Deletion of Lkb1 in Renal Tubular Epithelial Cells Leads to CKD by Altering Metabolism

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ABSTRACT

Renal tubule epithelial cells are high-energy demanding polarized epithelial cells. Liver kinase B1 (LKB1) is a key regulator of polarity, proliferation, and cell metabolism in epithelial cells, but the function of LKB1 in the kidney is unclear. Our unbiased gene expression studies of human control and CKD kidney samples identified lower expression of LKB1 and regulatory proteins in CKD. Mice with distal tubule epithelial-specific Lkb1 deletion (Ksp-Cre/Lkb1fl/lox) exhibited progressive kidney disease characterized by flattened dedifferentiated tubule epithelial cells, interstitial matrix accumulation, and dilated cystic-appearing tubules. Expression of epithelial polarity markers β-catenin and E-cadherin was not altered even at later stages. However, expression levels of key regulators of metabolism, AMP-activated protein kinase (Ampk), peroxisome proliferative activated receptor gamma coactivator 1α (Ppargc1a), and Ppara, were significantly lower than those in controls and correlated with fibrosis development. Loss of Lkb1 in cultured epithelial cells resulted in energy depletion, apoptosis, less fatty acid oxidation and glycolysis, and a profibrotic phenotype. Treatment of Lkb1-deficient cells with an AMP-activated protein kinase (AMPK) agonist (A769662) or a peroxisome proliferative activated receptor alpha agonist (fenofibrate) restored the fatty oxidation defect and reduced apoptosis. In conclusion, we show that loss of LKB1 in renal tubular epithelial cells has an important role in kidney disease development by influencing intracellular metabolism.


Renal tubular epithelial cells (TECs) display strict apico-basal polarity. They allow a highly regulated uptake or excretion of substances at its apical surface, while keeping a closed, impenetrable surface through the formation of tight intercellular junctions in the basolateral membrane. The establishment and maintenance of TEC polarity is incompletely understood. The liver kinase B1 (LKB1) is an important regular of polarity. Early studies indicated that single intestinal epithelial cells polarize in a cell-autonomous fashion in response to LKB1 expression. The LKB1 or STK11 gene encodes an evolutionarily conserved serine/threonine protein kinase. Following LKB1 expression, intestinal epithelial cells reorganized their cytoskeleton to form an apical brush border, demonstrating LKB1’s critical role in establishing epithelial polarity. On the other hand, the effect of LKB1 on cell polarity appears to be cell type specific and deletion of LKB1 did not alter polarity of lung epithelial and pancreatic cells.

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LKB1 was originally identified as a tumor suppressor. Mutations of LKB1 in humans cause Peutz–Jeghers syndrome (PJS).\(^3,^4\) PJS is a rare disease inherited in an autosomal dominant fashion and characterized by predisposition to benign hamartomatous polyps of the intestine and mucocutaneous pigmentation abnormalities. Molecular pathways of LKB1-induced tumor development are not fully understood, as LKB1 is broadly expressed,\(^3,^6\) but the changes appear to be organ specific. Downstream activation of mammalian target of rapamycin (mTOR) signaling appears to play an important role in the process. Several early case reports described polycystic disease development in patients with PJS,\(^7,^8\) however the incidence of kidney in these patients has not been characterized.

Activation of LKB1 occurs via allosteric binding of LKB1 to STE20–related adaptor and mouse protein 25.\(^9,^{10}\) Phosphorylation on Ser428 may also increase LKB1’s activity.\(^11,^{12}\) LKB1 can phosphorylate more than 13 different target proteins.\(^13\) The best-known substrate of LKB1 is AMPK, a master regulator of cellular and organismal metabolism.\(^14\) In some, but not all cells, mTOR is also regulated by LKB1.\(^15,^{16}\)

LKB1 serves as a critical metabolic checkpoint and arrest cell cycle in response to low intracellular ATP, such as low nutrient availability. LKB1 influences both glucose and lipid metabolism. The metabolic effect of LKB1 is mostly mediated by AMPK. In most cells, AMPK inhibits lipogenesis and this effect has been proposed to be important for the tumor suppressor function of LKB1.\(^17\) In some cancer types, LKB1 (via AMPK) can modulate glycolysis via the phosphorylation of phosphofructo kinase. LKB1 controls several transcriptional regulators of metabolism including Foxo3, Hnf4α, and Pparg1α, having a long-term influence on metabolism. Some effects of LKB1 are independent of AMPK and they are mediated via microtubule affinity-regulating kinase, synapases of the amphid defective kinase, and salt-inducible kinase.\(^18–^20\)

Kidney fibrosis is the histologic manifestation of CKD. Kidney fibrosis is characterized by accumulation of matrix and inflammatory cells. Epithelial cells are progressively lost in CKD and the remaining cells undergo dedifferentiation.\(^21–^23\) As the kidney is a primary epithelial organ the loss of functional epithelial cells has been proposed to be the cornerstone of functional decline. Several groups proposed that epithelial cells actually become fibroblasts in fibrosis via a process called epithelial to mesenchymal transition.\(^21,^{22}\) During epithelial to mesenchymal transition epithelial cells reduce the expression of apico–basal polarity markers (e-cadherin) and acquire mesenchymal markers including vimentin and alpha smooth muscle actin. The dedifferentiation process is governed by the activation of transforming growth factor beta/Smad, Notch, and Wnt pathways.\(^24–^26\) Recent studies indicate that metabolic alterations are also important in kidney fibrosis development. Large-scale unbiased studies indicated concerted decrease in expression of genes related to glucose and fatty acid oxidation both in patients and in mouse models. Pharmacological activation of the peroxisome proliferative activated receptor alpha (PPARα) pathway or genetic expression of Pparg1α decreased fibrosis development in multiple models.\(^27–^29\)

The primary aim of this study was to examine the role of LKB1 in renal TECs, as defects in metabolism and polarity have been proposed to play a role in kidney disease development.

