabolic macromolecules like amino acids, glucose, and insulin are depleted. Previous studies indicate that reductions in intracellular nutrient levels can alter mTOR signaling not only to activate cell cycle progression but to provide sufficient quantities of macromolecules required for increases in cellular mass during cell division[42,43]. It has also been implied that the mTOR pathway coordinates the breakdown of extracellular proteins can be a source of the substrates needed to fuel mitochondrial bioenergetics and inhibit cell death[44].

mTOR is composed of two multiprotein signaling complexes, mTORC1 and mTORC2. mTORC1 which consists of mTOR, RAPTOR, a localization and scaffolding protein, mLST8/GβL, a stabilization and catalytic subunit, and two inhibitory subunits,

PRAS40 and DEPTOR[45,46]. mTORC2 also consists of mTOR, mLST8/GβL, and DEPTOR but instead of RAPTOR, mTORC2 encompasses RICTOR and two regulatory subunits, Sin1 and Protor1/2[45,46]. In response to growth factors, nutrient fluctuations, and energy levels the mTOR complexes phosphorylate various downstream targets such as ribosomal protein S6 kinase (S6K) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1)[42,43] via activation of two families of small guanosine triphosphatases, RAS and Ras homolog enriched in brain (RHEB). These effectors produce the signals that promote protein translation and synthesis, cytoskeletal remodeling, generation of biomass, and other fundamental processes required for cellular growth and division.

Mutations in Hamartin (TSC1) or Tuberin (TSC2) in humans result in Tuberous Sclerosis Complex, a multisystemic disorder characterized by benign tumors called hamartomas, partly due to the hyperactivation of mTOR signaling[46]. The phosphorylation of AMPK and AMPK-related kinases by LKB1 is reported to activate the TSC1/2-mTOR signaling pathway through the direct phosphorylation of the TSC1/TSC2 complex[41]. The TSC1/TSC2 complex can also activated by various other upstream regulators including the the Insulin receptor (INR) and Akt pathways[46].

When Tsc1 is specifically ablated from the collecting ducts in mice, the epithelial tubules exhibited increases in potassium levels (hyperkalemia) and aldosterone (hyperaldoseronism) resulting in the imbalance of acid-base homeostasis in the kidneys and circulating blood, known as metabolic acidosis[47]. This is attributed to activation of mTOR signaling and decreased expression of Na^+/K^+-ATPase in the collecting ducts causing a disproportion of sodium and potassium channel activity[47].

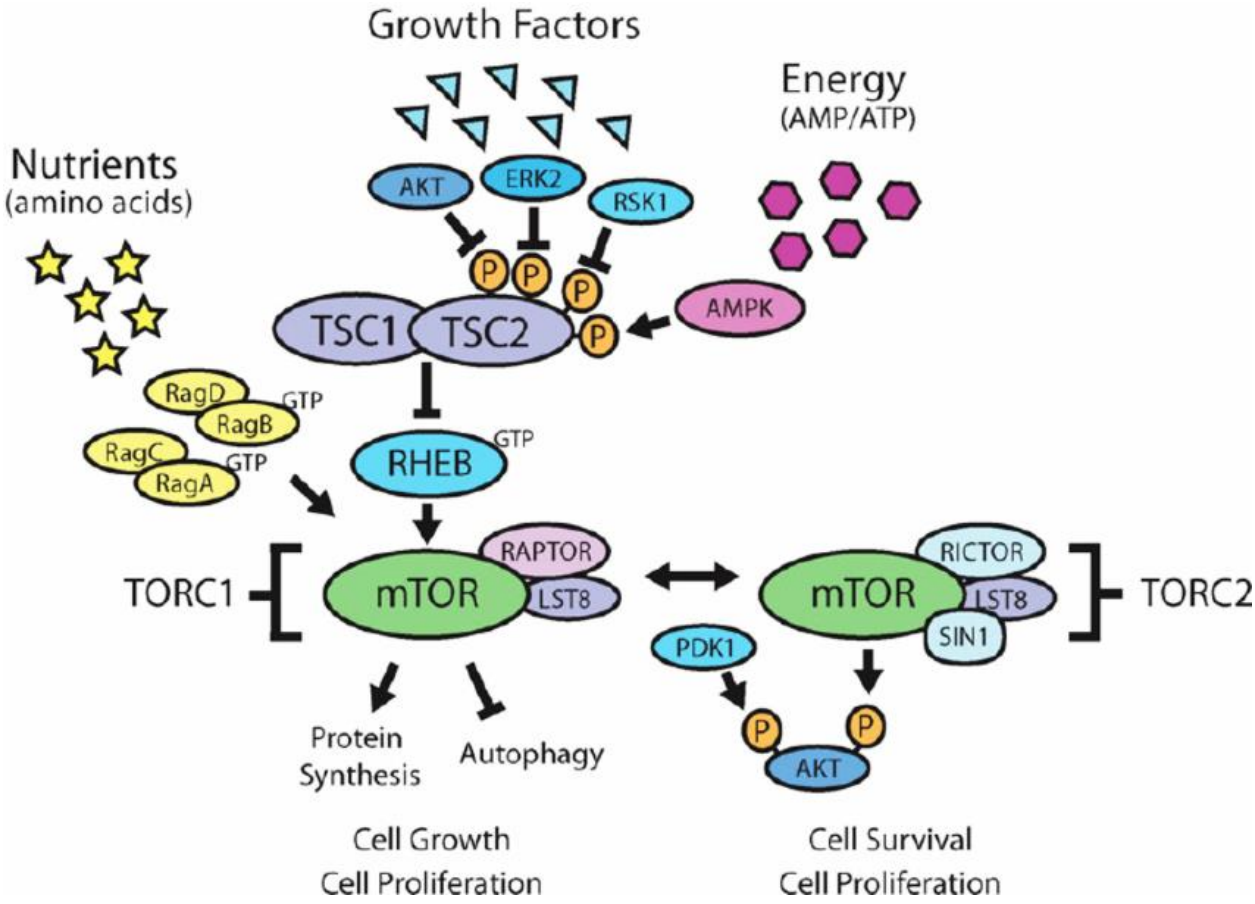

Figure 1-4: mTOR signaling pathway. Nutrients, growth factors, and energy molecules are sensed and integrated by the mTOR pathway. TSC1 and TSC2 are negative regulators in the mTOR signaling cascade and suppress cell growth. Tuberous Sclerosis Complex is caused by mutations in either the TSC1 or TSC2 genes that activate the mTOR pathway and stimulate cellular growth. Illustration from MacKeigan, JP and Kruger, DA, Neuro-Oncology, 2015.

Polycystic Kidney Disease

Polycystic kidney disease (PKD) is an inherited disorder and one of the most prevalent causes of end-stage renal disease (ESRD), affecting 1 in 400 to 1 in 1000 births every year in the United States[48]. PKD is characterized by cystic malformations within the kidney, and often the pancreas, liver, and lungs as well, but most morbidities result from cystic burden, rupture, or infection within the kidneys. Common manifestations of PKD are systemic hypertension, hematuria, nephromegaly, increased urine osmolality and sodium excretion, and lower serum HDL-cholesterol, which are typically identified through physical or radiological examination.

The majority of PKD patients have mutations in polycystin-1 or polycystin-2, encoded by *PKD1* and *PKD2* genes in humans[49,50]. Polycystin-1 (PC1) is approximately 460kDa and consists of 11 predicted transmembrane domains that are organized into a large extracellular N-terminal domain (approx. 3000 amino acids) and a short cytoplasmic C-terminal domain (approximately 200 amino acids)[51]. Polycystin-2 (PC2) is approximately 110kDa and consists of 6 predicted transmembrane domains[51]. There are two forms of PKD, autosomal dominant PKD (ADPKD) and autosomal recessive PKD (ARPKD). Perceptible symptoms of ADPKD are commonly observed in adults in their third to fifth decade of life, but characteristics indicative of ADPKD may arise as early as embryonic development. Mutations in *PKD1* account for approximately 85% of ADPKD patients while mutations in *PKD2* compose the other 15%, mostly due to the larger size of *PKD1*, making it more mutable than its counterpart[51,52]. ARPKD is a more severe form of PKD that typically manifests in utero. Due to the early inception of the symptoms, the

mortality rate is higher than ADPKD, with most patients surviving less than one year of life[53,54]. Both ADPKD and ARPKD exhibit a broad spectrum of phenotypes that partially results from variance in the age of presentation.

The loss of *PKD1* or *PKD2* results in the overproliferation of epithelial cells, loss of apicobasal polarity, loss of cellular identity/morphology, and defects in solute transporter location and function[51,55-57]. *PKD1*, *PKD2*, and *PKHD1*, the gene that encodes for fibrocystin/polyductin[53], can all be localized to the primary cilia, vestigial hair-like organelles that act as sensory antennae of renal epithelial cells[58,59]. The primary cilia are important in the coordination of multiple external signals during renal development and homeostasis[58,60]. The mTOR signaling pathway is directly suppressed in normal primary cilia and is aberrantly activated when ciliary function is disrupted in PKD. Correspondingly, many PKD patients have a large chromosomal deletion that includes the *PKD1* gene and an adjacent *TSC2* gene, that when mutated, causes the entrapment of the PKD1 protein in the Golgi apparatus instead of the cell membrane of the primary cilia[47]. Deviation of one or more of these processes leads to the formation of epithelial cyst that progressively expand in a subset of tubules.

Cyst initiation typically results from two hits, the first hit is a germline mutation in *PKD1*, *PKD2*, or *PKHD1*, coupled with at least one acquired or somatic mutation. As a renal tubule transitions into a cyst, proteins are generated that are characteristically associated with immature epithelia. Over time, the lumen of these cysts increase in volume due to the inability to properly manage fluid secretion leading to the accumulation of unabsorbed glomerular filtrate and transepithelial solute[61]. As the cysts fill with fluid

and begin to dilate within the kidney tubules, there is also a gradual yet significant increase in cell proliferation within the epithelial cyst cell walls. As the disease advances, cysts continue to increase in size and number until the kidneys become immensely enlarged with multiple cysts surrounded by thick bands of fibrotic tissue that gradually replace normal renal parenchyma impeding renal function. As the disease proceeds into its most terminal stages the increase in cyst burden, rupture, and infection eventually lead to ESRD and renal failure.

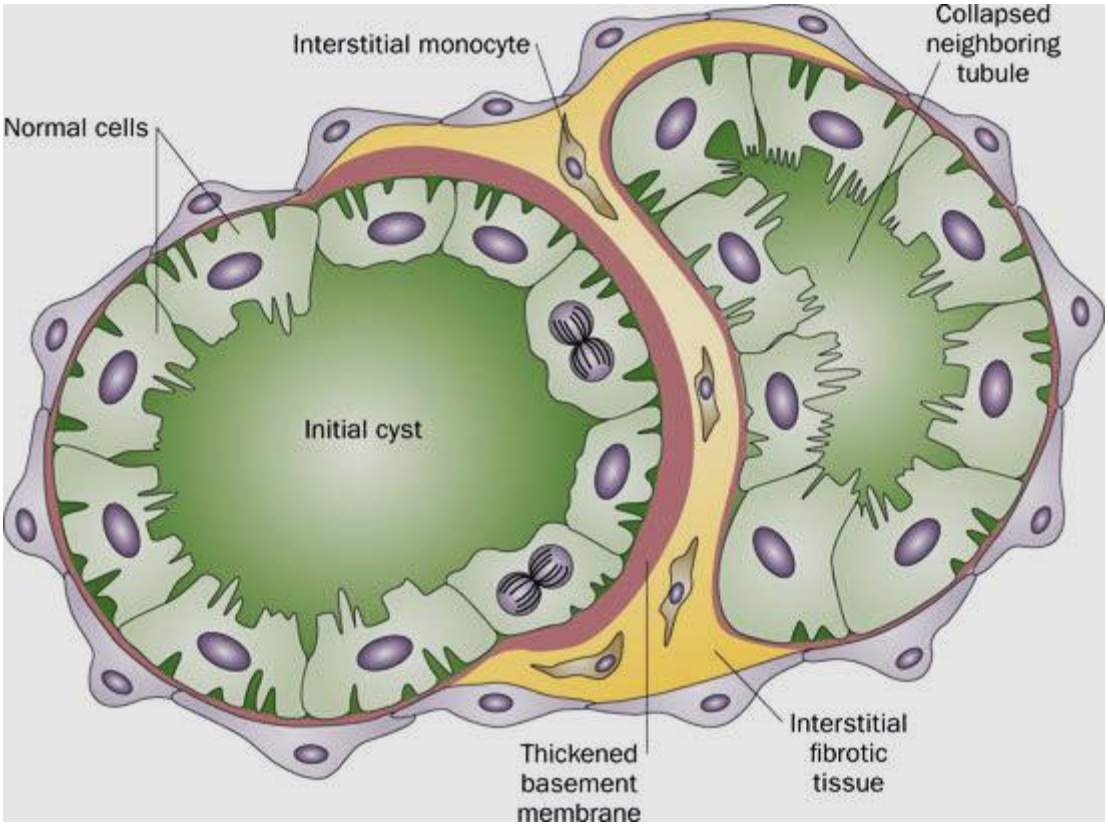

Figure 1-5: Cyst initiation in PKD. Parent epithelial tubule cells rapidly divide generating new cells that lack proper cell identity and differentiation. Fluid accumulation from irregular solute transport leads to collagen, macrophage, and fibroblast deposits, thickening the tubule basement membrane and extracellular matrix. After the tubule begins to dilate, most cysts ultimately resolve into isolated fluid-filled sacs separated from the parental tubule. Illustration from Grantham, JJ, Science and Medicine 9, 128-139 (2003).

There are several challenges remaining in understanding the role of the polycystins and fibrocystin/polyductin in normal cellular physiology and pathological conditions. Interaction between PC1 and PC2 within renal epithelial cells is mediated by a coiled coil domain present in the C-terminal tail of PC1, but the regulation of this interaction is still largely misunderstood[51]. PC1 and PC2 physically interact with several other proteins that regulate their participation in signal transduction and membrane trafficking, but how these associations are mediated still remains unclear.

One of the major obstacles is defining the in vivo function of *PKD1*, *PKD2*, and *PKHD1* at the cellular and molecular levels. This is due in part to the fact that numerous signaling factors downstream of polycystin signaling have yet to be identified and characterized[62], so it is unfeasible to determine which factors should be therapeutically targeted for activation or inhibition. The identification of downstream effectors is also important to determine where the mechanistic failure occurs and if there are secondary pathways or networks that are involved during cyst initiation and progression. It is also problematic to model PKD, a structurally three-dimensional disease, using cells grown in a monolayer as there are no current protocols substantiated in transformation or direct differentiation. Using cellular based assays alone to study PKD creates obstacles in determining which mechanism is responsible for primary cyst formation from the parent renal tubule as well as de novo cysts that are not attached to any other epithelia.

Furthermore, current treatments for PKD are predominantly diuretics designed to limit cyst expansion through via obstruction of the Vasopressin 2 receptors located in principal cell basolateral membranes responsible for water reabsorption. However,

though the anti-diuretics that have U.S. approved for treatment for PKD have no adverse effects on renal morphology or function, their usage is limited to severe side effects on other organ systems; so obtaining novel PKD treatments that can be administered for long-term use are in dire need.

CHAPTER TWO

Lkb1 deficiency confers glutamine dependency in polycystic kidney disease

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Abstract

Polycystic Kidney Disease (PKD) is a common genetic disorder characterized by the growth of fluid-filled cysts in the kidneys. Several studies reported that the serine-threonine kinase Lkb1 is dysregulated in PKD. Here we show that genetic ablation of Lkb1 in the embryonic ureteric bud has no effects on tubule formation, maintenance, or growth. However, co-ablation of Lkb1 and Tsc1, an mTor repressor, results in an early developing, aggressive form of PKD. We find that both loss of Lkb1 and loss of Pkd1 renders cells dependent on glutamine for growth. Metabolomics analysis suggests that Lkb1 mutant kidneys require glutamine for non-essential amino acid and glutathione metabolism. Inhibition of glutamine metabolism in both Lkb1/Tsc1 and Pkd1 mutant mice significantly reduces cyst progression. Thus, we identify a role for Lkb1 in glutamine metabolism within the kidney epithelia and suggest that drugs targeting glutamine metabolism may help reduce cyst number and/or size in PKD.

Introduction

Polycystic Kidney Disease (PKD) is one of the most common lethal genetic disorders in humans affecting an estimated 600,000 people in the United States and 12 million people worldwide. The disease is characterized by organ overgrowth and the formation of fluid-filled cysts that displace/replace normal renal tissue eventually leading to end-stage renal disease (ESRD) and renal failure. PKD is similar to neoplastic disorders in that the formation and enlargement of cysts is largely due to increased cellular proliferation and growth, abnormal cell polarity, and altered cellular metabolism[2,52,56,63-65].

The majority of PKD patients have inherited a genetic mutation in one of the polycystin genes, *PKD1* or *PKD2*[58]. Several other genes/pathways involved in kidney development have been implicated in PKD, but the precise cause of cyst formation still remains relatively unclear. Most PKD patients begin to exhibit symptoms between 30 and 50 years of age. However, the age of onset and severity of progression varies greatly and can begin as early as childhood/adolescence. This spectrum of individuals/cases suggests additional modifications from genetic, epigenetic, or environmental factors contribute to disease pathology and represent a prospective set of therapeutic targets to inhibit or slow cyst progression.

Several classes of drugs have been identified as having potential benefit in autosomal dominant polycystic kidney disease (ADPKD) in animal models. However, available treatments such as Tolvaptan that alleviate cyst enlargement through the

regulation of water and sodium transport, have systemic side effects that restrict usage[57,66]. Additional therapies that target cystic/neoplastic tissue while not interfering with normal renal maintenance/function are needed.