**RESULTS**

**Decreased LKB1 Expression in Fibrotic Kidney Samples**

First we examined the expression pattern of LKB1 in control and fibrotic kidneys by analyzing transcript levels of LKB1 and associated genes in 95 microdissected human kidney samples.\(^27,^{30,31}\) CKD was defined based on the eGFR < 60 ml/min/1.73 m\(^2\) for ≥ 3 months.\(^32\) Among these, 41.1% of the samples met the criteria for CKD (Supplemental Table 1). As expected, CKD samples showed significant glomerulosclerosis and interstitial fibrosis. Microarray analysis was performed on microdissected tubule samples using the Affymetrix platform.\(^27,^{30,31}\) LKB1 transcript level did not show statistically significant differences when control and CKD samples were compared. This may be less surprising as LKB1 is ubiquitously expressed. On the other hand, the expression of the allosteric activator of LKB1, the calcium binding protein 39 (CAB39, homolog of mouse protein 25) positively correlated with eGFR (Figure 1A). Protein expression of CAB39L, analyzed by immunohistochemistry, confirmed the transcript level data. In healthy kidneys CAB39L was expressed both in proximal and distal tubules in the nucleus and in the cytoplasm (Supplemental Figure 1). Its expression was significantly decreased in advanced stages of CKD (Figure 1B). Transcript levels of an important LKB1 target, AMP-activated alpha 2 catalytic subunit (AMPKα2) also correlated with eGFR (Figure 1C), raising the possibility that LKB1’s activity is regulated in CKD.

Recently, a phosphorylation-mediated activation of LKB1 has been described.\(^11\) This encouraged us to analyze phospho-LKB1 levels by immunostaining in control and CKD human kidney samples. Double immunostaining with the proximal tubule marker LTL indicated that phospho-LKB1 is mostly expressed in distal tubules in healthy control kidney samples (Figure 1D, upper panel). Nuclear phospho-LKB1 expression (Figure 1D, lower panel) and percentage of positive nuclear staining were significantly lower in CKD kidney samples (Figure 1E).

Taken together, we observed decreased expression of LKB1 binding partner (CAB39L), phosphorylated LKB1, and LKB1 targets in distal tubule cells when patient samples with kidney fibrosis were compared with controls, suggesting the dysregulation of the LKB1 pathway in kidney fibrosis and raising the possibility that it might play a role in disease development.

**Distal Tubule-Specific LKB1 Deletion Results in Progressive Tubulointerstitial Damage**

To investigate the functional role of LKB1 in the kidney, we generated mice with tubule epithelial–specific deletion of Lkb1. We crossed mice expressing Cre recombinase under the control of the cadherin 16 promoter with mice harboring floxed alleles of Lkb1 to generate Ksp-Cre/Lkb1\(^{floxed}\) mice. Littermate floxed
homozygous animals (*Lkb1*^fl/fl^ without Cre) were considered controls. Transcript levels of *Lkb1* in whole kidney tissue of *Ksp-Cre/Lkb1*^fl/fl^ mice were reduced by 60% when compared to control animals (Figure 2A).

*Ksp-Cre/Lkb1*^fl/fl^ mice were born at the expected Mendelian ratio and did not show gross abnormalities at birth and at 5 weeks of age (Figure 2B). By 14 weeks, fibrotic changes started to appear focally accompanied by dilated tubules (Figure 2C). By 27 weeks of age the kidney became grossly enlarged; the normal renal architecture was lost and animals developed severe fibrosis. Renal TECs were simplified and the lumen was dilated (Figure 2, D and E) and occasionally cystic appearing. The interstitium was widened with accumulation of matrix and activated myofibroblasts, whereas glomeruli remained intact. In contrast, *Lkb1* deletion in proximal tubules using the *Pepck-Cre/Lkb1*^fl/fl^ mice did not result in fibrosis development (Supplemental Figure 2).

In line with the morphologic alterations, quantitative PCR analysis showed that transcript levels of fibrosis markers such as *Col1a1*, *Col3a1*, and fibronectin were significantly increased in 14-week *Ksp-Cre/Lkb1*^fl/fl^ mice compared to control mice (Figure 3A). Sirius-red staining confirmed the severe fibrosis development in the *Ksp-Cre/Lkb1*^fl/fl^ mice (Figure 3, B–D). In addition to the fibrosis we also observed cystic dilatations. These cystic dilatations started to occur around 14 weeks of age and were more prominent around the fibrotic regions. At 14 weeks of age, about 3% of the tubules were affected by microcystic changes (Supplemental Figure 3) and even at 27 weeks of age less than 10% of tubules were occupied by cysts (Supplemental Figure 3). Kidneys were characterized by marked interstitial proliferation, but proliferation was less prominent in tubule epithelial cells.(Supplemental Figure 4). Epithelial deletion of *Lkb1* was also sufficient to induce macrophage infiltration and increased F4/80 expression (Figure 3A). TECs appeared simplified and even occasionally dysplastic, but we did not observe preneoplastic or neoplastic lesions in mice with TEC-specific *Lkb1* deletion. In summary, mice with tubule epithelial deletion of *Lkb1* are mostly characterized by progressive tubule dilation and fibrosis.

**No Obvious Polarity and Ciliary Abnormalities in *Ksp-Cre/Lkb1*^fl/fl^ Mice**

Next, we examined the molecular mechanism of LKB1-induced renal disease. As LKB1 is a critical determinant of cell polarity, we analyzed the expression epithelial polarity markers in mice with conditional deletion of *Lkb1*. To this end, we examined the localization of basolateral proteins, β-catenin and E-cadherin. Overall, there was no change in transcript levels of *Cdh1* and *Zo-1* in tubule-specific *Lkb1* null mice when compared with

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**Figure 1.** Expression of LKB1 and related genes in patients with kidney fibrosis. (A) Correlation between eGFR and transcript levels of CAB39L (determined on microarrays). (B) Immunostaining of control and diseased human kidney samples with CAB39L. (C) Correlation between eGFR and transcript levels of protein kinase AMP-activated alpha 2 catalytic subunit (*PRKAA2*) (determined on microarrays). (D, upper panel) Representative image of double immunofluorescence staining of phospho-LKB1 (red) and lotus lectin (green) in control human kidney sample. (D, lower panel) Representative image of phospho-LKB1 immunostaining of control and CKD human kidney samples. (E) The expression of phospho-LKB1 was quantified and presented as a percentage of positive nuclei. Phospho-LKB1 expression was significantly lower in CKD samples compared with healthy controls.
control animals (Supplemental Figure 5A). Immunofluorescence labeling showed that basolateral localization of β-catenin and E-cadherin was maintained, indicating no obvious polarity defects in tubule-specific Lkb1 null mice (Supplemental Figure 5B).

Recently, LKB1 has been identified as part of the ciliary proteome. Therefore, we next tested for the presence of cilia by acetylated tubulin staining in kidney samples. Immunofluorescence staining indicated that primary cilia were present without gross abnormalities both in 5- and 14-week Ksp-Cre/Lkb1flox/flox mice (Supplemental Figure 5C). In summary, Lkb1 null TECs failed to show obvious polarity or ciliary defects.

**Metabolic Alterations in Tubule Epithelial Specific Lkb1 Knock-Out Mice**

Failing to identify significant polarity differences, we examined metabolic changes in cells and mice with Lkb1 deletion. Most described effects of LKB1 are mediated by AMPK, a master regulator of metabolism. Therefore, we next compared AMPK levels in TECs in the presence or absence of Lkb1.

To this end, we cultured primary TECs from wild-type (WT) and WT/Lkb1flox/flox mice and infected them with Ad5CMVCre-eGFP adenovirus to delete Lkb1 (Figure 4A). Fluorescence microscopy indicated more than 90% infection efficiency (data not shown) and about 90% reduction in Lkb1 level following Cre infection of Lkb1flox/flox cells (Figure 4B).

Western blot analysis showed significant reduction (about 50%) in phospho-AMPK expression in cultured primary tubule cells with Lkb1-deletion compared with control cells (Figure 4C). Decreased expression of phospho-AMPK in the absence of Lkb1 was also observed in vivo in mice with tubule-specific Lkb1 deletion by immunohistochemistry (Figure 4D). AMPK is an important negative regulator of mTOR. However, we found no significant increase in phospho-mTOR or its effector S6-kinase levels in Lkb1-deficient cells (Supplemental Figure 6). These results indicate that AMPK but not mTOR is an important target of LKB1 in TECs.

We found that LKB1 controlled the expression of several transcription factors that play an important role in regulating metabolism. Quantitative PCR and western blot analyses

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**Figure 2.** Loss of Lkb1 in renal tubule cells causes severe structural damage in the kidney. (A) Quantitative real-time PCR analysis of Lkb1 and Cre transcript levels in kidneys of control and Ksp-Cre/Lkb1flox/flox mice. (B–E) Gross morphology (E) and representative images of periodic acid-Schiff-stained kidney sections from control and Ksp-Cre/Lkb1flox/flox mice at (B) 5, (C) 14, and (D, E) 27 weeks of age.
indicated significant decreases in Ppara and Ppargc1a expression in vitro in Lkb1 null cells (Figure 4, C and E). We found that mRNA levels of Ppara and Ppargc1a were also decreased in vivo in Ksp-Cre/Lkb1^{flox/flox} mice (Figure 4F). Immunohistochemistry analysis corroborated the lower protein expression of Ppara and Ppargc1a in Ksp-Cre/Lkb1^{flox/flox} kidneys compared with control animals (Figure 4G).

**Lkb1 Deficiency Alters Fatty Acid and Glucose Metabolism of TECs**

Differences in Ampk, Ppara, and Ppargc1a levels in the Lkb1 knock-out mice prompted us to examine further the effect of LKB1 on fatty acid and glucose utilization. AMPK is a known regulator of fatty acid utilization by influencing acyl-CoA-carboxylase (ACC) levels, a critical enzyme in fatty acid metabolism that produces malonyl-CoA. Malonyl-CoA is an inhibitor of carnitine palmitoyl-CoA transferase 1, the key rate-limiting enzyme in fatty acid oxidation. We found that phospho-ACC levels (Figure 5A) were decreased in Lkb1 null cells. Overall, these observations raised the possibility of lower fatty acid oxidation in the absence of Lkb1.

Transcript levels of rate-limiting enzymes in the β-oxidation pathway, such as carnitine palmitoyl transferase (Cpt1, Cpt2) and acyl-CoA oxidase (Acox1), were significantly decreased in kidneys of Ksp-Cre/Lkb1^{flox/flox} mice compared to control animals (Figure 5B). The decrease was already evident by 14 weeks of age, prior to the development of significant fibrosis. Consistent with the in vivo findings, mRNA expressions of Cpt1, Cpt2, and Acox1 were significantly decreased in vitro in the absence of Lkb1 (Figure 5C). Upon measuring fatty acid utilization using the Seahorse bioanalyzer, we found that palmitate-induced oxygen consumption rate was significantly lower in Lkb1 null cells, when compared with control cells (Figure 5D), indicating a defect in fatty acid oxidation.

The significant decrease in fatty acid oxidation was associated with increased accumulation of intracellular lipids. Increased lipid accumulation was evident by increased oil-red-O positive staining in Lkb1-deficient cells (Figure 5E) and in kidneys of mice with Lkb1 deletion (Figure 5F).

Next, we analyzed levels of enzymes and transporters related to glucose utilization in 5- and 14-week-old control and Lkb1 knock-out animals (Figure 6A) and in control and Lkb1 null TECs (Figure 6B). The transcript level of phosphofructokinase, the rate-limiting enzyme in glycolysis, was decreased, but did not reach statistical significance in vivo or in vitro. Transcript levels of pyruvate kinase (Pki), glucose transporter 1 (Glut1), and hexokinase (Hk) were significantly reduced in vitro in absence of Lkb1.

Next, we studied glucose utilization using the Seahorse bioanalyzer and found that glucose-induced extracellular
Figure 4. Lower expression of metabolic regulators in tubule epithelial-specific Lkb1 knock-out mice and in cells with Lkb1 deletion. (A and B) Lkb1 mRNA levels (A) and LKB1 protein levels (B) in cultured cells. (C) Representative western blots followed by quantification analysis of P-AMPK, PPARα and PGC1α, and beta actin levels in control (CON) and Lkb1-deficient tubule cells. (D) Representative images from P-AMPK immunostaining of control and Ksp-Cre/Lkb1fl/flox/lox kidney samples. (E and F) Transcript levels of Ppara and Ppargc1a in control and Lkb1-null cells (E) and control and 14-week-old Ksp-Cre/Lkb1fl/flox/lox mice. (G) Representative images of PPARα and PGC1α immunostaining of control and 27-week Ksp-Cre/Lkb1fl/flox/lox kidneys.
acidiﬁcation (ECAR), a measure of glycosis, was actually significantly lower in cells lacking Lkb1 (Figure 6C).