Current models suggest that transformed/proliferative tissue undergoes metabolic reprogramming that results in enhanced glycolytic flux[67-71]. A possible explanation for this phenomenon is that glycolysis and coincident activities such as an anaplerotic TCA cycle provide precursors for biomass assimilation in growing cells. Understanding how metabolism is (mis)regulated in PKD may lead to the development of therapeutics that can reduce increased cell proliferation, one of the principal drivers of PKD.

Lkb1/Stk11 is a serine/threonine kinase that was first identified as the causative genetic factor for Peutz-Jeghers syndrome[10,11] and has since been shown to act as a tumor suppressor in multiple cell types[20]. Lkb1 seems to function in a context-dependent manner that is uncharacteristic of other tumor suppressors. Lkb1 and its primary target, AMP-Kinase, have been shown to regulate energy metabolism in several organ systems[72-75], and mutations in Lkb1 have been identified in several sporadic epithelial cancers[76]. Although Lkb1 activity has been shown to be abrogated in PKD[65,77], the precise role that Lkb1 plays in cystogenesis is still uncertain.

Utilizing a combination of in vitro and in vivo techniques, we have found that loss of Lkb1 alone is not sufficient to induce cyst formation. However, ablation of Lkb1 enhances cystic growth and accelerates the loss of normal renal parenchyma in kidneys with activated mTor signaling (Tsc1 mutants). We find that loss of Lkb1 activity renders cells dependent on glutamine for their growth. Similar results are observed in an

orthologous model of PKD (Pkd1 mutants). In these kidneys, glutamine is required to maintain pools of nonessential amino acids and glutathione. Excitingly, treatment of mouse models of PKD, including Pkd1 mutants, with drugs that block glutaminolysis mitigates cyst formation in vivo suggesting a potential avenue for PKD therapeutics.

Results

Loss of Lkb1 from the collecting ducts does not initiate PKD

Lkb1/Stk11 is a serine/threonine kinase that has been reported to regulate cell proliferation, polarity, mTor activity, and energy metabolism[19,21,22,36,65,78], all of which are perturbed in polycystic kidney disease. Furthermore, recent data showed that Lkb1 activity was attenuated in cells lacking Pkd1[65,77], the gene mutated in the majority of cases of human PKD, suggesting that ablation of Lkb1 in the kidney epithelia may be sufficient to cause PKD. A recent study found that deletion of Lkb1 utilizing the Ksp-Cre deleter, which is active throughout the kidney epithelia with highest levels in the distal nephron, led to late-onset tubular atrophy and fibrosis and occasional cyst formation[22]. Somewhat surprisingly, deletion with HoxB7-Cre, a line that is active in the embryonic and adult collecting ducts, had no discernible phenotype upon histological analysis (Fig. 2-1, a-h). To determine if Lkb1 was efficiently deleted, we stained mutant and wild-type kidneys with an antibody to Lkb1. Lkb1 protein levels were significantly decreased in HoxB7-Cre; Lkb1$^{flox/flox}$ collecting ducts at P1 suggesting efficient deletion (Fig. 2-2).

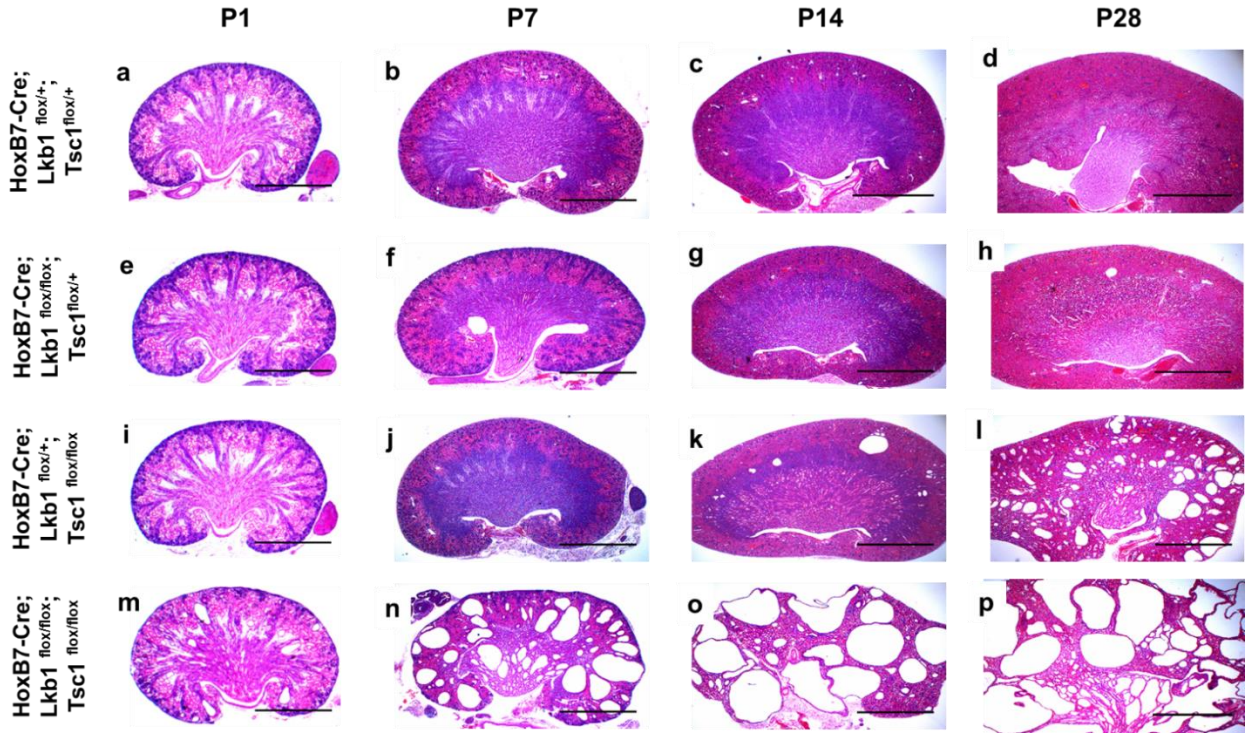

Figure 2-1: Deletion of Lkb1 enhances cyst progression of Tsc1 mutant kidneys. Representative images of hematoxylin and eosin stained sections of Wild-type (a,b,c,d) Lkb1 mutant (e,f,g,h), Tsc1 mutant (I,j,k,l), and Lkb1/Tsc1 double mutant (m,n,o,p) kidneys at post-natal day 1, 7, 14, and 28. Lkb1 single mutant kidneys displayed no cyst formation. n>5 for each genotype. Scale bars equal 20mm

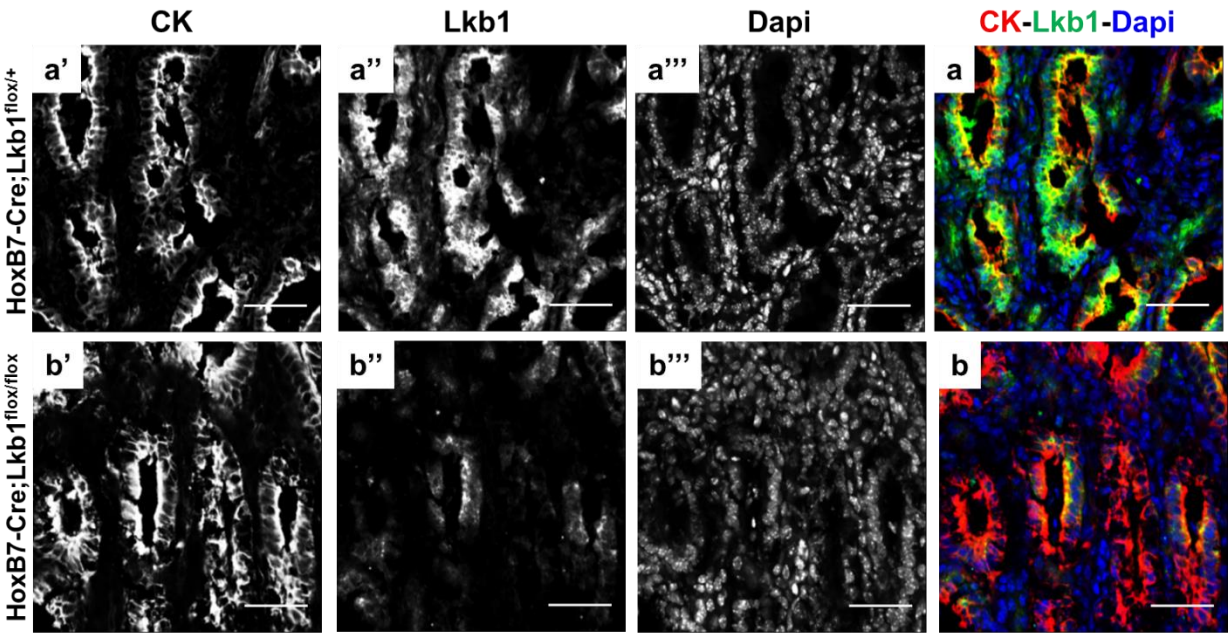

Figure 2-2: Hoxb7-Cre efficiently deletes Lkb1. Sections through HoxB7-Cre; $Lkb1^{flox/+}$(a) or HoxB7-Cre; $Lkb1^{flox/flox}$ (b) P1 kidneys stained with antibodies to cytokeratin (red), Lkb1 (green) and the nuclear marker DAPI (blue). Individual channels for cytokeratin are in a' and b', Lkb1 a" and b" and Dapi in a"' and b"'. Scale bars equal 50 microns.

We next sought to determine if any of the processes reported to be regulated by Lkb1 in other contexts were perturbed in mutant collecting ducts without resulting in a significant mutant phenotype. HoxB7-Cre; $Lkb1^{flox/flox}$ and HoxB7-Cre; $Lkb^{flox/+}$ kidneys were stained with indicators of cell proliferation (proliferating cell nuclear antigen/PCNA), cell death (ACTIVE caspase-3), epithelial polarity (aPKC, ZO-1α) and cilia (acetylated α-tubulin). Lkb1 mutant collecting ducts showed no significant differences in staining pattern of any of these proteins relative to control littermates (Fig. 2-3, 2-4, 2-5).

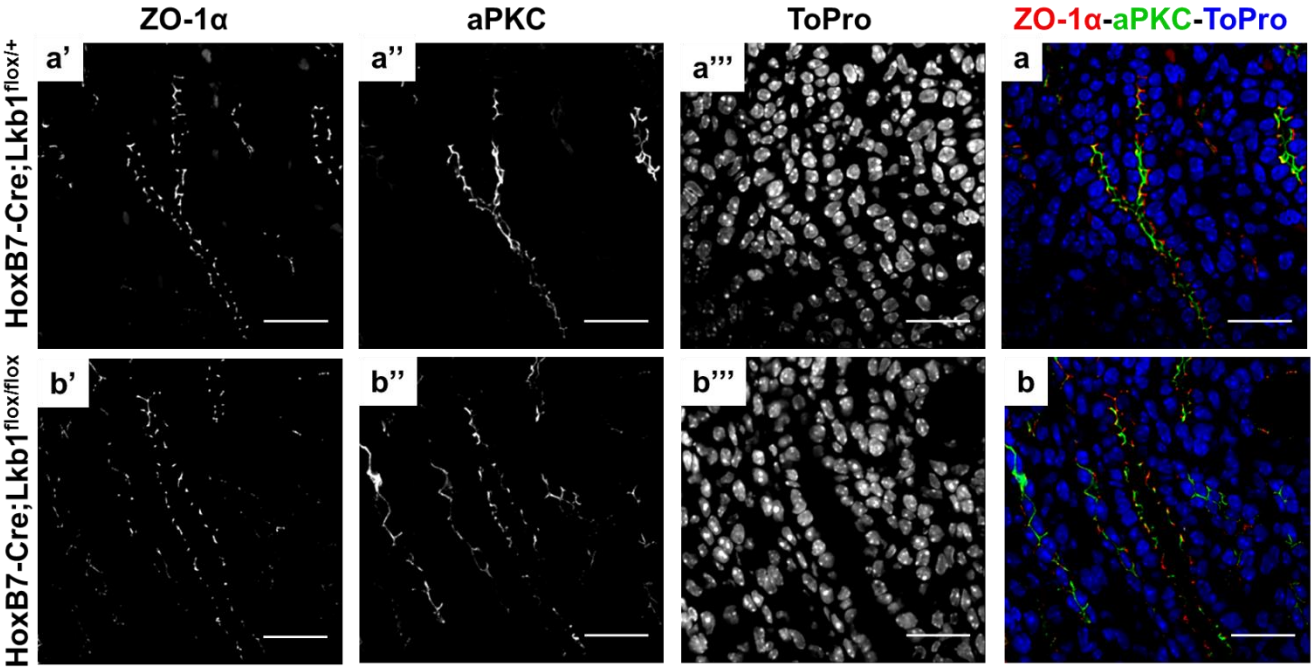

Figure 2-3: Cell polarity is unaffected in Lkb1 mutant collecting ducts. Sections through HoxB7-Cre; $Lkb1^{flox/+}$(a) or HoxB7-Cre; $Lkb1^{flox/flox}$ (b) P1 kidneys stained with antibodies to ZO-1α (red), aPKC (green) and the nuclear marker DAPI (blue). Individual channels for ZO-1α are in a' and b', aPKC a" and b" and DAPI in a"' and b"'. Scale bars equal 50 microns.

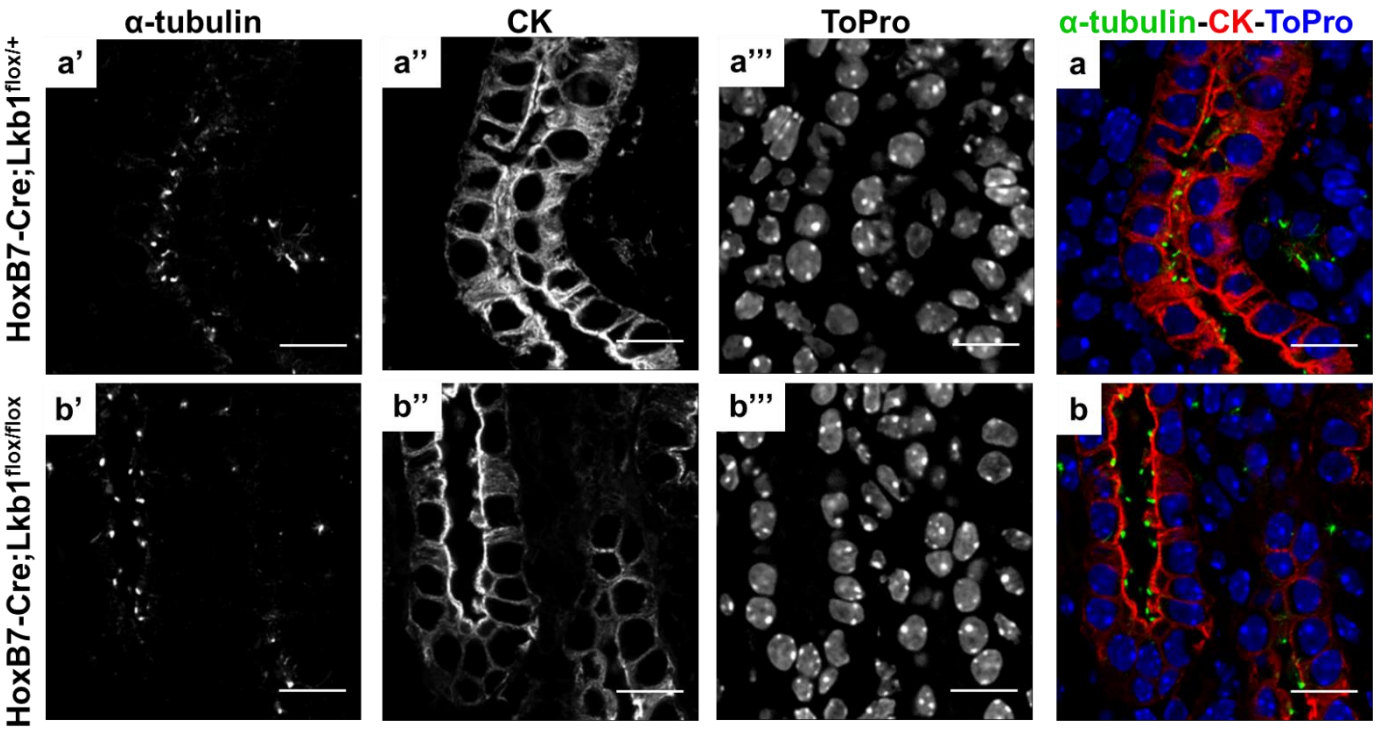

Figure 2-4: Deletion of Lkb1 from the collecting ducts does not result in loss of cilia. Sections through HoxB7-Cre; Lkb1$^{flox/+}$(a) or HoxB7-Cre; Lkb1$^{flox/flox}$ (b) P1 kidneys stained with antibodies to cytokeratin (red), acetylated α-tubulin (green) and the nuclear marker ToPRO (blue). Individual channels for cytokeratin are in a' and b', α-tubulin a'' and b'' and ToPRO in a''' and b'''. Scale bars equal 5 microns.

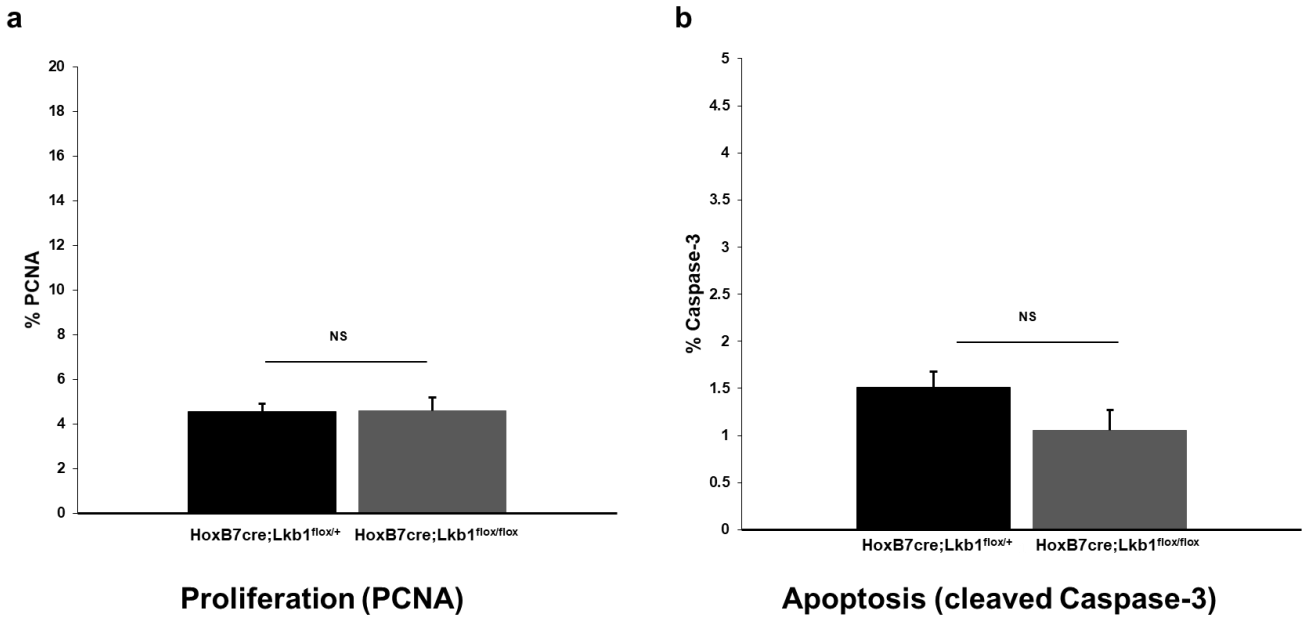

Figure 2-5: Deletion of Lkb1 does not affect proliferation or apoptosis within the collecting ducts. (a) Quantification of cell proliferation via proliferation cell nuclear antigen (PCNA) within the collecting ducts of P1 HoxB7-Cre; Lkb1$^{flox/+}$ (n=5) and HoxB7-Cre; Lkb1$^{flox/flox}$ (n=5) sectioned kidneys. (b) Quantification of cell death via active caspase-3 within the collecting ducts of P1 HoxB7-Cre; Lkb1$^{flox/+}$ and HoxB7-Cre; Lkb1$^{flox/flox}$ sectioned kidneys. Statistical analysis via Mann-Whitney U-test. NS, not significant. Error bars shown as mean +/- standard error of the mean (SEM).

Ablation of Lkb1 enhances cystic growth in Tsc1 mutants

It had previously been suggested that loss of Lkb1 activity in Pkd1 mutant kidney epithelia promoted activation of the mTorc1 pathway and a switch in cellular metabolism towards glycolysis, a phenomenon referred to as the Warburg effect[77]. The relationship of Lkb1 loss to mTor pathway status is complicated. There is evidence indicating that mTorc1 activation in PKD was a direct result of loss of Lkb1 activity[77], while other reports suggest that activation of mTorc was independent of Lkb1 and that these two pathways cooperated to regulate pAMPK levels[19]. To determine whether the mTor pathway was perturbed in Lkb1 mutants, wild-type and Lkb1 mutant P1 kidneys were co-stained with cytokeratin, a marker of the collecting duct epithelia and an antibody specific to the phosphorylated form of S6 ribosomal protein, an indicator of mTor activity (Fig. 2-6). No changes in phospho-S6RP levels were detected in Lkb1 mutant collecting ducts. Thus deletion of Lkb1 alone from the collecting ducts has no effect on mTor signaling.

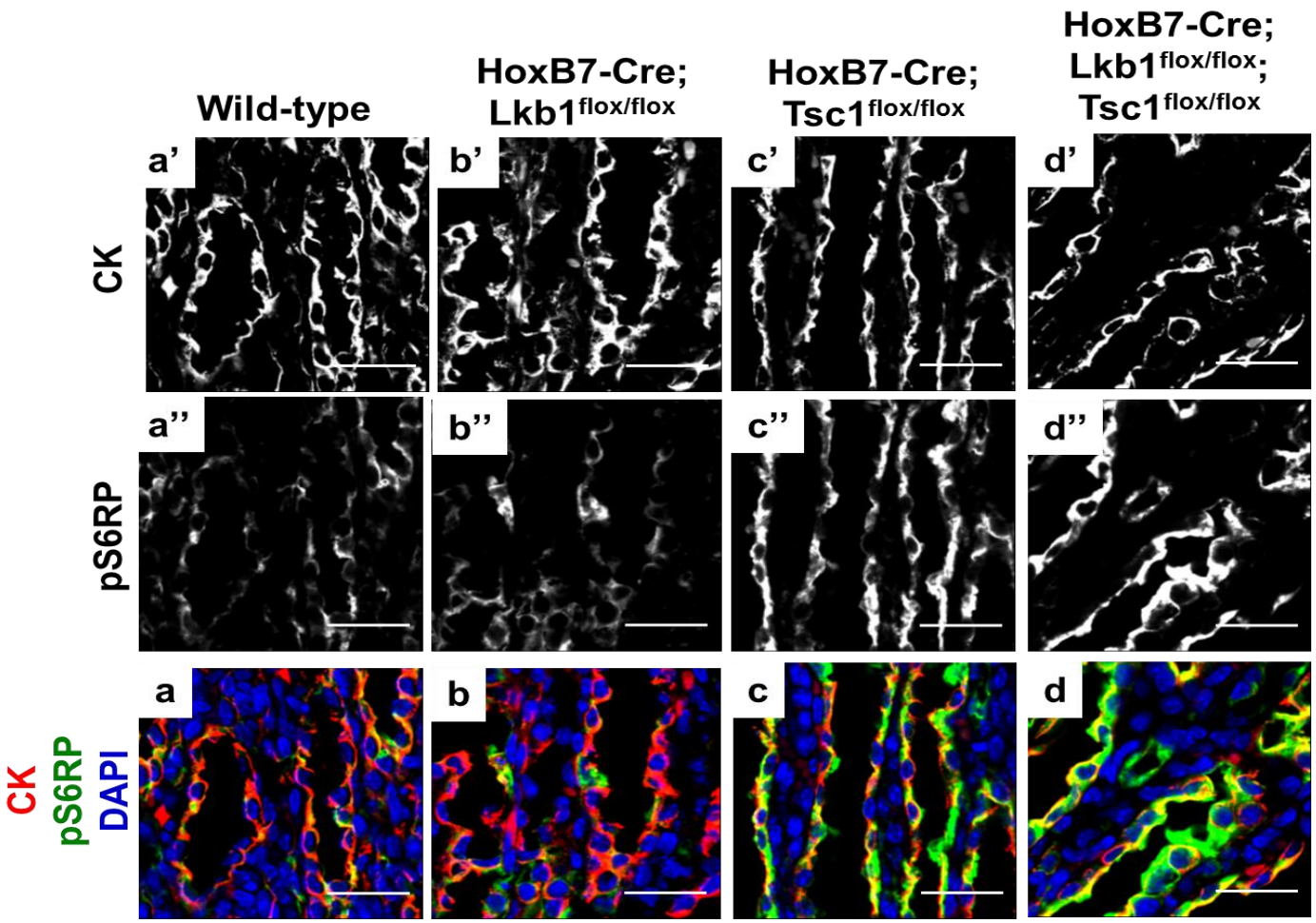

Figure 2-6: Lkb1 mutant collecting ducts show no signs of mTOR pathway activation. Sections through HoxB7-Cre; $Lkb1^{flox/+}$; $Tsc1^{flox/+}$ (n=5) (a), HoxB7-Cre; $Lkb1^{flox/flox}$ (n=5) (b), HoxB7-Cre; $Tsc1^{flox/flox}$ (n=5) (c), and HoxB7-Cre; $Lkb1^{flox/flox}$; $Tsc1^{flox/flox}$ (n=5) (d) P7 kidneys stained with antibodies to cytokeratin (red), phospho-S6RP (green), and the nuclear marker DAPI (blue). Individual channels for cytokeratin are in a', b', c', and d'; phospho-S6RP a'', b'', c'', and d''. Scale bars equal 50 microns.

One possible explanation for the lack of mTor activation in Lkb1 mutant collecting ducts is that perhaps Lkb1 does not act upstream or is not the only upstream determinant of this pathway in this cell type. Indeed, Rowe et al. suggested that Lkb1 acted in parallel to the mTor pathway to cause cyst formation in Pkd1 mutants. To investigate this possibility, we analyzed the effect of Hamartin deletion in the collecting ducts. Hamartin, the protein coded for by Tsc1, cooperates with Tuberin, to form a GAP that represses mTor activity. Tsc1 deletion should be sufficient to activate the mTor pathway. Indeed, in contrast to the Lkb1 mutants, HoxB7-Cre; $Tsc1^{flox/flox}$ mice had a late progressing PKD with cysts first appearing between 10 and 14 days of age (Fig. 2-1, i-l). The cysts grew progressively larger as did kidney volume with vast majority of mutants not surviving past 12 months of age. Characterization of the Tsc1 mutant collecting ducts showed that mutant cells were highly proliferative and had a significant increase in mTor activity as indicated by increased levels of phosphorylated S6 ribosomal protein (Fig. 2-6). Thus the mTor pathway can be activated in the collecting ducts and its activation is sufficient to drive cystogenesis.

To determine whether the Lkb1 and mTor pathways act in parallel, we generated kidneys with collecting ducts lacking both Lkb1 and Tsc1. Interestingly, ablation of Lkb1 in Tsc1 mutant kidneys greatly accelerated the onset and progression of cystogenesis. Lkb1/Tsc1 double mutant kidneys exhibited cysts as early as embryonic day 15.5 (compared to P14 for Tsc1 mutants and no cysts in Lkb1 mutants. See Fig. 2-1, i-p). Cyst formation progressed rapidly over the next several weeks and by P28, little normal renal

parenchyma remained (Fig. 2-1, m-p). The vast majority of HoxB7-Cre; $Tsc1^{flox/flox}$; $Lkb1^{flox/flox}$ mutants died by 8 months of age.

Lkb1 mutant embryonic kidneys require glutamine for growth

We next sought to determine how loss of Lkb1 enhanced cystogenesis in Tsc1 mutants. Previous studies reported that Pkd1 mutant cells were glycolytic[2,65,78], resulting in increased dependence on glucose as a metabolic substrate and that this metabolic switch was at least in part due to loss of Lkb1 activity. We thus hypothesized that Lkb1 deletion altered the metabolic profile of mutant cells, increasing their preference for glucose. To test this, we wished to specifically deprive the kidneys of glucose. As the cystic phenotype was evident in Lkb1/Tsc1 double mutants as early as E15.5, we hypothesized that whatever role Lkb1 was playing, it was first required in the embryonic collecting ducts. Thus, we could use embryonic organ culture as a bioassay to examine metabolite dependence in a modified synthetic lethal screen (synthetic sickness).

To determine if Lkb1 mutant cells were more glucose-dependent than controls, we cultured E12.5 HoxB7-Cre; $Lkb1^{flox/flox}$ kidneys in glucose depleted media and assessed growth of the mutant cells. This was accomplished by quantifying UB proliferation rates and branch number. Although Lkb1 mutant ureteric buds grown in the absence of glucose did show defects in their growth rate, there was no significant difference from mutants grown in control (glucose containing) media (Fig. 2-7). In fact, Lkb1 mutants grown in the absence of glucose had higher growth rates than wild-type kidneys grown in the absence of glucose. Thus, Lkb1 deficiency does not enhance glucose dependence,

suggesting that alternative nutrients may fuel energy and biosynthetic metabolism in these mutants.

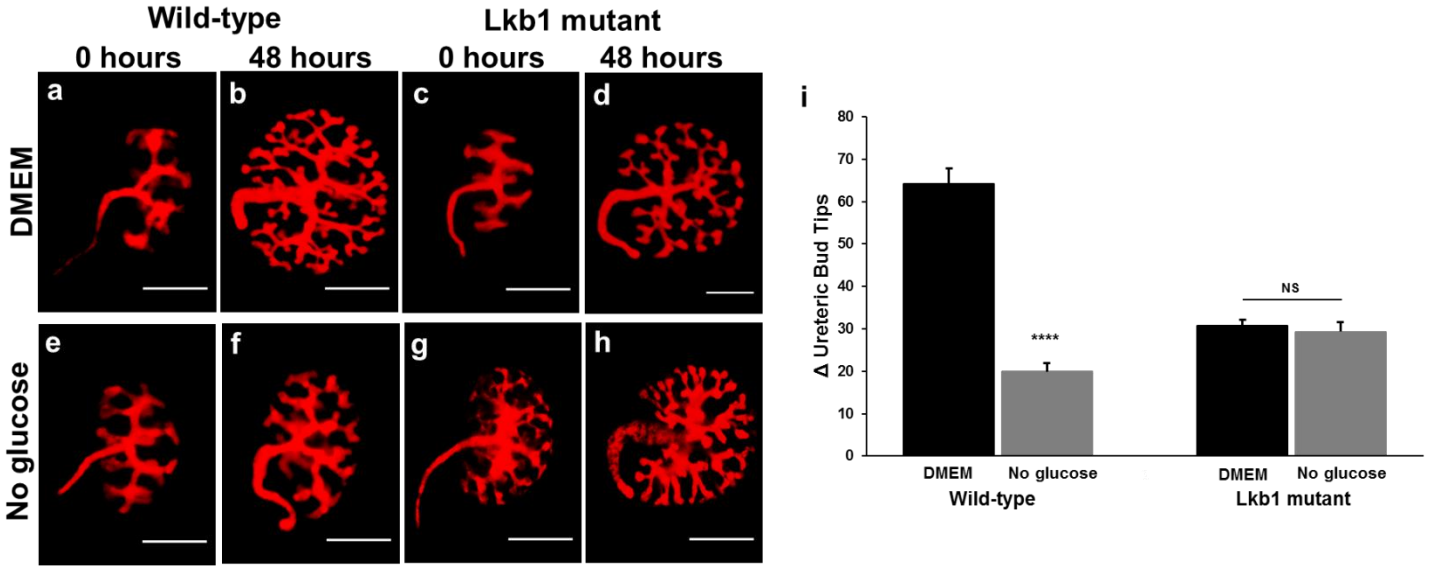

Figure 2-7: Lack of glucose does not alter collecting duct growth in cultured Lkb1 mutant kidneys. Live images of E12.5 HoxB7-Cre; RosaTomato (n=10) (a,b,e,f) and HoxB7-Cre; Lkb1$^{flox/flox}$; RosaTomato (n=10) (c,d,g,h) kidneys after 0 (a,e,c,g) or 48 (b,f,d,h) hours of culture in control media (DMEM, a-d) or DMEM lacking glucose (e-h). Quantification of the change (Δ) in branch number for wild-type and Lkb1 mutant collecting ducts (i). n=10 for each genotype under each condition. Statistical analysis via Mann-Whitney U-test.****$p < 0.0001$, NS, not significant. Error bars shown as mean +/- standard error of the mean (SEM). Scale bars equal 30 microns.

Because of its ability to donate both nitrogen and carbon, glutamine serves as a precursor in the generation of amino acids/proteins, nucleotides and lipids and participates in other metabolic processes[79-81]. It has previously been shown that proliferating cells require high levels of glutamine, and that some oncogenic mutations enhance glutamine consumption in a manner similar to the over-utilization of glucose during the Warburg effect[43,82-84]. To determine if the embryonic collecting ducts were utilizing glutamine as an energetic source, we cultured e12.5 wild-type and Lkb1 mutant kidneys in glutamine deficient media and quantified UB branching/proliferation. Although wild-type collecting ducts grew normally in the absence of glutamine, Lkb1 mutant kidneys displayed a significant decrease in ureteric bud branching and cell proliferation indicating that loss of Lkb1 resulted in glutamine dependence during cell proliferation (Fig. 2-8, 2-9).