As our results indicated a deﬁcit both in fatty acid oxidation and glycosis, we therefore visualized mitochondria by mto

tracker staining. Whereas control cells had a well preserved ﬁlamentous mitochondrial network, Lkb1 null cells showed a punctate mitochondrial staining pattern (Figure 7A), indicating a potential mitochondrial deﬁcit. Distorted mitochondrial struc

ure was further conﬁrmed by electron microscopy (Figure 7B). As we observed a signiﬁcant deﬁcit both in fatty acid and glucose utilization we measured intracellular ATP content in control and Lkb1 null cells. We found that cellular ATP content was lower in the absence of Lkb1 (Figure 7C).

Taken together, these ﬁndings indicate that mitochondrial structure and fatty acid and glucose utilization were altered in Lkb1-deﬁcient kidneys and TECs, resulting in lower ATP levels.

Lkb1 Deletion Results in Increased Apoptosis

Next, we examined cell death as decreased energy levels and mitochondrial alterations are usually associated with increased apoptosis rate. Quantitative PCR analysis indicated that mRNA expression levels of Bcl2 (anti-apoptotic gene) were signiﬁcantly lower in Lkb1 null kidneys and tubule cells obtained from these animals (Figure 8A). Western blot analysis conﬁrmed the decreased BCL2 levels in renal TECs with Lkb1 deletion (Figure 8B). Increased levels of cleaved caspase-3 in cultured renal TECs with Lkb1 deletion (Figure 8B) further substantiated the increased apoptosis rate. The increased apoptosis rate and higher cleaved caspase-3 expression was also evident in renal TECs of kidneys of Ksp-Cre/Lkb1^{flox/flox} mice (Figure 8C).

In addition to apoptosis, Lkb1 deletion was associated with dedifferentiation of TECs evident by the increased expression of Col1a1, Col3a1, and Acta2 (Figure 8D), likely explaining the ﬁbrotic phenotype observed in vivo (Figures 2 and 3). In summary, these ﬁndings suggest that Lkb1 deﬁciency results in increased cell death and dedifferentiation most likely due to energy depletion.

AMPK and PPARα Agonists Attenuate Lkb1 Deletion-Induced Epithelial Dedifferentiation and Apoptosis

Next, we tested whether lower AMPK and PPARα levels play functional roles mediating the effect of LKB1. To examine this hypothesis we treated Lkb1 null TECs with the AMPK agonist (A769662) and the PPARα agonist fenofibrate. The drug dosage was titrated to restore levels of the rate-limiting enzyme levels in the fatty acid oxidation pathway, Cpt1, Cpt2, and Acox1 (Figure 9A) (100 μM A769662 and 1 μM fenofibrate). Western blot analysis conﬁrmed that these drugs fully restored protein expression of phospho-ACC (Figure 9B). Oxygen consumption studies indicated that palmitate oxidation was increased in cells treated with AMPK or PPARα agonists in the absence of Lkb1 (Figure 9C), suggesting that these pathways play a functional role in mediating LKB1’s effect.

We next evaluated whether AMPK or PPARα agonists can also restore the glycolytic capacity of the Lkb1 null cells. Glucose utilization related transcripts (Figure 9D) were not different following A79 or fenofibrate treatment. Seahorse Bioanalyzer–based glucose utilization studies conﬁrmed that neither the Ampk nor the PPARα agonists could restore glucose-induced ECAR in Lkb1 null cells (Figure 9E).

To prove that AMPK and PPARα and downstream fatty acid utilization play a critical role in mediating the effect of LKB1, we examined functional outcomes in Lkb1 null cells after AMPK or PPARα agonist treatments. The epithelial marker E-cadherin expression was signiﬁcantly lower in cells with Lkb1 deletion and A769662 and fenofibrate restored Cdh1 expression. Expression of Acta2, Col1a1, Col3a1, and Fn1 were signiﬁcantly increased in Lkb1 null cells, and fenofibrate and A769662 were able to ameliorate the increase (Figure 9F). Furthermore, pretreatment with A769662 and fenofibrate decreased the Lkb1 deletion–induced high apoptosis rate (Figure 9G) evident by the expression of cleaved caspase-3. In summary, the metabolic effect of LKB1 appears to be at least partly mediated by AMPK or PPARα as pharmacological agonists of these pathways protected cells from apoptosis and dedifferentiation.

DISCUSSION

In summary, here we report that genetic deletion of Lkb1 in distal tubule epithelial cells caused severe structural damage in the kidney, which was associated with dedifferentiation of tubule epithelial cells, accumulation of interstitial matrix, and inﬁammatory cells. This phenotype most resembles tubulointerstitial ﬁbrosis, observed in patients with CKD, which is the ﬁnal common pathway to ESKD development. As the expression of phosphorylated LKB1 and LKB1 co-activator CAB39L is decreased in patient samples with kidney ﬁbrosis, these observations raise the possibility that LKB1 actually plays a role in ﬁbrosis development.

Even though LKB1 has been identiﬁed as an important component of the ciliary complex and cilia plays an important role in cystic disease development, we did not observe bona ﬁde cystic disease in our animals. Tubule epithelial cells showed signiﬁcant dilatation and occasional microcystic changes; however, these changes did not reach the severity observed in animal models of polycystic kidney disease. Microcystic change and a swiss-cheese appearance of the kidney is described in severe ﬁbrosis. Furthermore, cilia structure was present in tubule epithelial cells even in the absence of LKB1. Future studies might determine smaller changes in cilia length and width, as well as potential functional defects. Mice with tubule-speciﬁc Lkb1 deletion did not develop renal cell carcinoma or excessive proliferation and precancerous lesions, even though Lkb1 is an important tumor suppressor and decreased LKB1 expression has been demonstrated in renal cell carcinoma. It is likely that additional mutations or activation of mTOR are necessary for renal cell cancer development.