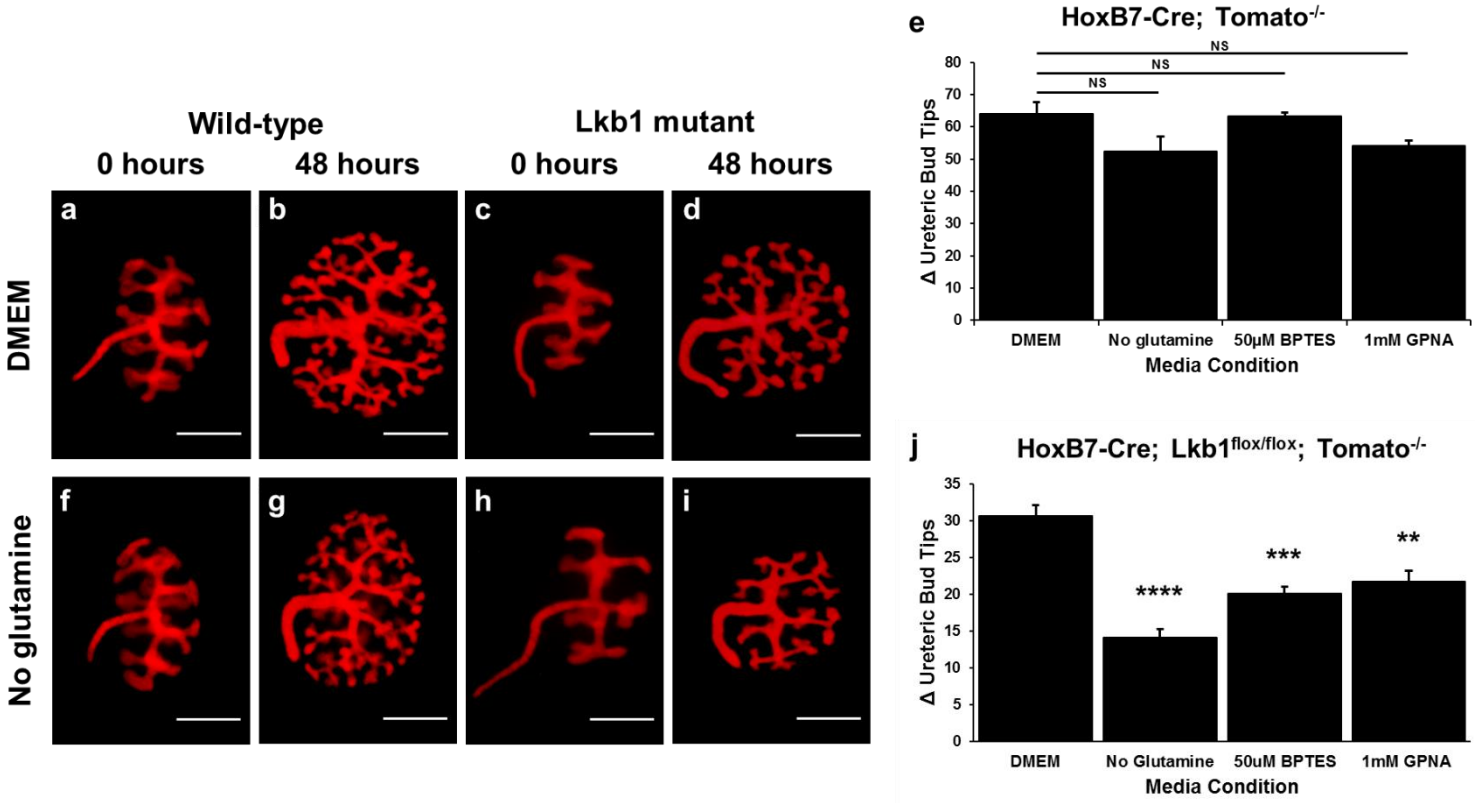

Figure 2-8: Perturbation of glutamine metabolism causes growth defect in embryonic Lkb1 mutant collecting ducts. Live images of E12.5 HoxB7-Cre;RosaTomato (n=10) (a,b,f,g) and HoxB7-Cre;Lkb1$^{flox/flox}$;RosaTomato (n=10) kidneys after 0 (a,f,c,h) or 48 (b,g,d,i) hours of culture in control media (DMEM, A-D) or DMEM lacking glutamine (f-i). Quantification of the change in branch number (number of ureteric bud tips after 48 hours – ureteric bud tips at 0 hours) for HoxB7-Cre;RosaTomato (e) or HoxB7-Cre;Lkb1$^{flox/flox}$;RosaTomato (j) kidneys grown in complete DMEM, DMEM-glutamine, DMEM + 50μM concentration of the glutaminase inhibitor BPTES, or 1mM concentration of the SLC1A5 glutamine transporter inhibitor GPNA. n=10 for each genotype under each condition. Statistical analysis via Mann-Whitney U-test **p < 0.01, ***p < 0.001, ****p < 0.0001, NS, not significant. Error bars shown as mean +/- standard error of the mean (SEM). Scale bars equal 30 microns.

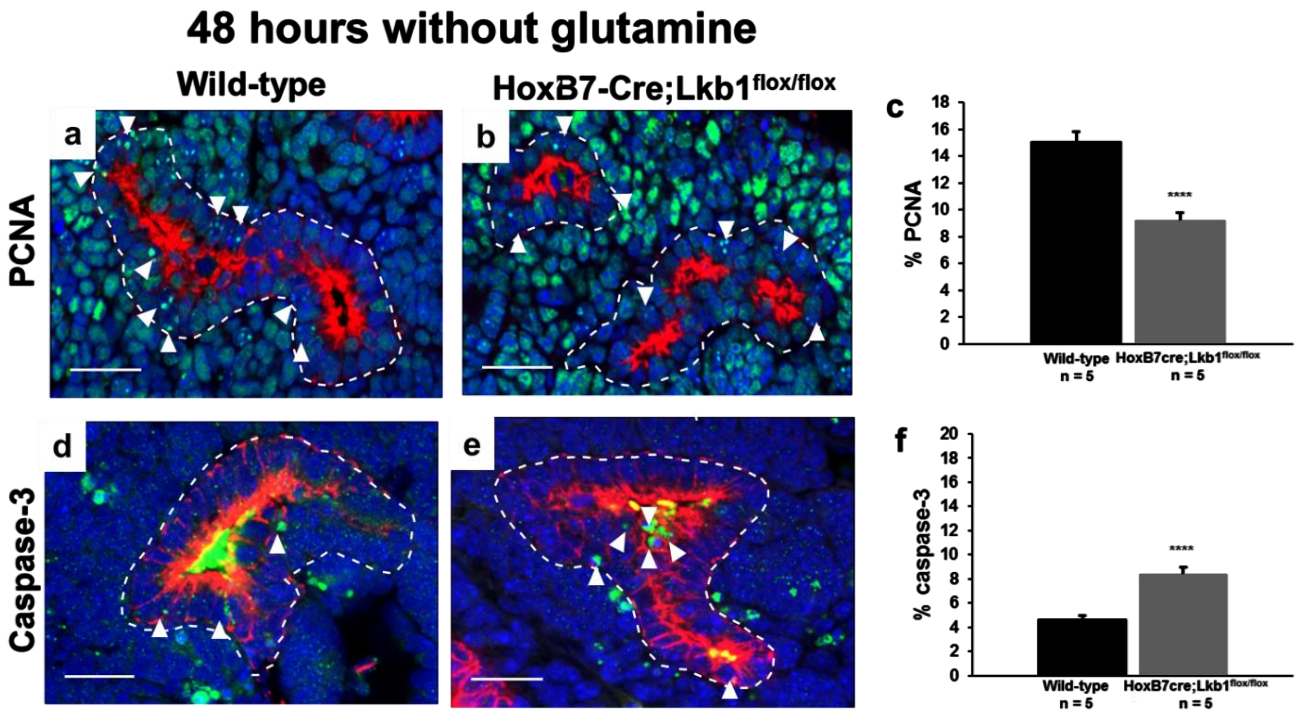

Figure 2-9: Glutamine withdrawal causes decrease in proliferation and increase in apoptosis in Lkb1 mutant kidneys. Sections through e12.5 Hoxb7-Cre;Lkb1$^{flox/+}$ (a,d) and Hoxb7-Cre;Lkb1$^{flox/flox}$ kidneys after 48 hours of culture in media lacking glutamine. Sections were stained with PCNA (green in a and b) or an antibody to capase-3 (d,e) and the collecting duct marker cytokeratin (CK, red) and DAPI (blue). The percentage of PCNA positive or Caspase-3 positive collecting duct cells was determined for 5 distinct kidneys and the average is presented in c and d, respectively. Statistical analysis via Mann-Whitney U-test. ****$p < 0.0001$. Error bars shown as mean +/- standard error of the mean (SEM). Scale bars equal 20 microns.

Previous studies have shown that activation of the mTor pathway also causes cells to become glutamine dependent. Although our in vivo analysis indicated that loss of Lkb1 did not affect mTor activity, we tested whether activation of this pathway could also lead to glutamine dependence under these conditions. To test this, we cultured Hob7Cre;Tsc1$^{flox/flox}$ kidneys with and without glutamine. Similar to wild-type cultures, while Tsc1 mutants did require glucose, they did not require glutamine for their growth (Fig. 2-10) in agreement with our previous observation that Lkb1 is not regulating mTor. Based on these observations, we hypothesized that the metabolism of glutamine could

be targeted as a therapeutic for PKD. Thus we sought to gain insight into the process(es) affected upon glutamine withdrawal.

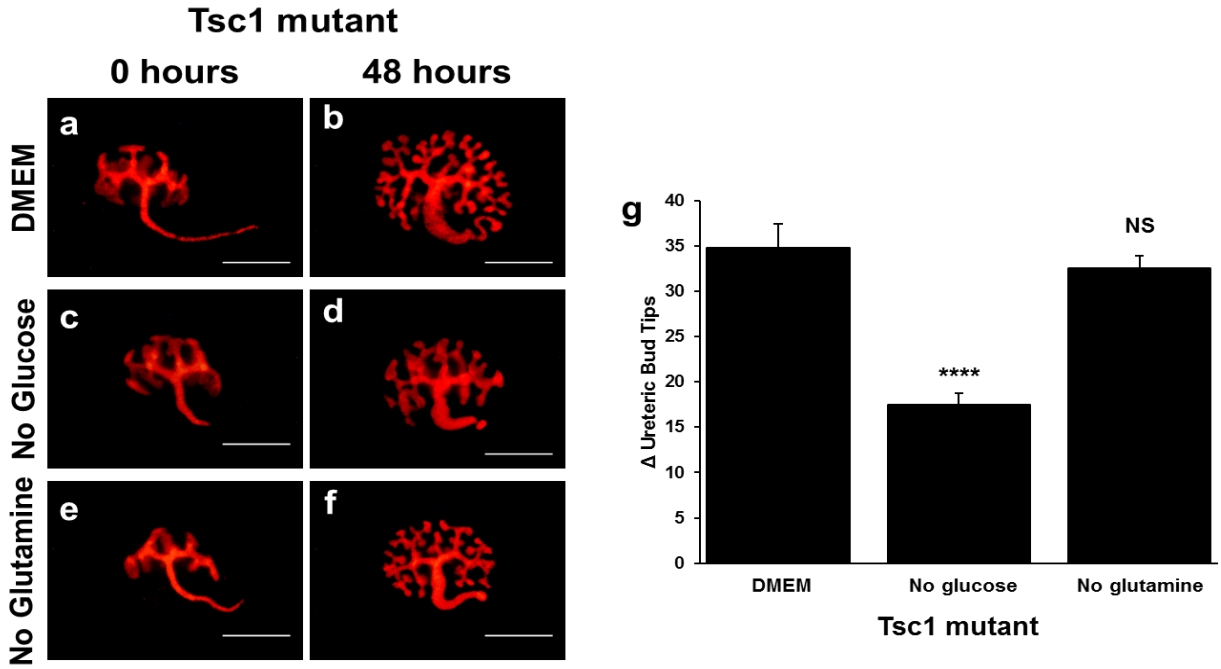

Figure 2-10: Absence of glutamine does not alter collecting duct growth in cultured Tsc1 mutant kidneys. Live images of e12.5 HoxB7-Cre;Tsc1$^{flox/flox}$;RosaTomato kidneys after 0 (a,c,e) or 48 (b,d,f) hours of culture in complete media (DMEM, a,b), glucose deficient media (c,d) or glutamine deficient media (e,f). Quantification of the change (Δ) in branch number for Tsc1 mutant collecting ducts (g). n=10 for each condition. Statistical analysis via Mann-Whitney U-test. ****p < 0.0001, NS, not significant. . Error bars shown as mean +/- standard error of the mean (SEM). Scale bars equal 30 microns.

Lkb1 mutants require NEAAs and glutathione for growth

Glutamine supplies a variety of metabolic pathways depending on the needs of the cell. In order to substantiate which pathway(s) contributes to collecting duct proliferation within wild-type and Lkb1 mutant kidneys, we used established metabolites and inhibitors of the glutamine/glutamate metabolism pathway in our ex vivo culture system.

We first sought to establish whether glutamine uptake was necessary for branching in the Lkb1 mutant kidneys. To accomplish this we supplemented the culture media with GPNA, an inhibitor of Slc1a5, the primary glutamine transporter, to block glutamine uptake into the cell[79,85].

Inhibition of Slc1a5 resulted in a significant decrease in ureteric bud branching in Lkb1 mutant (but not wild-type) kidneys (Fig. 2-8), indicating that glutamine uptake by the cell was necessary for growth in Lkb1 mutants.

Once inside the cell, glutamine can be metabolized and utilized in various metabolic processes. To narrow down the requirement for glutamine in Lkb1 mutants, we tested whether inhibition of glutaminase, the enzyme that converts glutamine into glutamate, would lead to a defect in branching in Lkb1 mutants. Similar to what was observed upon withdrawal of glutamine from the media, treatment of Lkb1 mutant kidneys with the glutaminase inhibitor, BPTES[86,87], resulted in decreased ureteric bud branching relative to wild-type treated kidneys (Fig. 2-8), suggesting that glutamine must be metabolized in Lkb1 mutant kidneys to promote growth.

As glutamine derivatives can contribute to multiple biosynthetic processes in the cell, we utilized an unbiased approach to determine which metabolic pathways were deficient in Lkb1 mutants. A metabolomics screen of embryonic wild-type and Lkb1 mutant kidneys in complete media and media lacking glutamine was performed. Surprisingly, these results showed that there was no change in TCA cycle intermediates in Lkb1 mutant kidneys cultured in the absence of glutamine suggesting that the role of Lkb1 loss in cystic enhancement is not due to defects in this pathway. However, we did see significant deficiencies in nucleotide, amino acid, and glutathione metabolism precursors (Fig. 2-11).

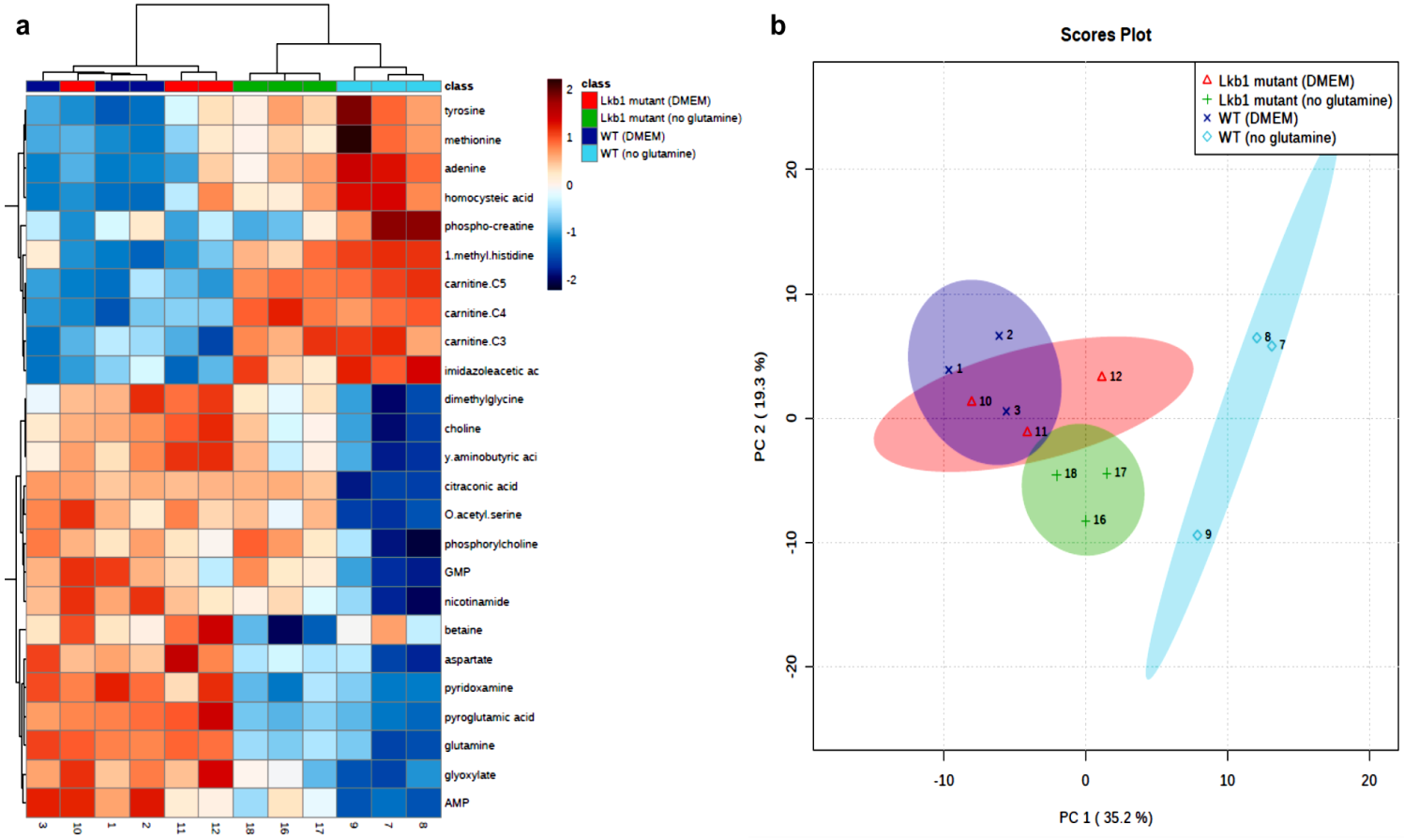

Figure 2-11: Metabolomics

Heatmap showing relative abundance of the 25 most significantly affected metabolites extracted from samples of cultured kidneys from embryonic day 13.5 wild-type and Lkb1 mutant kidneys cultured in control media (DMEM) and DMEM – glutamine. The colorbar presented is a log2 scale (a). Principal Component Analysis (PCA) of wild-type and Lkb1 mutant kidneys in the presence and absence of glutamine (b).