We found that LKB1’s effect was mostly mediated by AMPK and downstream metabolic changes as the AMPK agonist
Figure 5. Alterations in fatty acid oxidation in renal TECs in absence of Lkb1. (A) p-ACC western analysis (representative and quantification) of control and Lkb1 null cells. (B and C) The mRNA expression of carnitine palmitoyl-transferase 1 (Cpt1), carnitine palmitoyl-transferase 2 (Cpt2), and acyl-coA oxidase 1 (Acox1) in control and Ksp-Cre/Lkb1^{fl/fl} kidneys (B) and control and Lkb1-deficient cells (C). (D) Oxygen consumption rate of control and Lkb1-deficient cells, when indicated palmitate (180 μM), etoxomir (40 μM), and oligomycin (1 μM) was added. The right panel shows quantification from multiple measurements. Representative images of oil-red-O staining of control and Lkb1-deficient TECs (E) and control and Ksp-Cre/Lkb1^{fl/fl} mice (F) and ImageJ-based quantification of the oil-red-O staining.
A769662 had a protective effect on renal TECs. Loss of Lkb1 resulted in impaired glucose utilization, including lower enzyme levels and altered glucose-induced ECAR. This is in keeping with recent results showing that AMPK can regulate glucose transporters and enzymes involved in glucose oxidation. Our data, on the other hand, indicate that lower glucose utilization plays only a minor role in the phenotype development because the PPARα agonist fenofibrate and AMPK agonist A769662 did improve apoptosis and de-differentiation, even though the glycolytic defect was not restored.

We propose that the decreased fatty acid utilization induced by Lkb1 deletion plays an important functional role in the phenotype development as we observed protection from apoptosis and dedifferentiation following A769662 and fenofibrate treatment. This is consistent with the known role of PPARα in regulating fatty acid utilization while not having an effect on glucose utilization. Previous publications and our recent study indicate that fatty acids are the major energy source in the kidney. Nevertheless, it should be noted that fatty acid oxidation and apoptosis was not completely recovered by A769662 and PPARα agonist evidenced by the Seahorse analysis in spite of almost full normalization of phospho-ACC expression and the transcript levels of genes involved in the β-oxidation pathway. These findings could imply that additional mechanisms besides AMPK could mediate the effect of LKB1. Such an AMPK independent effect has recently been described in the muscle during exercise. Furthermore, our results cannot fully exclude the possibility that Lkb1 deletion also results in a broader mitochondrial dysfunction, as was also evident by altered mitochondrial morphology and the decreased expression of peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (PGC1α), a key regulator of mitochondrial biogenesis. Recent studies suggest that PGC1α is downregulated in bone marrow and skeletal muscle cells in the absence of Lkb1 deletion. Future studies will dissect LKB1-mediated differences in mitochondrial function and fatty acid oxidation in renal TECs.

Figure 6. Alterations in glucose utilization in renal TECs in absence of Lkb1. (A and B) Transcript levels of Glut1 (glucose transporter1), Hk (hexokinase), Pfk (phosphor-fructokinase), and Pk (pyruvate kinase) in control and Ksp-Cre/Lkb1<sub>fl/fl</sub> kidneys (A) and control and Lkb1-deficient cells (B). (C) ECAR of control and Lkb1-deficient TECs. When indicated glucose (10 mM), 2–4 dinitrophenol (2–4 DNP, 100 μM), 2-deoxyglucose (2-DG, 100 mM), and rotenone (1 μM) were added. The right panel summarizes results from three independent experiments. DNP, dinitrophenol.

Growing evidence suggests the key role of metabolism in fibrosis development. Our unbiased gene expression studies indicated a concerted downregulation of metabolic pathways in kidney fibrosis. We found that decreased fatty acid oxidation plays an important role in fibrosis development and pharmacological activation of Ppara or genetic overexpression of Ppargc1a ameliorated fibrosis development.27 Several studies indicate that the AMPK, PPARα, and PGC1α pathways are important in fibrosis development.29–51 Data from the Dufield group suggest that microRNA 21 is an important upstream regulator of the pathway.52

In conclusion, this study showed that selective deletion of Lkb1 in the kidney induced severe injury over time, characterized by dilated tubules and interstitial fibrosis. Our data indicate that loss of AMPK activity and lower fatty acid oxidation and energy depletion play an important role in the phenotype development, indicating that restoring LKB1 activity in CKD could offer therapeutic benefit.

CONCISE METHODS

Generation of Mice Lacking Lkb1 in the Renal TECs
Mice harboring Lkb1-floxed alleles have been described previously.53 Lkb1fl/fl mice were crossed to transgenic mice expressing Cre recombinase under the cadherin 16 promoter (Ksp-Cre).54 Transgenic mice were identified by genomic PCR analysis using transgene-specific primers. The presence of a WT Lkb1 and a floxed Lkb1 allele was detected using primers Lkb36/Lkb39 and Lkb39/Pcrs5. The primer sequences of Lkb36, Lkb39, and Pcrs5 are as follows: Lkb36, 5′-GGGCTTCACCTGTTGCGACCTGT-3′; Lkb39, 5′-GAGATGGGTACCAGGAGTTGGGGCT-3′; and Pcrs5, 5′-TCTAACAATGCAGGCTCATCGTATCCCTCGGC-3′. The presence of the Cre recombinase was detected using primers forward 5′-GCATAACCAGTGAAACAGCATTGCTG-3′ and reverse 5′-GGACATGGCCAGGGATCGCCAGGCG-3′. Animal care and experiments were performed in accordance with the National Institutes of Health guidelines and approved by the Animal Care Committee at Perelman School of Medicine, University of Pennsylvania.

Quantitative Real-Time PCR Analysis
We compared transcript levels of Lkb1, genes related to β-oxidation, glycolysis, apoptosis, and dedifferentiation (Acta2, Col1a1, and Col3a1) by quantitative real-time PCR. The detailed method is described in the supplemental material.

Western Blot Analysis
We examined protein expression levels of LKB1, p-AMPK, p-ACC, PPARα, PGClα, BCL2, and cleaved caspase-3 using western blot analyses. The detailed method and information on antibodies are available in the supplemental material.