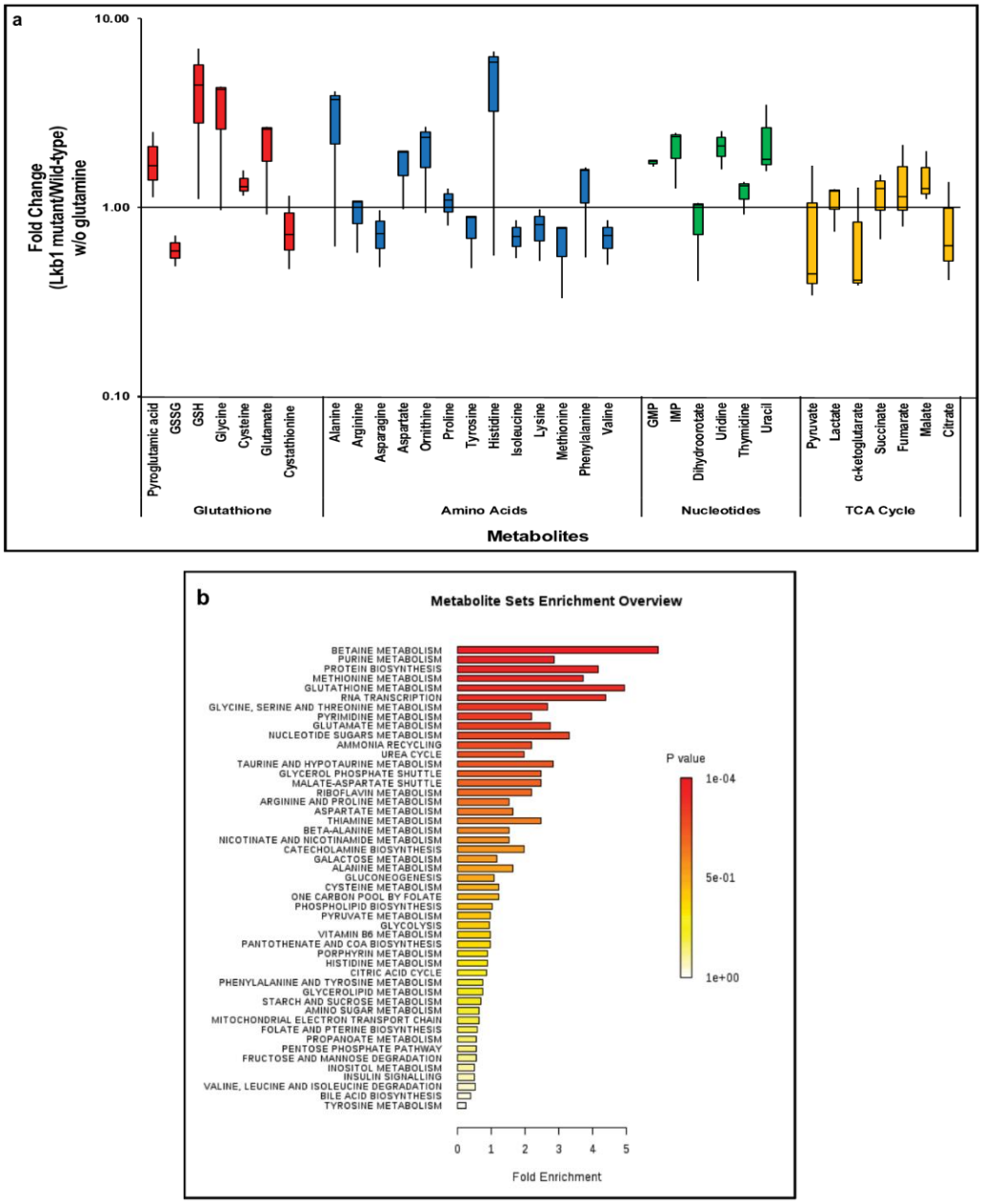

Figure 2-12: Metabolomics Enrichment Analysis

Box plot of fold changes of key metabolites in glutathione, amino acid, nucleotide, and TCA cycle metabolic pathways during glutamine withdrawal (a). Variable importance (VIP) scores were calculated and compared between wild-type and Lkb1 kidneys under control and glutamine-free conditions. Metabolites with a VIP score over 1.00 were considered to be significant in determining the classes used for metabolite set enrichment analysis (MSEA) (b). Metabolomics was performed on 3 independent samples containing 20 intact e13.5 kidneys per sample cultured in the presence and absence of glutamine. Statistical analysis via student's t-test. Error bars shown as mean +/- standard error of the mean (SEM).

To determine whether any of these deficiencies played a causal role in the growth defects observed, we cultured Lkb1 mutant kidneys in glutamine-free media supplemented with essential amino acids, non-essential amino acids, or glutathione. A mix of essential amino acids (Sigma) provided minimal but statistically significant enhancement of Lkb1 mutant ureteric bud growth. The addition of oxidized glutathione (GSSG) was capable of rescuing Lkb1 mutant kidneys to control media conditions. However, augmentation with non-essential amino acids or reduced glutathione increased ureteric bud branching to levels comparable to what is observed in wild-type kidneys cultured in complete media suggesting that defects in one or both of these biosynthetic pathways may be contributing to the growth defects observed in Lkb1 mutant kidneys (Fig. 2-13).

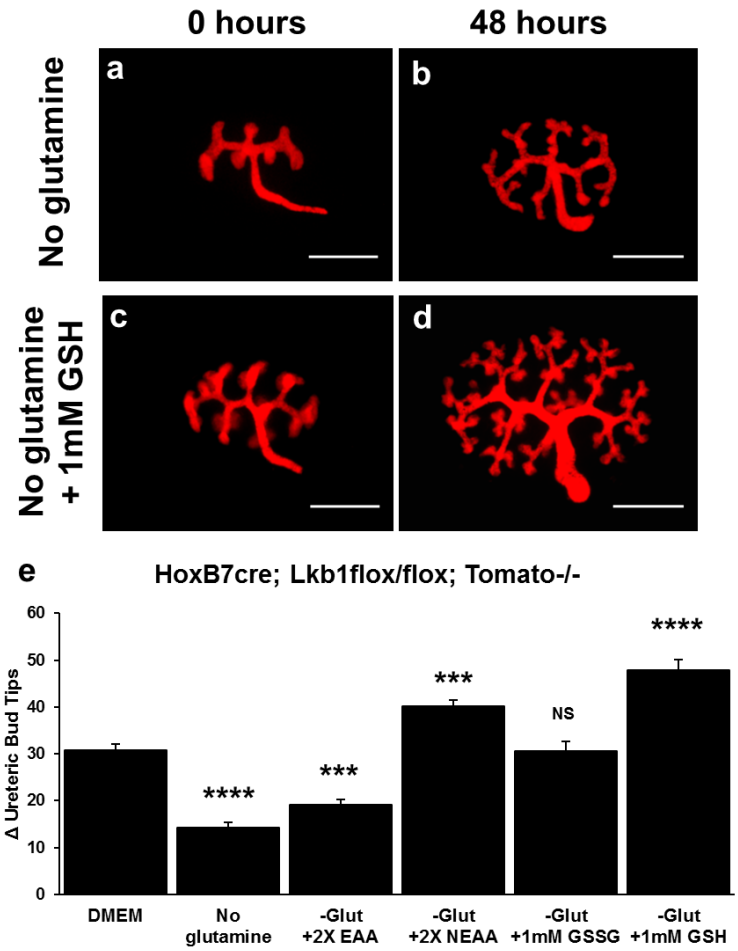

Figure 2-13: Exogenous sources of amino acids or glutathione rescues growth defect of Lkb1 mutant cells in the absence of glutamine. Live images of e12.5 HoxB7-Cre;Lkb1$^{flox/flox}$;RosaTomato kidneys after 0 (a,c) or 48 (b,d) hours of culture in glutamine deficient media (a,b) or glutamine-deficient media supplemented with 1mM GSH (c,d). (e) Quantification of the change (Δ) in branch number (number of ureteric bud tips after 48 hours – ureteric bud tips at 0 hours) for e12.5 HoxB7-Cre;Lkb1$^{flox/flox}$;RosaTomato kidneys grown in DMEM, DMEM-glutamine, DMEM-glutamine + 2x essential amino acids, DMEM-glutamine + 2x non-essential amino acids, DMEM-glutamine + 1mM oxidized glutathione (GSSG), or DMEM-glutamine + 1mM reduced glutathione (GSH). Note that supplementation NEAAs, GSSG, or GSH is capable of restoration of Lkb1 mutant collecting duct branching equal to or above levels seen in control media. All statistical comparisons are relative to DMEM conditions. n=10 for each condition. Statistical analysis via Mann-Whitney U-test, ***$p < 0.001$, ****$p < 0.0001$, NS, not significant. Error bars shown as mean +/- standard error of the mean (SEM). Scale bars equal 30 microns.

Pkd1 mutants require NEAAs and glutathione for growth

Although previous studies have suggested that Pkd1 mutants have decreased Lkb1 activity[65,77], a similar glutamine dependence has not been shown. To determine If Pkd1 mutants also show a dependence on glutamine, E12.5 HoxB7-Cre;Pkd1$^{flox/flox}$ kidneys were cultured for 48 hours in complete media or media lacking glutamine and cell proliferation rates and UB branching were quantified. Similar to what was observed in Lkb1 mutants, we observed a significant decrease in Pkd1 mutant ureteric bud branching when glutamine was withdrawn from the culture media suggesting that Pkd1 mutant kidneys also require glutamine for their growth (Fig. 2-14). To determine if this glutamine dependence required glutaminolysis, we first sought to determine whether glutaminase activity was required. Similar to what was observed with Lkb1 mutants, addition of BPTES to the culture media inhibited growth in Pkd1 mutants but not in wild-type cultured kidneys. Further, analogous to what we observed in Lkb1 mutant kidneys, an exogenous source of essential amino acids or oxidized glutathione (GSSG) was sufficient to increase proliferation and branching compared to glutamine withdrawal, but neither was capable of increasing growth to control media conditions. In contrast, the supplementation of non-essential amino acids or reduced glutathione (GSH) to glutamine-free media was capable of returning Pkd1 mutant kidney ureteric bud growth to control levels (Fig. 2-14).

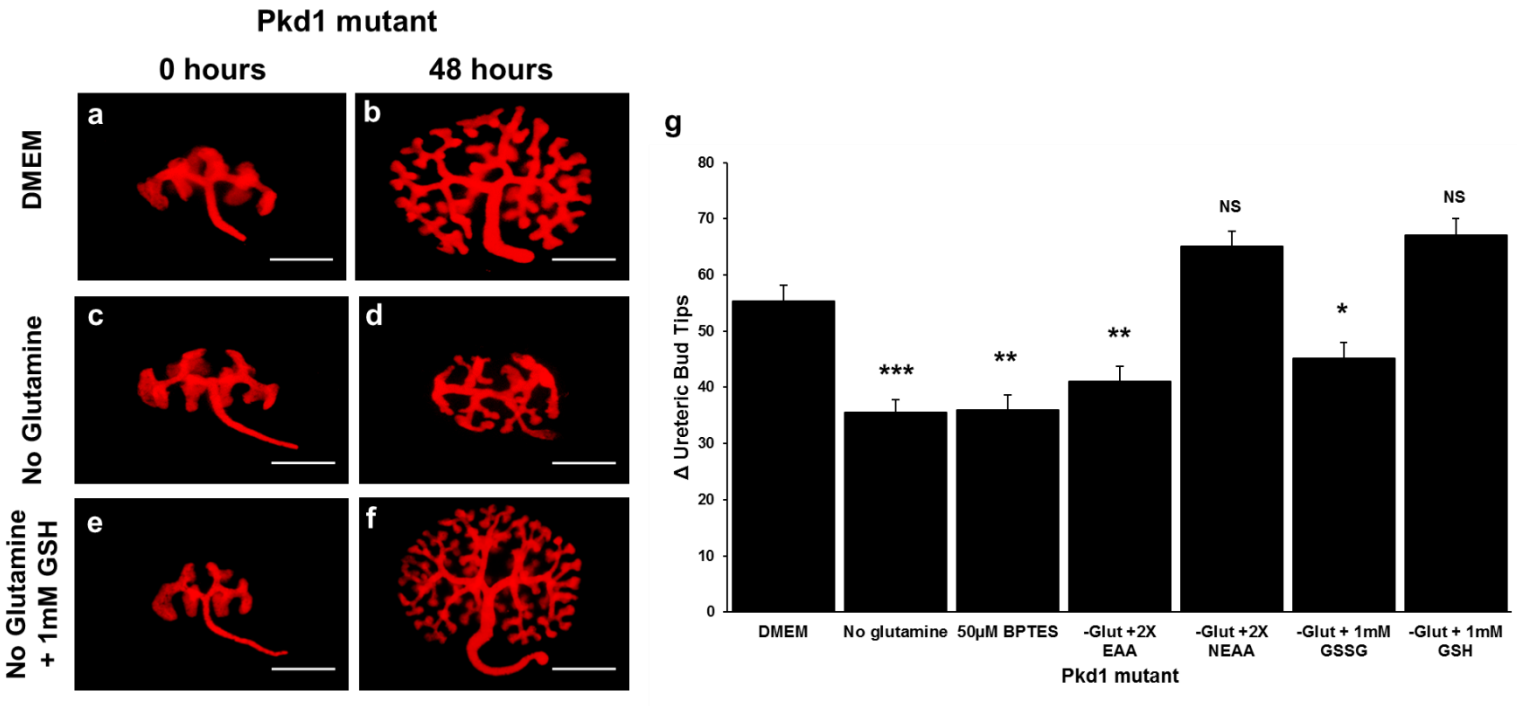

Figure 2-14: Embryonic Pkd1 mutant collecting ducts are dependent on glutamine for growth. Live images of e12.5 HoxB7-Cre;Pkd1$^{flox/flox}$;RosaTomato kidneys after 0 (a,c,e) or 48 (b,d,f) hours of culture in complete media (DMEM, a,b), glutamine deficient media (c, d) or glutamine-deficient media supplemented with 1mM GSH (e,f). (g) Quantification of the change (Δ) in branch number (number of ureteric bud tips after 48 hours – ureteric bud tips at 0 hours) for E12.5 HoxB7-Cre;Pkd1$^{flox/flox}$;RosaTomato kidneys grown in DMEM, DMEM-glutamine, DMEM+BPTES, DMEM-glutamine + 2x essential amino acids, DMEM-glutamine + 2x non-essential amino acids, DMEM-glutamine + 1mM oxidized glutathione (GSSG), or DMEM-glutamine + 1mM reduced glutathione (GSH). Statistical analysis is relative to Pkd1 mutant grown in complete media (DMEM). . n=10 for each media condition. Statistical analysis via Mann-Whitney U-test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, NS, not significant. Error bars shown as mean +/- standard error of the mean (SEM). Scale bars equals 30 microns.

Inhibition of glutaminase represses cyst progression in vivo

Our data suggest that glutaminolysis is necessary for proliferation in Lkb1 and Pkd1 mutant embryonic kidneys. As Lkb1 activity appears to be lost in Pkd1 mutants and as Lkb1 can suppress cystic growth in adult kidneys, we hypothesized that we may be able to target pathological growth in cystic kidneys in vivo by targeting glutamine metabolism. To test this, we administered the glutaminase inhibitor, BPTES (100mg/kg), to pregnant females carrying litters of HoxB7-Cre;Lkb1$^{flox/flox}$;Tsc1$^{flox/flox}$ embryos beginning at e15.5. Unfortunately, the administration during embryogenesis lead to spontaneous termination of the pregnancy. To overcome this issue, we administered the drug to nursing mothers from post-natal day 1 to post-natal day 10. Kidneys were harvested from HoxB7-Cre;Lkb1$^{flox/flox}$;Tsc1$^{flox/flox}$ mutants and subsequently sectioned, stained and their cystic burden quantified. We observed a significant decrease in cystic index in mutant pups whose mothers were administered BPTES versus ones whose mothers were administered the vehicle alone (Fig. 2-15, a-c).

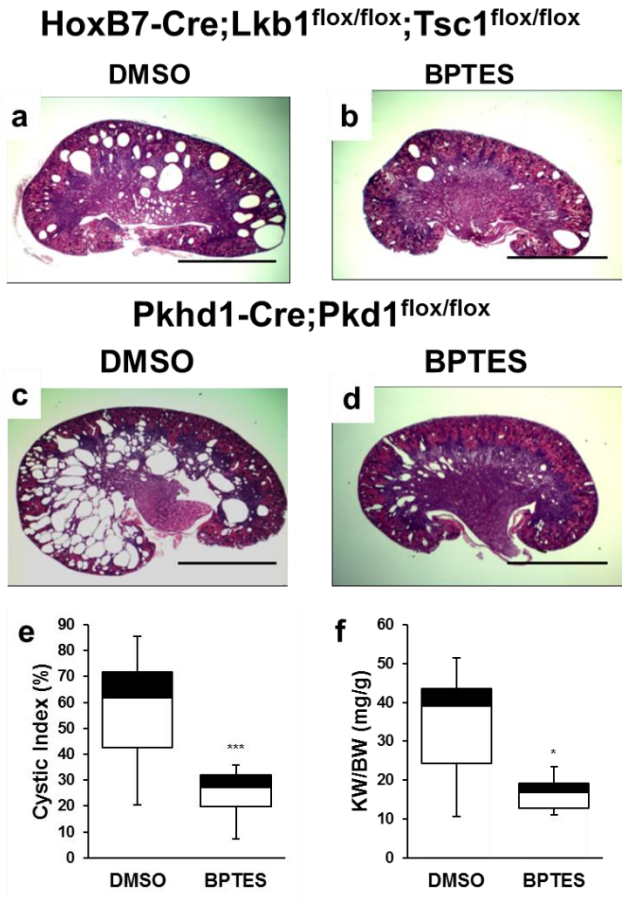

Figure 2-15: Inhibition of glutamine metabolism slows cyst progression in vivo. Representative examples of H and E stained sections from post-natal day 10 HoxB7-Cre; Lkb1^{floxflox}; Tsc1$^{flox/flox}$ (a,b); and Pkhd1-Cre; Pkd1$^{flox/flox}$ (c,d) kidneys that were treated with 5% DMSO (a,c) or 100mg/kg body weight of the glutaminase inhibitor, BPTES (b,d). Cystic indices for DMSO vs BPTES-treated Pkhd1-Cre; Pkd1$^{flox/flox}$ kidneys shows a significant reduction in the number of cysts in BPTES-treated animals (e) Kidney-to-body weight ratio of DMSO vs BPTES-treated Pkhd1-Cre; Pkd1$^{flox/flox}$ kidneys (f). HoxB7-Cre;Lkb1$^{flox/flox}$;Tsc1$^{flox/flox}$ (DMSO-treated, n=16; BPTES-treated, n=16). Pkhd1-Cre; Pkd1$^{flox/flox}$ (DMSO-treated, n=14; BPTES-treated, n=14). Statistical analysis via Mann-Whitney U-test. *$p < 0.05$, ***$p < 0.0001$**.** . Error bars shown as mean +/- standard error of the mean (SEM). Scale bars equals 20mm.

Although our data suggest that the Lkb1/Tsc1 mutants mimic the etiology of human PKD, we wanted to test the therapeutic potential of glutaminase inhibitors in an orthologous model of the disease. To accomplish this, we generated mice in which Pkd1 was deleted with Pkhd1Cre. Previous analysis showed that this Cre line is primarily active in the postnatal collecting ducts[60] allowing us to determine whether the glutamine dependence was only relevant to embryonic stages or also in a post-natal state.

Pkhd1-Cre;Pkd1$^{flox/flox}$ pups were administered glutaminase inhibitor through the breast milk and harvested 10 days later. Similar to what was observed with the Lkb1/Tsc1 double mutants, administration of the glutaminase inhibitor significantly decreased the kidney-to-body weight ratio and cystic burden in Pkhd1-Cre;Pkd1$^{flox/flox}$ pups (Fig. 2-15, d-g). Although serum creatinine levels become elevated in older mutants, we were unable to detect significant differences in creatinine levels between untreated wild-type and mutant mice (treated or untreated) at P10 (Fig. 2-16).

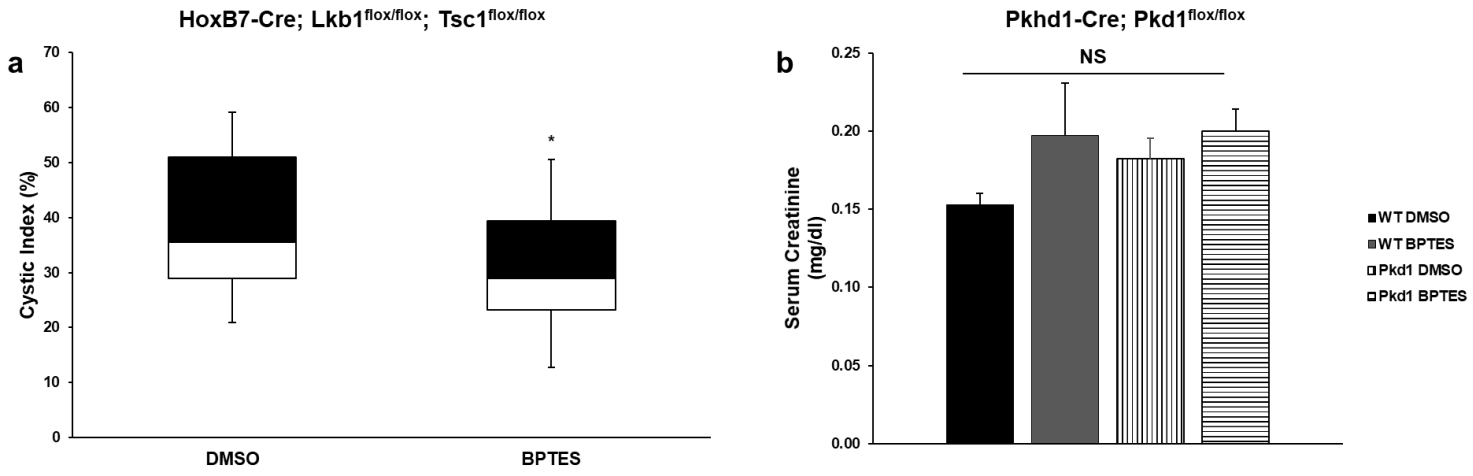

Figure 2-16: Glutaminase inhibitor, BPTES, has no effect on serum creatinine of Pkd1 mutant kidneys. Comparison of cystic indices for DMSO vs BPTES-treated HoxB7-Cre; Lkb1$^{flox/flox}$; Tsc1$^{flox/flox}$ kidneys shows a significant reduction in the number of cysts in BPTES-treated animals (a). Quantification of serum creatinine of wild-type and Pkhd1-Cre; Pkd1$^{flox/flox}$ kidneys treated with DMSO or the glutaminase inhibitor, BPTES (b). Statistical analysis via Mann-Whitney U-test. *$p < 0.05$, NS, not significant. Error bars shown as mean +/- standard error of the mean (SEM).

Glutamine is essential for growth of adult Lkb1 mutant cells

To this point, our studies have focused on embryonic and early post-natal kidneys. However, the majority of human PKD patients do not develop cysts until 30 to 50 years of age. To determine if our findings would be relevant to the adult kidney, we generated cell lines from the collecting duct of 6-to-8 week old $Lkb1^{flox/flox}$ and $Pkd1^{flox/flox}$ mice. Individual lines were transfected with adenoviruses expressing ubiquitous Cre to generate mutant cell lines.

In contrast to what we observed with our intact embryonic kidney cultures, control, Lkb1 mutant, and Pkd1 mutant cells all died within 48 hours if glutamine was completely withdrawn from the media (Fig. 2-17, a). This is in agreement with previous studies showing that SV-40 immortalization alone was sufficient to cause glutamine addiction. To determine if mutant cells were more sensitive to glutamine deprivation, we cultured cells in different concentrations of glutamine. Similar to what was observed in our ex vivo models, isolated Lkb1 mutant collecting duct cells were more dependent on glutamine than control cells, and this dependence led to a significant decrease in proliferation in a concentration-dependent manner (Fig. 2-17, b). The growth defect of Lkb1 mutant cells in glutamine deprived conditions could be rescued by the addition of an exogenous source of non-essential amino acids (-glutamine), as well as reduced glutathione (Fig. 2-17, b-c). Addition of nucleotides/nucleosides (Millipore) to glutamine-depleted media was unable to rescue the proliferation defect of Lkb1 mutants (Fig. 2-17, c). However, isolated adult Pkd1 mutant cells did not respond in a similar manner to glutamine withdrawal as

Pkd1 embryonic cultures. Pkd1 mutant cells had no significant decrease in proliferation compared to control cells under low glutamine conditions. Further, while the addition of non-essential amino acids or reduced glutathione to low glutamine media (0.25mM) rescued the Lkb1 mutants, it actually caused a significant decrease in proliferation of both wild-type and Pkd1 mutant cells (Fig. 2-17, b,d). These data suggest that while loss of Lkb1 renders embryonic and adult collecting duct cells glutamine dependent, loss of Pkd1 results only in glutamine dependence in the embryonic/earl post-natal stage but not in adults. This may explain why deletion of Pkd1 from adult kidneys leads to a very slow progressing cystic disease.

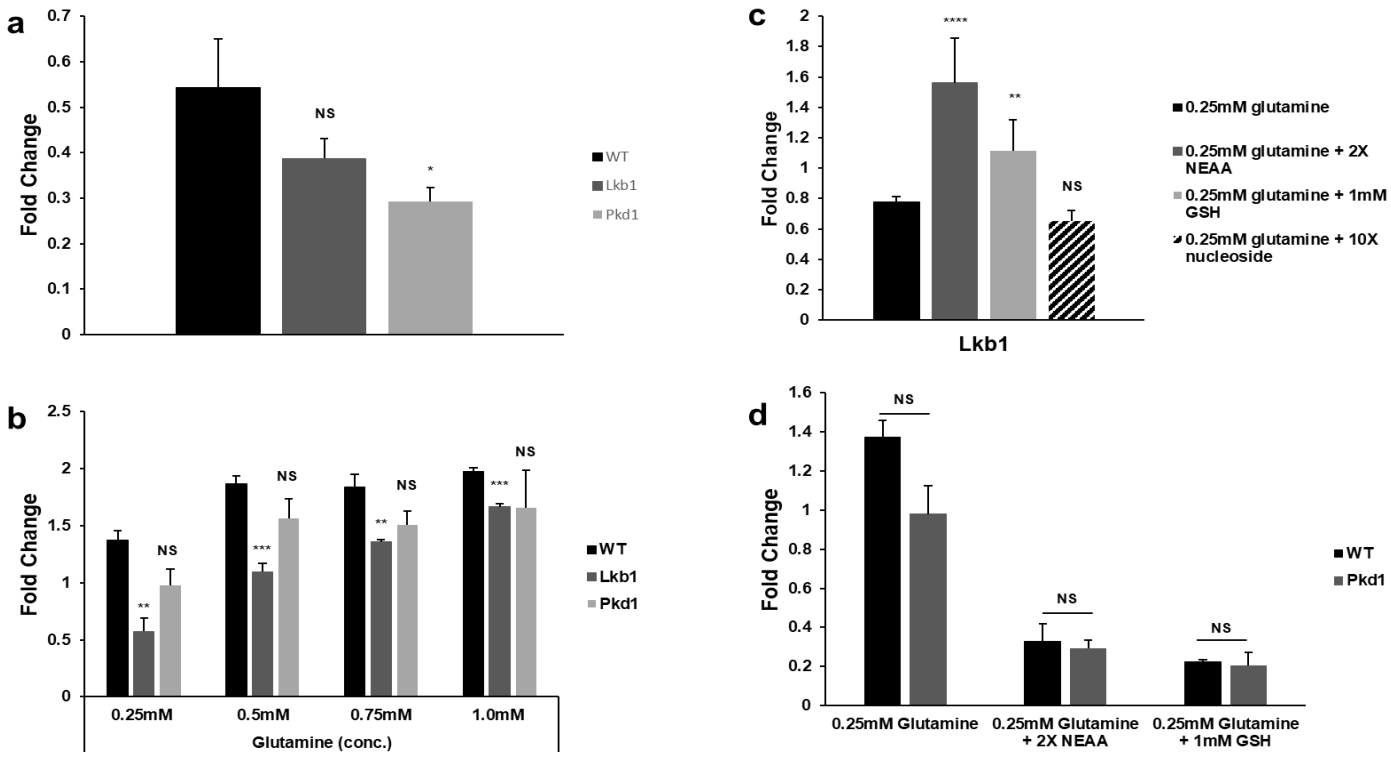

Figure 2-17: Decreases in glutamine concentration causes decrease in proliferation in isolated Lkb1 mutant collecting duct cells. Quantification of fold change in growth from T=0 to T=48 hours in DMEM lacking glutamine (a). Quantification of fold change in specified (0.25mM-1.0mM) glutamine concentration in control (WT), Lkb1 mutant, and Pkd1 mutant inner medullary collecting duct (IMCD) cells (b). Quantification of fold change of Lkb1 mutant (c) and Pkd1 mutant (d) IMCD cells in low glutamine (0.25mM), low glutamine with non-essential amino acid mix (without glutamine), low glutamine with 1mM reduced glutathione (GSH), and low glutamine with nucleoside mix. Statistical analysis via student's t-test. *p <0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, NS, not significant. Error bars shown as mean +/- standard error of the mean (SEM).

Discussion

There have been many attempts to therapeutically target increased cell proliferation in human PKD, although to date, no therapeutics have been approved for human use in the U.S. Here, we show that embryonic kidneys lacking Pkd1 require glutamine for cell proliferation and tissue growth. Our data suggest that this is at least in part due to loss of Lkb1 activity as Lkb1 mutants show a similar glutamine dependence and co-ablation of Lkb1 enhances cyst formation in Tsc1 mutants. Importantly, ablation of Tsc1 alone does not lead to glutamine dependence (not shown). Inhibition of glutaminolysis both in ex vivo organ culture and in vivo slows cell growth and blocks cyst progression. Thus, we have revealed a novel metabolic pathway that can be targeted for PKD therapy.

We acknowledge that PKD is not solely caused by increased proliferation[88] and defects in solute transport most likely contribute to the disease. However, therapies that can slow proliferation should have a significant impact on this disease and can potentially be coupled with therapies that regulate tubular secretion. We feel that the data collected in this study will have a direct and significant impact on PKD treatment.

Mechanistically, our data suggests that Lkb1 normally acts downstream of Polycystin1 to suppress cell proliferation within the kidney epithelia. However, it is important to note that our data does not suggest that defects in glutamine metabolism contribute to PKD. This is highlighted by the fact that loss of Lkb1 alone is not sufficient to cause cyst formation. The metabolic alterations themselves are not cyst causing. Instead, our data suggests that loss of Lkb1 activity alters some aspect of normal

metabolism rendering mutant cells more dependent on glutamine than wild-type. In other words, glutamine metabolism is an Achilles' heel in PKD. Although usually considered a non-essential amino acid, glutamine is actually a conditionally essential amino acid, meaning under certain conditions, the body is not able to synthesize sufficient amounts to fulfill metabolic requirements. Indeed glutamine appears to be essential for rapid cell growth and many cancers require high rates of synthesis and/or import for their growth/survival[80,82,83,89,90]. This conditional dependence makes targeting glutamine dependence particularly attractive as it should only affect the diseased cells leaving normal, healthy tissue unaffected (as we observed in our ex vivo and in vivo studies).

In other pathological conditions, alterations in various molecular processes have been reported to underlie glutamine dependence. Our metabolomic analysis indicates that in Lkb1 mutants, glutamine is required for non-essential amino acid and glutathione synthesis. Ex vivo culture experiments revealed that the defect in growth caused by the absence of glutamine can be completely rescued with an exogenous source of non-essential amino acids (NEAA) and reduced glutathione (GSH), while the addition of essential amino acids shows only moderate enhancements in growth, relative to NEAA and GSH.

The changes in the NEAA and glutathione metabolic pathways may be related. Glutathione, an antioxidant that prevents cellular damage from reactive oxygen species (ROS), consists of three non-essential amino acid precursors (glutamate, glycine, and cysteine), and the production rates of these amino acids are strictly controlled by cells to optimize glutathione utilization for redox reactions. The ability of the oxidized form of

glutathione to rescue at low efficiency may be the result of the cells converting it back to the reduced form. It is possible that the absence of glutamine slows the production of one or more of the required glutathione precursors and increases the vulnerability of mutant cells to energetic stress and ROS. However, it is possible that this has nothing to do with ROS and glutathione is simply able to be metabolized into some other metabolite (such as glutamate and cysteine) that is necessary for growth. Further investigation into what genetic, epigenetic, and environmental alterations occur within mutant cells during glutamine withdrawal is needed to determine how these metabolic shifts cause significant changes in cellular behavior including cell stability, proliferation, and repair. It is still not clear where and how Lkb1 is functioning to regulate metabolism in the collecting ducts. We found that deletion of Lkb1 does not affect the levels of pAMPK in collecting duct cells in vivo or in vitro under otherwise normal conditions; thus the substrate of Lkb1 within the collecting ducts is still not clear. Indeed it is not even certain that this role requires the kinase activity of this protein.

A final important observation that comes from this study is that organ culture can be used as a bioassay for PKD therapeutics. In the past, this was not considered to be the case as Pkd1 and Pkd2 mutant kidneys do not form cysts when cultured ex vivo. Although addition of cyclic AMP agonists lead to tubule dilation, the physiological relevance of this treatment is unclear[52]. Here, we show that growth of the embryonic kidney can be used as a surrogate for pathological growth, and this can be easily visualized by monitoring branching morphogenesis. By performing a modified synthetic lethal screen (synthetic sickness), we were able to identify a crucial role for glutamine in

kidney growth, the biological process in which it was acting, and identify potential therapeutics.

Because our studies were performed in embryonic and early post-natal kidneys, it is possible that these findings may not extend to the adult disease. In fact, while deletion of Lkb1 in adult collecting duct cell lines resulted in the same metabolic changes as seen in the embryo/post-natal collecting duct, Pkd1 mutants behaved similar to wild-type in terms of dependence on glutamine and response to non-essential amino acids and glutathione. This could be interpreted to mean that Pkd1 deletion in adult kidneys does not lead to glutamine dependence. However, another possibility is that ablation of Pkd1 in adult cells is not sufficient to alter metabolism without some additional stress. Indeed, previous work has shown stark differences in PKD pathology depending on the developmental stage in which Pkd1 is ablated[91]. Ablation of Pkd1 in adult kidneys leads to a very slow progressing disease relative to ablation in embryos/early post-natal animals. The lack of glutamine dependence in adult Pkd1 mutant cells may reflect this developmental switch. However, given that ablation of Lkb1 from the adult does lead to glutamine dependence, this model would predict that adult ablation of Pkd1 does not affect Lkb1 activity. Importantly, Polycystin1 had only been shown to regulate Lkb1 activity in embryonic fibroblasts and early postnatal kidneys[92,93]. To our knowledge, it has not been analyzed in mutant adult kidneys. It will be interesting to determine if Lkb1 activity plays a role in this developmental switch. A second possibility is that Lkb1 and/or glutamine only play a role in immature tubules and they have no role in adult PKD. Studies to address these questions are underway. Interestingly, cells derived from an autosomal

recessive form of PKD in rats also show glutamine dependence suggesting that glutamine metabolism may represent a therapeutic target for multiple forms of PKD[94].

In summary, we have identified a metabolic alteration in PKD caused at least in part to deficits in Lkb1 activity that makes cells sensitive to glutamine withdrawal. We believe that a greater insight into the precise molecular role of Lkb1 and metabolism in cyst progression will advance our understanding of PKD etiology as well as identifying additional therapeutic targets.

CHAPTER THREE

DISCUSSION

What are the metabolic bottlenecks in PKD?