Histology, Immunohistochemistry, and Immunofluorescence
We also performed Sirius-red staining, immunohistochemistry, and immunofluorescence studies to examine morphologic changes, fibrosis, and expression of p-AMPK, PPARα, PGClα, and cleaved caspase-3. The detailed method is described in the supplemental material.
Figure 8. Loss of Lkb1 results in apoptosis and increased expression of fibrosis markers. (A) Quantitative PCR-based transcript level of Bcl2 in control and Ksp-Cre/Lkb1\textsuperscript{fl/fl} mice and Lkb1-deficient cells. (B) Western blot analysis (representative images and quantification) of control and Lkb1-deficient tubule epithelial cells with antibodies against BCL2 (B-cell CLL/lymphoma 2), cleaved caspase-3, and beta actin. (C) Representative immunostaining for cleaved caspase-3 of control and Ksp-Cre/Lkb1\textsuperscript{fl/fl} mice. (D) Transcript levels of profibrotic markers such as Col1a (collagen 1a1), Col3a1 (collagen 3a1), and Acta2 (alpha smooth muscle actin) in control and Lkb1-deficient cells.
Figure 9. AMPK and PPARα agonists attenuated cell injury by restoring fatty acid oxidation in Lkb1-null cells. (A) Relative mRNA level of carnitine palmitoyl-transferase 1 (Cpt1), carnitine palmitoyl-transferase 2 (Cpt2), and acyl-coA oxidase 1 (Acox1) in control and Lkb1 null TECs treated with increasing dose of A769662 (A76) or fenofibrate. (B) Western blot analysis of P-ACC and beta actin in control Lkb1 null TECs treated with A769662 (A76) or fenofibrate. (C) Palmitate-induced oxygen consumption rate measured by Seahorse bioanalyzer of control and Lkb1 null TECs. When indicated cells were also treated with A769662 (A76) or fenofibrate. (D) Relative mRNA levels of glucose transporter 1 (Glut1), hexokinase (Hk), phosphofructokinase (Pfk) and pyruvate kinase (Ppk) of control, Lkb1 null
Oil-Red-O Staining
Oil-red-O working solution was made with 60% 3 mg/ml oil-red-O (#O0625; Sigma-Aldrich, St. Louis, MO) in isopropanol. Frozen sections were fixed in 10% formalin, dipped with 60% isopropanol, and then stained with oil-red-O working solution for 15 minutes at room temperature. After rinsing with 60% isopropanol and washing with distilled water, sections were mounted with glycerin jelly. Quantification of oil-red-O was performed using ImageJ v1.49 (National Institutes of Health, Bethesda, MA; online at http://rsbweb.nih.gov/ij) as previously described.55

Primary Cell Cultures
We isolated TECs from WT, WT/Lkb1<sup>flx/flx</sup> mice, and Ksp-Cre/Lkb1<sup>flx/flx</sup> mice. Cells were cultured in RPMI media (Gibco, Grand Island, NY), 10% FBS (Gibco), 100 units/ml penicillin G, 2.5 μg/ml amphotericin B, and 20 ng/ml of epidermal growth factor (all from Sigma-Aldrich). Briefly, kidneys were dissected visually, placed in 1 ml ice-cold Dulbecco’s phosphate buffered saline (DPBS) (Cellgro) and minced into pieces of ~1 mm<sup>3</sup>. Fragments were transferred to collagenase solution (1 mg/ml in DPBS; Sigma-Aldrich) and digested for 60 minutes at 37°C. Afterwards, the supernatant was sieved through a 100 μm nylon mesh. Samples were centrifuged for 10 minutes at 3000 rpm. The pellet was resuspended in sterile red blood cell lysis buffer (8.26 g NH₄Cl, 1 g KHCO₃, 0.037 g EDTA/l dH₂O) and incubated on ice for 10 minutes, followed by centrifugation for 10 minutes at 3000 rpm. The pellet was washed twice with DPBS before plating in 10 cm dishes.

Infection of Kidney TECs and Treatment
When cell confluence reached 70%–80%, the media were changed with serum-free RPMI and WT and WT/Lkb1<sup>flx/flx</sup> cells were infected with Ad5CMV-eGFP (Ad-GFP) and Ad5CMVCre-eGFP (Ad-Cre-GFP) (University of Iowa Gene Transfer Vector Core, Iowa City, IA) at 4×10<sup>10</sup> plaque forming units/ml. There were no differences in transcript levels of Lkb1 and genes involved in -oxidation and glycolysis when we compared WT cells infected with Ad-GFP, WT cells infected with Ad-Cre-GFP, and WT/Lkb1<sup>flx/flx</sup> cells infected with Ad-GFP (Supplemental Figure 7). Therefore, we used WT cells infected with Ad-Cre-GFP as a control group. At the same time, infected cells were treated with 100 μM A769662 (#A1803; LC Laboratories, Woburn, MA) or 1 μM fenofibrate (#F6020, Sigma-Aldrich). Infection efficiency was estimated under fluorescence microscopy by the presence of GFP-positive cells; it was approximately 90%. Cells were harvested and scraped off 48 hours post infection.

Measurement of ATP
Total ATP concentration was determined using an ATP colorimetric/fluorometric assay kit (Biovision, Mountain View, CA). Primarily cultured cells (1×10<sup>6</sup>) were lysed in 100 μl of ATP assay buffer and centrifuged (13,000g×2 minutes, 4°C). The supernatants (50 μl) were added to 96-well plates and mixed with 50 μl of the reaction mix (ATP probe, ATP converter, developer mix in ATP assay buffer). The plates were incubated at room temperature for 30 minutes in the dark. We determined absorbance of the samples at 570 nm using a micro-plate reader.

Mitochondrial Visualization
Cells were plated at 1×10<sup>5</sup> cells/well onto 12 mm petri dishes (WillCo-dish). After the virus infection, cells were stained with 200 nM MitoTracker Red FM dye for 30 minutes and visualized with a Leica TCS SP8 confocal microscope (Leica SP5, Goettingen, Germany). Images were acquired with LAS software.

Electron Microscopic Examination
Mitochondrial structure was further examined by standard transmission electron microscopy. Primary TECs were fixed with a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde overnight, washed, dehydrated, and embedded in resin according to standard procedures. Mitochondria were examined by a JEOL 1010 microscope (Tokyo, Japan).

Measurement of Oxygen Consumption Rate and Extracellular Acidification Rate
A Seahorse Bioscience X24 extracellular flux analyzer was used to measure the rate change of dissolved oxygen in medium immediately surrounding adherent cells cultured in an XF24 V7 cell culture microplate (Seahorse Bioscience). Mouse primary TECs cultured in RPMI 1640 supplement with 0.5% FBS were seeded in XF24 V7 cell culture microplate at 1.0×10<sup>4</sup> cells per well. Oxygen consumption rates (pmol/minute) and extracellular acidification rate (mpH/minute) were assessed at baseline and after the addition of palmitate conjugated BSA (180 μM) or 10 mM glucose followed by the addition of the carnitine palmitoyltransferase-1 inhibitor etomoxir (40 μM) or the mitochondrial uncoupler, 2–4 dimethyloctylamine (100 μM) or glyclycerol inhibitor 2-deoxyglucose (100 mM). The final state was determined after the addition of the ATP synthase inhibitor oligomycin (1 μM) or rotenone (1 μM).

Human Kidney Samples
Kidney samples were obtained from routine surgical nephrectomies. Samples were de-identified and the corresponding clinical information was collected by an individual who was not involved in the research protocol. The study was approved by the Institutional Review Boards of the Albert Einstein College of Medicine Montefiore Medical Center (IRB#2002–202) and the University of Pennsylvania. Histologic analysis was performed by an expert pathologist (IRB#815796).

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cells and Lkb1 null cells treated with A769662 or fenofibrate. (E) Glucose-induced ECAR in control, Lkb1 null cells, and Lkb1 null cells treated with A769662 or fenofibrate. (F) Transcript levels of cadherin 1 (Cdh1), alpha smooth muscle actin (Acta), fibronectin (Fn1), collagen 1α1 (Col1α1), and collagen 3α1 (Col3α1) of primary mouse TECs in the presence and absence of Lkb1 and treated with A769662 (A76) or fenofibrate (Feno). (G) Western blot analysis (representative image and quantification) for BCL2 (B-cell CLL/lymphoma 2), cleaved-caspase 3, and beta-actin of control and Lkb1 null cell treated with A769662 or fenofibrate.
Microarray
Tissue was placed into RNALater and manually microdissected at 4°C for glomerular and tubular compartments as described earlier. Dissected tissue was homogenized and RNA was prepared using RNAeasy mini columns (Qiagen, Valencia, CA) according to the manufacturer’s instructions. RNA quality and quantity was determined using Lab-on-Chip Total RNA PicoKit (Agilent BioAnalyzer, Santa Clara, CA). Only samples without evidence of degradation were used. Biotinylated cRNA were prepared according to the standard Affymetrix protocol from 6 μg total RNA (Expression Analysis Technical Manual, 2001, Affymetrix). Following fragmentation, 10 μg of cRNA were hybridized for 16 hours at 45°C and GeneChips were washed and stained in the Affymetrix Fluidics Station 400. The data were scanned using a GeneChip scanner in accordance with the Affymetrix protocol. Data were normalized in GeneSpring software using the RMA16 summarization algorithm. Baseline is the median of all samples.

Statistical Analyses
Statistical analysis was performed using the statistical package SPSS for Windows version 11.0 (IBM SPSS, Chicago, IL). All results are presented as mean±SD. To analyze the difference between two groups, Student’s t test was used. Bonferroni correction was used when more than two groups were present. P values <0.05 were considered statistically significant.

ACKNOWLEDGMENTS
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DISCLOSURE
The laboratory of Dr. Susztak received research support from Boehringer Ingelheim and Biogen Idec for projects that are not related to this work.

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transition. EMBO J 23: 1155–1165, 2004


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<table>
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**Table S2.** Sequences of oligonucleotide primers used for qPCR test

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Supplemental figures

Figure S1. CAB39L expression in healthy human kidney samples

Representative images of double immunofluorescence staining of CAB39L (red) and distal tubule marker PNA (green) (arrow head). CAB39L was expressed both in PNA positive (distal) and PNA negative (proximal) tubules. CAB39L expression was also observed in the cytoplasm (lower panel).
Figure S2. *Lkb1* deletion in the proximal tubules did not result in kidney fibrosis

Representative images of PAS-stained kidney sections from control and *Pepck-Cre/Lkb1*\textsuperscript{flox/flox} mice at 27 weeks of age
Figure S3. Cysts are not prominent in kidneys of $Ksp$-$\text{Cre}/\text{Lkb1}^{\text{fl/fl}}$ mice

Quantification of dilated tubules (TD), microcysts, and cysts in control and $Ksp$-$\text{Cre}/\text{Lkb1}^{\text{fl/fl}}$ mice
Figure S4. Cell proliferation in $Ksp$-$Cre/Lkb1^{fl/fl}$ mice

(A and B) Immunohistochemistry images of (A) Ki-67 and (B) PCNA in control and $Ksp$-$Cre/Lkb1^{fl/fl}$ mice. Note the increased proliferation of interstitial cells.
Figure S5. No apparent polarity or ciliary abnormalities in \( Ksp-Cre/Lkb1^{\text{flox/flox}} \) mice

Transcript levels of cadherin 1 (\( Cdh1 \)) (A) and zonular occludens-1 (\( Zo1 \)) (B) in control and 5 and 14-wk old \( Ksp-Cre/Lkb1^{\text{flox/flox}} \) mice. (B) Basolateral localization of \( \beta \)-catenin and E-cadherin was not altered in 14-wk old \( Ksp-Cre/Lkb1^{\text{flox/flox}} \) mice. These markers were co-stained with peanut agglutinin (PNA), a distal tubule marker. (C) Immunostaining of acetylated tubulin (red) demonstrated that primary cilia were present and normal appearing in mutant mice, both at week 5 and 14.