The onset and developmental timetable of PKD as well as the precise mechanism for cyst generation and progression remains poorly understood. A widely established genetic mechanism proposes that though every cell in the kidneys of PKD patients contains the same germline mutation in either *PKD1* or *PKD2*, a second somatic "hit" is required for the focal cyst formation, resulting in cysts that are clones of the "two-hit" cells. Despite convincing evidence, this model does not completely explain evidence of incompletely penetrant mutations and inconsistencies observed in human PKD patients or PKD mouse models, including data that shows that the gene dosage of *PKD1* or *PKD2* may be dependent on specific developmental window. There is also evidence that individual cyst may arise due to reduced levels of PC1 or PC2 predispose renal epithelial cells to respond to conditions of stress, such as those associated with renal injury, that manifest into the hyperproliferative and malfunctioning cyst-lining cells. Moreover, the time of origin and severity of PKD, even among family members with the same germline mutation, suggests variations in intrinsic and extrinsic factors in acquisition of the cystic phenotypes observed in PKD.

One of the most striking features in cancer or other highly proliferative cells is the misappropriation of glucose and glutamine. Regulation of glucose, glutamine, and other nutrient uptake presents an essential weakness in cellular transformation because cells must attain mutations that allow the unrestricted and independent control of nutrient uptake in order to continuously drive cell cycle entry of proliferating cells. The shift from oxidative phosphorylation to aerobic glycolysis causes the majority of glucose molecules

to disengage from the TCA cycle despite an adequate presence of oxygen. The products of glucose catabolism are needed to significantly increase the generation of the building blocks needed to amplify cellular mass. Previous studies showed deviant glucose consumption in cystic kidneys[22,65]. Results from this study showed that glutamine metabolism is also abnormal in PKD. Embryonic kidneys deficient in Lkb1 or Pkd1 resulted in the arrested growth of collecting duct tubules in the absence of glutamine. Despite glucose and glutamine being two of the most important contributors of cellular metabolism, most cells maintain a metabolic flexibility to compensate for the depletion of either nutrient. When this flexibility is compromised, cells lack the capability to generate the diverse metabolites needed for cellular homeostasis and must scavenge the replacements from extracellular sources. Unbiased metabolic profiling of wild-type and Lkb1-defecient embryonic kidneys cultured in the presence and absence of glutamine suggested that the most prevalent metabolites altered in Lkb1 mutant cells are involved in amino acid and glutathione metabolism. Alterations in these pathways were substantiated by the ability to rescue the growth and branching defect of Lkb1 and Pkd1 mutant kidneys cultured without glutamine by supplementing the culture media with an exogenous source of amino acids or glutathione.

What is the potential function of nucleotide metabolism in cystic proliferation in vivo?

Metabolomic analysis also revealed biosynthesis of nucleotides as one of the metabolic pathways differentially regulated between our wild-type and Lkb1 mutant kidneys during culture without glutamine. This was not surprising because nucleotides play a vital role in several processes in cell growth and proliferation as components of deoxyribonucleic acid/ ribonucleic acid (DNA/RNA), energy transfer coenzymes adenosine triphosphate/ guanosine-5'-triphosphate (ATP/GTP), metabolic redox cofactors nicotinamide adenine dinucleotide/ flavin adenine dinucleotide (NAD/FAD), and cellular second messengers cyclic adenosine monophosphate/ cyclic guanosine monophosphate (cAMP/cGMP). Preliminary observations divulged no insight into the role of nucleotides biosynthesis or degradation in the growth defect seen in Lkb1 mutant kidneys during glutamine withdrawal. The effect on nucleotide metabolism may be a secondary effect from inadequate amino acid metabolism as the de novo synthesis of purine and pyrimidine compounds present in nucleotides requires glutamine, glycine, and aspartate in several steps in their synthesis. Determining how nucleotide metabolism is involved in the defects in proliferation detected in glutamine-deprived Lkb1 and Pkd1 mutant kidneys may give greater insight into the precise mechanism of normal and cystic growth of the collecting ducts.

How does non-essential amino acid metabolism influence cystic growth?

Amino acids are obligatory for cell survival. Highly proliferative transformed cells have a remarkably increased demand for amino acids to support rapid cell division. Essential amino acids cannot be synthesized within the cell and must be obtained from external sources. Non-essential amino acids are readily synthesized within cells, but due to the fast proliferation rate of transformed cells, even these amino acids need to be acquired from peripheral metabolic pathways to meet the increased demand for nutrients.

Glutamine, a non-essential amino acid, is the second-most consumed metabolite of rapidly dividing cells because it can be utilized as both a carbon source for TCA cycle intermediates and a nitrogen source for nucleotides, hexoamines, and other amino acids[89,95]. Results from this research show that the absence of glutamine has many downstream consequences in amino acid and glutathione metabolic signaling. Correspondingly, exogenous sources of non-essential amino acids or reduced glutathione resulted in the most significant restoration of growth in glutamine-deprived Lkb1 and Pkd1 mutant embryonic kidneys in culture.

Most non-essential amino acids obtain their carbon backbone from glycolysis and TCA cycle intermediates and their nitrogen from ammonia generated from glutamine catabolism or other amino acids present in excess[42,46,96]. Determining which amino acids are directly or indirectly affecting cell proliferation in Lkb1 and Pkd1 mutant collecting ducts, and their site of formation, must be investigated to advance our knowledge of the network of metabolic pathways performed in these mutants and to ascertain a more precise mechanism for the role of Lkb1 within the mammalian kidney.

Is defective amino acid transport participating in cystic growth?

The vertebrate kidney serves a critical role in maintaining body homeostasis by filtering the blood. Most essential nutrients including glucose, amino acids, phosphates, and bicarbonate are reabsorbed within the proximal tubules of the kidney after filtration. Surprisingly, though the absolute levels of nutrient transport occurs in more upstream segments, like the proximal tubules, the variability of transport rates of the remaining unfiltered nutrients in the distal convoluted tubule and collecting ducts is significantly higher under normal conditions.

All amino acids are hydrophilic and cannot cross the plasma membrane of epithelial cells within the proximal tubules or collecting ducts without selective transport proteins in which each transporter is specialized for different classes of amino acids. Most amino acid transporters are differentially expressed and have both tissue-specific and development-specific expression patterns. In normal cells, the regulation of amino acid transporters is still relatively unclear, but it has been well established that growth factors and various signaling pathways are involved and instructive to this process. It is likely that the differential regulation of these transporters rely on the same pathways to increase amino acid transport in transformed cells. If the increase in selective amino acid transporters is cell-dependent, administration of transport inhibitors should be capable of blocking the function of these transporters should have detrimental effects in cystic cells while having little effect on normal cells.

Preliminary data using SLC1A5 transporter inhibitor, L-γ-Glutamyl-p-nitroanilide (GPNA), or SLC3A1/SLC7A9 transporter inhibitor, Sulfasalazine, inhibited Lkb1 mutant

collecting duct growth in a similar manner to the inhibition of the glutamine transporter SLC1A5 (Figure 3-1). In contrast, inhibition of L-type amino acid transporters, SLC7A8/SLC3A2 and SLC7A7/SLC3A2, showed no significant change in Lkb1 mutant growth (Figure 3-1). These data suggest that defects in the transport of specific amino acids may contribute to the substandard growth of glutamine deprived Lkb1 mutant kidneys. Exploration into which transporters are important for Lkb1 and Pkd1 mutant epithelial cell proliferation may provide additional comprehension into the mechanism of Lkb1's role in development and polycystic kidney disease.

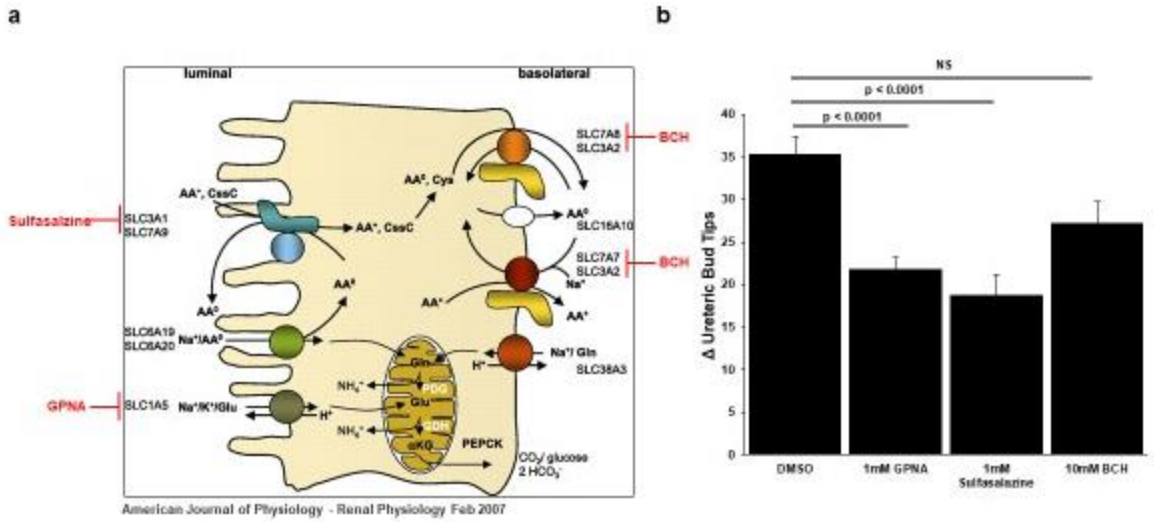

Figure 3-1: Targeting amino acid transport. (a) Schematic of amino acid transporters with known inhibitors. (b) Quantification of the change (Δ) in branch number for embryonic Lkb1 mutant collecting ducts grown in complete DMEM + 1mM DMSO (control), 1mM concentration of the SLC1A5 glutamine transporter inhibitor GPNA, 1mM concentration of the SLC3A1/SLC7A9 transporter Sulfalsalzine, and 10mM concentration of the SLC7A8/SLC3A2 and SLC7A7/SLC3A2 inhibitor 2-amino-2-norbornanecarboxylic acid (BCH). n=10 for each genotype under each condition. Statistical analysis via Mann-Whitney U-test.****p < 0.0001, NS, not significant. Error bars shown as mean +/- standard error of the mean (SEM).

How does glutathione metabolism influence cystic growth?

Reactive oxygen species (ROS), small molecules containing highly unstable oxygen free radicals, are by-products of cellular metabolism and the partial reduction of oxygen, perform a significant role in cell homeostasis by stimulating responses to hormone signals, G-protein coupled receptors, and activating ion channels, transcription factors, kinases, and phosphatases[97,98]. ROS molecules are products of cytokines, NADPH oxidases (NOX), cyclooxygenases (COX), lipooxygenases, xanthine oxidases, cytochrome P450 enzymes, and mitochondrial energy production. ROS have an important function in normal cell signaling, survival, and homeostasis[97-99]. ROS producing enzymes must be proficiently regulated so ROS do not accumulate and cause transformative or oncogenic modifications. These ROS producing enzymes, depending on their site of origin, can cooperate with various other signaling molecules to utilize ROS as second messengers in different signaling cascades. These ROS are capable of moderating diverse intracellular signal transduction pathways to control cell proliferation and differentiation.

The best described ROS molecules are hydrogen peroxide (H_2O_2) and superoxide anions ($O_2^{\bullet -}$). Superoxide anions are derived from mitochondrial complex I and III when an overabundance of electrons from energy production are passed directly to free oxygen molecules. These superoxide anions are then converted into hydrogen peroxide via superoxide dismutase. H_2O_2 oxidizes nucleic acid and protein residues. Elevated ROS levels in tissue is often due to oxidative stress, inflammation, fibrosis, or injury[100]. Increased ROS levels lead to genomic instability and oncogenesis through the damage of DNA, proteins, and lipids as well as inhibiting signaling pathways involved in cell cycle

arrest, apoptosis, and tumor suppression[97-99]. Increases in ROS production also causes post-translational modifications of several amino acids including methionine, histidine, and cysteine[97-99]. When ROS quantities are too high, it inflicts damage on the cellular and mitochondrial membranes triggering senescence and apoptosis of impaired cells. Determining which reactive oxygen species population(s) are causative in our observed embryonic Lkb1 and Pkd1 culture phenotypes may lead to the possible identification of a new class of PKD targetable therapeutics.

Since the highest manufacturer of ROS in the cell is the mitochondria, evaluating any mitochondrial dysfunction and analyzing mitochondrial membrane potential within normal and cystic renal cells could ascertain the source, concentration, and molecules involved in ROS formation in PKD. One of the notable techniques in cellular metabolism and kinetics analysis is the live-cell Seahorse XF Analyzer. This analysis provides real-time quantitative measurements of oxygen consumption rates (OCR), extracellular acidification rates (ECAR), and mitochondrial respiration rates under normal culture conditions and under energetic stress; and could corroborate the changes in redox metabolism within intact collecting duct cells as well as isolated mitochondria in real-time.

Preliminary data suggests that reductions in glutathione, a critical cytoprotectant and antioxidant within the cell, is a potential contributor to the defective growth of Lkb1 mutant kidneys in the absence of glutamine (Figure 2-13, 3-2). The glutathione pathway includes glutathione, glutathione reductase, glutathione peroxidase (GPX), and glutathione-S-transferase (GST). Glutathione is comprised of three amino acids, glutamate, glycine, and cysteine. Glutathione is typically in its reduced state (GSH). GSH

and its oxidation product, GSSG, together preserve the reducing environment of the cell through the detoxification of reactive oxygen species. There are two requisite ATP-dependent enzymes involved in GSH synthesis: glutamate-cysteine ligase (GCL) and glutathione synthetase (GSS). GCL catalyzes the first step of glutathione biosynthesis which couples the amino residue of cysteine with the carboxylic residue of glutamate to form a unique peptide bond resistant to cleavage from peptidases excluding gamma-glutamyl transpeptidase. The enzymatic activity of GCL is the rate-limiting step of glutathione synthesis and determines glutathione biosynthetic capacity and cellular levels. The homodimeric enzyme GSS forms the final glutathione product by catalyzing the condensation of gamma-glutamylcysteine with glycine.

Since cysteine availability is the primary determinant of GSH levels, several tissue-specific transporters are involved in the import of the reduced form of cysteine as well as the oxidized form of cysteine, cystine, which is the prevalent form found in circulation. One of the transporters responsible for the import of cystine, X_C, has been shown to be upregulated in many cancer cells in patients and tumor cell lines. X_C, which is composed of two components, X_{CT} (SLC7A11) and CD98hc (SLC3A2), acts as an antiporter of glutamate and cystine in a sodium (Na^+)- and Chloride (Cl^-)-independent manner. The promoter region of X_{CT} subunit contains an amino acid response element that is directly regulated by nuclear factor erythroid 2–related factor 2 (NRF2) and ETS-1 when amino acids are exhausted or oxidative stress occurs within a cell through the activation of transcriptional regulator ATF4. When localized within the nucleus, NRF2 is a principal driver of cellular survival by activating redox sensitive gene expression activating

cytoprotective and redox resistant properties. ETS1 is a transcriptional repressor associated with the proliferation, epithelial-to-mesenchymal transition, and angiogenesis of epithelial cancers. High ETS1 expression is also correlated with the metastasis and drug resistance of several epithelial tumor cells originating from colorectal, lung, cervical, ovarian, breast, and gastric subtypes. Surveying signaling transduction pathways known to govern ROS activity within normal renal cells, and defining which of these pathways, if any, are differentially regulated in cystic renal cells will aid in establishing the role of Lkb1 in oxidative stress within the kidney.

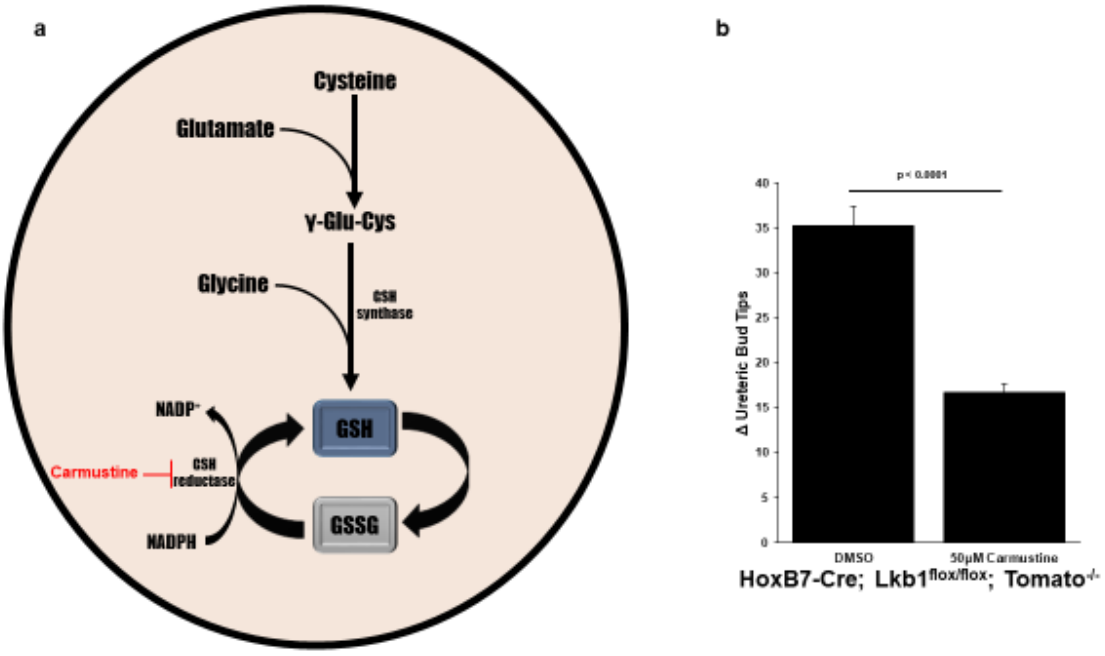

Figure 3-2: Inhibition of glutathione reductase suppresses Lkb1 mutant growth (a) Simplified schematic of glutathione metabolism. (b) Quantification of the change (Δ) in branch number for embryonic Lkb1 mutant collecting ducts grown in complete DMEM + 50μM DMSO (control) or 50μM concentration of the glutathione reductase inhibitor Carmustine. n=10 for each genotype under each condition. Statistical analysis via Mann-Whitney U-test.****p < 0.0001, NS, not significant. Error bars shown as mean +/- standard error of the mean (SEM).