![Image of Figure S5](image-url)
Figure S6. The mTOR pathway is not activated in Ksp-Cre/Lkb1$^{flax/flax}$ mice

(A) Transcript level of Mtor and Hif1a in control and Ksp-Cre/ Lkb1$^{flax/flax}$ mice. (B) Representative immunostaining of p-mTOR and p-S6K levels in control and Ksp-Cre/ Lkb1$^{flax/flax}$ mice. (C) Western blot analysis for P-mTOR and P-S6K and beta actin of control and Lkb1-deficient cells and control and Ksp-Cre/ Lkb1$^{flax/flax}$ mice.
Figure S7. Comparisons of different control groups

There were no differences in transcript levels of Lkb1, genes involved in β-oxidation and glycolysis between WT cells infected with Ad-GFP, WT cells infected with Ad-Cre-GFP, and WT/Lkb1\textsuperscript{floox/floox} cells infected with Ad-GFP. However, Lkb1 deficient cells (WT/Lkb1\textsuperscript{floox/floox} cells infected with Ad-Cre-GFP) had lower levels of Lkb1, Cpt1, Cpt2, Acox1, Ppara1, Pparag1a, Glut1, Hk, and Pk than these 3 groups.
Supplemental Methods

Tubular dilatation (TD)/Cyst Index

Using a grid of squares with each side measuring 13.625 μm, we quantified the number of dilated tubules and cysts per kidney stained with PAS. Each cross was marked with a dot and the number of dots in the tubular lumen was counted. The degree of dilatation was determined by the number of dots according to criteria as previously suggested by Bastos et al: (1) normal, one dot; (2) TD, two dots; (3) microcyst, three to ten dots; and (4) cyst, > 10 dots.

Quantitative real-time polymerase chain reaction analysis

We prepared total RNA from mouse kidney and from primary cultures of renal tubular cells using RNeasy Mini Kit (Qiagen). The quality was analyzed on agarose gels, and the quantity was measured using NanoDrop. We reverse-transcribed 1 μg total RNA using the cDNA Archival Kit (Applied Biosystems). Quantitative real-time polymerase chain reaction (qPCR) analysis was performed using a ViiA™ 7 Real-Time PCR System (Life Technologies), with SYBR Green Master Mix, 3-step standard cycling conditions, and sequence-specific primers. The sequences of primers used for the experiment are presented in Table S2. We examined the melting curve to verify that a single product was amplified. For quantitative analysis, all samples were normalized to Ubiquitin C gene expression using the ΔΔCT value method.

Western blot analysis

Cell lysates were prepared with RIPA lysis buffer containing protease inhibitor cocktail
(Complete Mini, Roche) and phosphatase inhibitor (PhosSTOP, Roche). Proteins were resolved on 5–15% gradient gels, transferred on to polyvinylidene difluoride membranes and probed with antibodies as below; LKB1 (#07-694, Millipore), phospho-(5'AMP-activated protein kinase) AMPK (Thr172) (#2535, Cell signaling), peroxisome proliferator-activated receptor-alpha (PPAR-α) (#ab8934, Abcam), phospho-acetyl-coenzyme A carboxylase (ACC) (#3661, Cell signaling), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) (#ab54418, Abcam), phospho-mammalian target of rapamycin (mTOR) (#5536, Cell signaling), phospho-p70 S6 Kinase (Thr389) (#9234, Cell signaling) and phospho-p70 S6 Kinase (Ser371) (#9208, Cell signaling), and β-actin (#A5316, Sigma-Aldrich). Anti-rabbit (#7074, Cell Signaling) or anti-mouse (#7076, Cell Signaling) IgG horseradish peroxidase (HRP) was used as a secondary antibody. Blots were detected by enhanced chemiluminescence (Western Lightning-ECL, Thermo Scientific).

**Histology, immunohistochemistry, and immunofluorescence**

We used formalin-fixed, paraffin-embedded kidney sections stained with periodic acid Schiff (PAS) for routine histologic examination. Slides were examined, and pictures were taken with an Olympus DP73 microscope.

To detect fibrosis, Sirius-red staining was performed by incubating slides in 0.1% Sirius Red F3B for 1 h, washing twice in acidified water, dehydrating three times in 100% ethanol, and then clearing in xylene. The sections were examined under a Nikon Eclipse E600 microscope equipped with a digital camera (Melville, NY). All images were submitted to ImageJ v1.49 (NIH, Bethesda, Maryland, USA; online at http://rsbweb.nih.gov/ij) to quantify the percentage of fibrosis compared to the total amount of tissue within an image.
For immunofluorescence staining, sections were deparaffinized and hydrated, followed by microwave antigen retrieval in 10 mM sodium citrate, pH 6.0. Sections were blocked in 5% normal goat serum for 30 min at room temperature and incubated in mouse anti–β-catenin antibody (1:100; #610154, BD Biosciences) and rabbit anti–E-cadherin antibody (1:100; #3195, Cell signaling) overnight at 4 °C. Anti-mouse Alexa Fluor 488 (1:200; #4408, Cell Signaling) and anti-rabbit Alexa Fluor 555 (1:200; #4413, Cell Signaling) were applied and nuclei were stained with DAPI. For double immunofluorescence staining using human kidney samples, sections were incubated with rabbit anti-phospho LKB1 (1:200; GTX30765, GeneTex) overnight and fluorescein lotus tetraglonolobus lectin (1:200; FL1321, Vector laboratories), a proximal tubule marker, was added for 60 minutes on the following day.

For fluorescence labeling of acetylated tubulin (#T741, Sigma-Aldrich), following antigen retrieval with citrate buffer, sections were blocked with AffiniPure Fab fragment anti-mouse IgG (#115-007-003, Jackson ImmunoResearch Labs), and incubated overnight with primary antibody. After washing, Cy3 conjugated anti-rabbit antibodies (Jackson) were applied, and the sections were mounted with fluoromount and visualized.

For immunohistochemical staining, sections were deparaffinized, hydrated in ethyl alcohol, and washed in tap water. Antigen retrieval was carried out in 1 mM EDTA, pH 8.0 by microwave for 15 min. Endogenous peroxidase activity was blocked in 3% H₂O₂ for 10 min. Sections were blocked in 0.2% fish skin gelatin for 60 min at room temperature and incubated overnight at 4 °C in rabbit anti–cleaved caspase 3 (1:100; #9664, Cell Signaling), rabbit anti-PGC-1α (1:750, #ab54418, Abcam), and rabbit anti-phospho LKB1 (Ser428) (1:200; GTX30765, GeneTex). Staining was visualized using peroxidase-conjugated antibodies to rabbit immunoglobulin using the Vectastain Elite kit and 3, 3-diaminobenzidine (DAB), as per manufacturer’s protocol (Vector Labs).
Supplemental References