Involvement of Downstream Targets of LKB1

Examination of the human kinome revealed that there are 12 protein kinases (BRSK1/2, NUAK1/2, QSK, SIK1/2, MARK1-4, SNRK) that are closely related to AMPK1/2 that are activated by T-loop phosphorylation at threonine or serine residues by LKB1. Thus far, we have not observed any notable changes in the protein expression of AMPK in our Lkb1 mutant kidneys in vivo under normal conditions, but additional analysis should be conducted to verify that AMPK1/2 is not involved during periods of energetic or oxidative stress. Several of other AMPK-related proteins have been shown to regulate processes that may contribute to the defects in growth that we observed in our ex vivo Lkb1 and Pkd1 mutant kidneys when glutamine was withdrawn from the culture media and may participate in cystogenesis. These proteins should be investigated further to determine which of these targets, if any, contribute to what we observe in our ex vivo cultures, and potentially, cyst progression. MARK3 has been shown to regulate cell cycle progression by mediating the binding of Cdc25 with14-3-3 protein through the phosphorylation of Cdc25 phosphatase. When this occurs, cell cycle entry is halted because Cdc25 is unable to activate the Cdc2/Cyclin B complex interaction required for progression into G2/M phase[101]. SIK1, SIK2, and SIK3, also known as QSK, have been implicated in cancer-related signaling pathways like cell cycle regulation, apoptosis, tumorigenesis, and metastasis through their interaction with several groups of proteins. Decreases in SIK1 expression have been shown to overlap with AMPK in the regulation of p53 expression and phosphorylation when cells are under cellular or energetic stress, including glucose withdrawal[102]. SIK2 along with QSK/SIK3 have been shown to repress

the Yorkie phosphorylation through the Hippo-Warts pathway, known to regulate cell proliferation and growth, in *Drosophila*, and QSK/SIK3 has also been shown to regulate mitotic exit in mouse fibroblasts[103]. When overexpressed, QSK/SIK3 boosts gene expression of cyclinDs and cyclinEs promoting cell proliferation in models for non-small cell lung carcinomas[104]. When downregulated, QSK/SIK3 enhances the pharmacological effects of conventional antimitotic reagents[103], while mice lacking QSK/SIK3 have defects in glucose and lipid metabolism homeostasis and adipocyte differentiation[105]. NUAK1, also known as ARK5, has been suggested to be a significant anti-apoptotic and tumor invasion factor in cancer during glucose deprivation by suppressing the activation of death receptors by caspase-8 and inducing membrane type-1 matrix metalloproteinase (MT1-MMP) promoting breast cancer cell survival via activation by AKT[106,107]. NUAK2, also known as SNARK, has also been shown to have anti-apoptotic properties in cancer cells during periods of cellular or energetic stress from UVB radiation, H_2O_2 oxidation, hyperosmosis from salt stress, glucose and glutamine deficits, and stress of the endoplasmic reticulum[108,109]. Mice lacking NUAK2 show reductions in melanoma progression by decreasing cell proliferation and migration and downregulating mTOR expression[110]. Studies in wild-type and LKB1-null mouse embryonic fibroblasts (MEFs) revealed that LKB1 constrains ROS production through the phosphorylation of p38 and c-Jun[111]. When LKB1 activity is lost in these cells, it sensitizes them to H_2O_2 exposure resulting from decreases in the expression of two antioxidant enzymes, superoxide dismutase-2 and catalase[111].

What role does cell lineage and tissue environment play in PKD progression?

Previously, Rowe et al. revealed the deletion of Pkd1 from all epithelial cells resulted in flaws in glucose metabolism during cystogenesis[65]. Results from this study and Soomro et al. showed defects in glutamine metabolism in PKD in a collecting duct cell specific manner[112]. However, these epithelial tubules are not isolated in the kidney, but are surrounded by other important structures that coordinate and reinforce renal function.

During normal renal development, the ECM undergoes several stages of active remodeling and facilitate ureteric bud and collecting duct morphogenesis and branching[63,113]. Atypical remodeling of the extracellular matrix (ECM) has been indicated as a prominent attribute of cyst formation in ADPKD and ARPKD kidneys. Studies indicate that accumulation and deposition of ECM proteins such as collagens and metalloproteinases (MMPs) are increased in cystic renal cells in vitro and in vivo and that inhibiting the production of these proteins significantly inhibits cystic growth[113-115].

Another important contributor, the endothelial cells that comprise the renal vasculature, are also a fundamental element of renal physiological function providing the network that carries circulating blood through the kidney. Vascular abnormalities are often found in PKD rat models and in human PKD patients producing an overall decrease vascular density and increased probability of aneurysm formation[116,117]. Further investigation into other cell types such as the renal stroma/interstitium and vasculature, their metabolic states, and the crosstalk between these cells, are imperative to determine

how the entire renal system manages and coordinates energy metabolism under normal and cystic conditions.

Role of Alpha-Ketoglutarate and other TCA cycle intermediates in PKD

Though we were unable to define the role of glutamine catabolism product and TCA cycle intermediate, alpha-ketoglutarate (αKG), in the rescue of our growth defect in Lkb1 and Pkd1 mutant cultures in the absence of glutamine, αKG may be a secondary yet vital component to our observations. αKG has also been demonstrated as an epigenetic modifier that regulates DNA methylation and histone modifications in mouse embryonic stem cells through the direct activation of Tet genes[118], ultimately altering pluripotency and proliferation states of these cells. Alpha-ketoglutarate has been shown to promote proliferation, differentiation, and branching morphogenesis of pancreatic-ductal progenitor cells in glutamine-independent conditions in three-dimensional culture[119]. Studies have shown that αKG is a principal precursor in the synthesis of oncometabolite 2-hydroxyglutarate in isocitrate dehydrogenase 1 (IDH1)-deficient acute myeloid leukemia and glioma mouse models[120,121]. Further analysis of these studies show that the utilization of αKG for the generation of 2-hydroxyglutarate limits αKG availability for other biological processes, transforming the entire metabolic landscape of these cell types contributing to oncogenic proliferation, yet render them more vulnerable to hypoxia or oxidative stress conditions[122,123]. Discovering which mechanism by which an exogenous source of alpha-ketoglutarate is able to yield a significant increase in Lkb1 and Pkd1 embryonic kidney growth in the absence of glutamine in our system could supply supplementary evidence into glutamine catabolism in normal and cystic collecting ducts.

Summary

This research, focused on the role of the serine/threonine kinase, LKB1, in developing renal collecting ducts. Current results indicate no direct role for LKB1 in normal renal tubule formation or maintenance. However, data suggests that LKB1 loss may act as an enhancer or modifier of glutamine metabolism during embryonic kidney growth and in polycystic kidney disease. Generating mice with a collecting duct-specific ablation of *Lkb1*, showed no abnormal renal phenotype, but co-ablation with a known PKD-causing gene, *Tsc1*, amplified the cystic burden and magnitude. Mice lacking *Lkb1* and *Tsc1* displayed a substantial change in the initial presentation, quantity, and dimension of cysts compared to mice that lacked *Tsc1* alone. Further investigation into the mechanism of Lkb1 in cyst enhancement showed the absence of Lkb1 within the collecting ducts during development resulted in aberrant glutamine utilization. Using in vitro cell culture and ex vivo embryonic kidney culture of wild-type and Lkb1-deficient collecting duct cells, a metabolic profile was generated to determine which modifications in metabolites occurs in the presence and absence of glutamine. In order to determine if the compulsory expenditure of glutamine within embryonic Lkb1 mutant kidneys also underlies enhanced cyst growth, an established inhibitor of glutamine catabolism, BPTES, was administered to two independent PKD mouse models. Administration of the inhibitor during prenatal development affected embryogenesis. However, administration to nursing mothers during postnatal development decreased cyst progression in both PKD models. Collectively, these data indicate glutamine, amino acid, and glutathione metabolic pathways as potential avenues for targetable therapeutics in polycystic kidney disease.

CHAPTER FOUR

METHODS

Generation of mouse lines

HoxB7-Cre, Pkhd1-Cre, Lkb1$^{flox/flox}$, Tsc1$^{flox/flox}$, and Pkd1$^{flox/flox}$ have been previously described (Yu et al., Development, 2002; Williams et. al, Am J Physiol Cell Physiol. 2014; Bardeesy et al., Nature, 2002; Meikle et al., Hum Mol Genet., 2005; Shibazaki et al., Hum Mol Genet. 2008). All mice were bred on a mixed genetic background. For each experiment, female mice 7−8 weeks of age were crossed with a male 9−10 weeks of age. For initial histological and immunohistological characterization experiments, 3−4 pregnant Lkb1$^{flox/flox}$, Tsc1$^{flox/flox}$, and Lkb1$^{flox/flox}$; Tsc1$^{flox/flox}$ females were crossed with one HoxB7-Cre; Lkb1$^{flox/+}$, HoxB7-Cre; Tsc1$^{flox/+}$, or HoxB7-Cre; Lkb1$^{flox/+}$; Tsc1$^{flox/+}$ male. For ex vivo culture experiments, plugs were checked and embryos were collected at embryonic day 12.5 for further analysis. Mice of both sexes with the desired genotype were randomly selected and the investigator was blinded to allocation. All animals were housed, maintained, and used according to National Institutes of Health (NIH) and Institutional Animal Care and Use Committees (IACUC) approved protocols at the University of Texas Southwestern Medical Center (OLAW Assurance Number D16-00296).

Mouse Genotyping

Primer sequences for PCR genotyping are as follows:

HoxB7-Cre	5′-GGTCACGTGGTCAGAAGAGG-3′
	5′-CTCATCACTCGTTGCATCGA-3′
Pkhd1-Cre	5′-AGGTTCGTTCACTCATGG-3′
	5′-TCGACCAGTTTAGTTACC-3′
RosaTomato	5′-AAGGGAGCTGCAGTGGAGTA-3′
	5′-CCGAAAATCTGTGGGAAGTC-3′
	5′-GGCATTAAAGCAGCGTATCC-3′
	5′-CTGTTCCTGTACGGCATGG-3′
Lkb1	5'-GGGCTTCCACCTGGTGCCAGCCTGT-3'
Pkd1	5′-CCGCTGTGTCTCAGTGTCTG-3′
	5′-CAAGAGGGCTTTTCTTGCTG-3′
Tsc1	5′-AGGAGGCCTCTTCTGCTACC-3′
	5′-CAGCTCCGACCATGAAGTG-3′
	5′-AGCCGGCTAACGTTAACAAC-3′

Hematoxylin & Eosin staining of sectioned kidneys

Kidneys were harvested, washed twice with phosphate-buffered saline (PBS) containing Ca^{2+} and Mg^{2+}, and fixed with 4% paraformaldyhde overnight. Tissues were then dehydrated through a series of graded ethanol washes to displace the water, infiltrated with paraffin wax, and embedded into wax blocks inside tissue cassettes until wax blocks were solidified. Sagittal sections of tissue were prepared from the wax blocks at 10μm per section onto glass slides using a microtome. Staining procedure: Slides were deparaffinized with xylene washes, followed by rehydration with graded ethanol washes, and then washed briefly with distilled water to remove residual ethanol. Slides were stained with Harris hematoxylin, washed with distilled water, differentiated in 1% acid alcohol, and washed with water again. Bluing of slides was performed using a saturated lithium carbonate solution, rinsed with water, and dipped in 95% alcohol repeatedly.

Slides were subsequently counterstained in an eosin-phloxine solution, dehydrated in a graded ethanol series, cleared with xylene, and mounted.

Immunofluorescence of sectioned kidneys

Post-excised kidneys were fixed with 4% paraformaldehyde, cryoprotected with 30% sucrose, and frozen embedded in OCT medium. Frozen sections were washed with PBS with 0.1% Triton-X or 1X TBS with 0.1% Tween-20. Slides were immersed and boiled in 10mM sodium citrate antigen retrieval buffer and blocked with a solution of 5% FBS/TBS-Tween-20 for 1 hour at room temperature followed by the application of primary antibodies diluted in blocking solution. Secondary antibodies were diluted in blocking solution (1:500 AlexaFluor®488-conjugated anti-rabbit IgG, 1:500 AlexaFluor®568-conjugated anti-mouse IgG) for 1 hour at room temperature. Nuclei were stained with DAPI or ToPro for 10 minutes, then mounted and sealed with a coverslip.

Ex vivo whole kidney culture

Organ culture of isolated embryonic day 12.5 (E12.5) metanephric kidneys were performed as previously described (Carroll et al., 2005, Karner et al., 2011).Embryonic day 12.5 metanephric kidneys were isolated and cultured on polycarbonate filters (pore-size of 0.8 μm) in a 24-well plate (Corning, Costar). They were cultured on an air-medium interface with media consisting of Dubelcco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (Sigma), and 1% antibiotic/anti-mycotic solution (Corning) with or without glucose and/or glutamine for 2 to 5 days. Bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulfide (BPTES), dimethyl 2-oxoglutarate (cell permeable alpha-ketoglutarate), Rapamycin, gamma-l-glutamyl-p-nitroanilide (GPNA), and ammonium

chloride (NH_4Cl) were all purchased from Sigma-Aldrich. All culture dishes were incubated in a fully humidified 37°C incubator with 5% CO_2.

Metabolomics

E13.5 HoxB7-Cre; RosaTomato and HoxB7-Cre; $Lkb1^{flox/flox}$; RosaTomato kidneys were harvested, cultured in control media (DMEM) or DMEM lacking glutamine for 24 h. Samples for each genotype, containing approximately 20 embryonic kidneys per sample, were generated for each media condition before processing. Kidneys were then washed twice with ice-cold saline, dried, transferred to an Eppendorf tube, and flash-frozen with liquid nitrogen. For metabolite purification, extraction, and analysis, kidneys were submerged in 500µl of cold methanol/water (50/50, v/v), subjected to three freeze/thaw cycles, vortexed, and centrifuged. The supernatant was transferred to a new tube, air-dried, and metabolites were reconstituted in 100µl 0.03% formic acid in analytical-grade water, vortexed, and centrifuged to remove cellular debris. The final supernatant was transferred to a high-performance liquid chromatography (HPLC) vial for metabolomics analysis via LC/MS (Agilent 6550 iFunnel LC/quadrupole-time of flight mass spectrometer (Mullen et al., Cell Reports, 2014). Pellets from each sample were used for protein quantification via BCA Protein Assay. Statistical differences were determined via Partial Least Squares-Discriminant Analysis (PLS-DA).

In Vivo Treatments

Pregnant or nursing dams were injected intraperitoneally once a day with BPTES (100 mg/kg) or vehicle (5% DMSO in PBS) from E15.5 until birth or beginning on post-natal day 1 until post-natal day 10. Tissues were then harvested from vehicle or BPTES-treated

HoxB7-Cre; Lkb1$^{flox/flox}$; Tsc1$^{flox/flox}$ or Pkhd1-Cre; Pkd1$^{flox/flox}$ neo-natal mice 12 hours after final injection. Post-excised kidneys were fixed with 4% paraformaldehyde overnight, dehydrated with a series of ethanol solutions, sectioned, and stained with Hematoxylin and Eosin as described above. Cystic indices were conducted by overlaying a grid composed of 160 0.1inch2 squares to H&E-stained sagittal kidney sections. Cystic index is presented as the percent of total kidney area that is occupied by cysts. HoxB7-Cre;Lkb1$^{flox/flox}$;Tsc1$^{flox/flox}$ (DMSO-treated, n=16; BPTES-treated, n=16). Pkhd1-Cre; Pkd1$^{flox/flox}$ (DMSO-treated, n=14; BPTES-treated, n=14).

Generation of Cell Lines

Inner medullary collecting duct cells were isolated under sterile conditions from renal papillae from 6-8 week old Lkb1$^{flox/flox}$, Tsc1$^{flox/flox}$, Lkb1/Tsc1$^{flox/flox}$, and Pkd1$^{flox/flox}$ mice. The papilla was considered to be the region of the medulla containing collecting tubules but no thick ascending limbs. Papillae were dissected, minced with a sterile razor blade, and transferred to a sterile culture tube containing 4mL of 0.1% collagenase in Krebs buffer (composition, in mM: 118 NaCl, 25 NaHC03, 14 glucose, 4.7 KCl, 2.5 CaC12, 1.8 MgSO+ and 1.8 KHnPOd), pH 7.3. Tubes containing fragmented tissue were incubated for 1.5 to 2.5 hours at 37°C at 5% CO_2. After incubation, tissues were gently agitated by using a sterile pipette 4-5 times. Cell suspensions were then filtered through 25mm pressure syringe filters and diluted with 2 volumes of distilled water to burst cells excluding collecting duct cells. The collecting duct cells were then pelleted by centrifugation at 100g for 10 minutes, and the supernatant was removed from the culture tubes. The cell pellets were resuspended in 10% bovine serum albumin in PBS

(composition: 151mM NaCl, 45mM KH_2P0_4, and 2.5mM NaOH), pH 7.2 and centrifuged again 100g for 10 minutes to pellet the cells. The cell pellets were resuspended in 1:! Mixture Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F-12 containing 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic and cells were placed at a density of 6-9x104 cells per well in a 24-well plate. Media was changed on day 1 for the cells to properly attach to the plastic and every 2 days afterward for cells to maintain cell growth and proliferation.

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