The SWI/SNF protein PBRM1 restrains VHL-loss-driven clear cell renal cell carcinoma

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Graphical Abstract

Highlights

- PBRM1 is a bona fide tumor suppressor in the pathogenesis of ccRCC
- PBRM1 prevents self-perpetuating amplification of HIF1/STAT3 signaling in Vhl−/− cell
- Loss of Vhl and Pbrm1 in mouse kidney results in multifocal, transplantable ccRCC
- In ccRCC, mTORC1 activation is the third driver event after loss of VHL and PBRM1

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In Brief

Nargund et al. present a three-step process in the pathogenesis of mouse and human clear cell kidney cancer. After the loss of VHL, the loss of SWI/SNF tumor suppressor protein PBRM1/BAF180 further activates HIF1/STAT3 signaling in mouse kidney and positions mTORC1 activation as the preferred third driver event.

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The SWI/SNF Protein PBRM1 Restraints VHL-Loss-Driven Clear Cell Renal Cell Carcinoma

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SUMMARY

PBRM1 is the second most commonly mutated gene after VHL in clear cell renal cell carcinoma (ccRCC). However, the biological consequences of PBRM1 mutations for kidney tumorigenesis are unknown. Here, we find that kidney-specific deletion of Vhl and Pbrm1, but not either gene alone, results in bilateral, multifocal, transplantable clear cell kidney cancers. PBRM1 loss amplified the transcriptional outputs of HIF1 and STAT3 incurred by Vhl deficiency. Analysis of mouse and human ccRCC revealed convergence on mTOR activation, representing the third driver event after genetic inactivation of VHL and PBRM1. Our study reports a physiological preclinical ccRCC mouse model that recapitulates somatic mutations in human ccRCC and provides mechanistic and therapeutic insights into PBRM1 mutated subtypes of human ccRCC.

INTRODUCTION

The estimated new kidney cancer cases diagnosed in the world and the United States every year are ~300,000 and ~63,000, respectively (Fitzmaurice et al., 2015; Siegel et al., 2016). Clear cell renal cell carcinoma (ccRCC) is the most common subtype (75%) (Hsieh et al., 2017b) and is lethal when metastasized (Rini et al., 2009). The Von Hippel-Lindau (VHL) tumor suppressor gene is the most frequently mutated gene in ccRCC (Gnarra et al., 1994; Linehan et al., 1995) and its complete loss constitutes an early, truncal oncogenic driver event. VHL is the substrate recognition of an E3 ligase that labels hypoxia-inducible factor (HIF) 1α and 2α with ubiquitin for degradation (Kaelin, 2007; Majmundar et al., 2010; Masson and Ratcliffe, 2014; Semenza, 2013). Thus, human ccRCC is highly vascular due to uncontrolled activation of HIFα targets that regulate angiogenesis. Thereby, anti-vascular endothelial growth factor (VEGF)/vascular endothelial growth factor receptor (VEGFR) agents are effective, first-line treatment for metastatic ccRCC (mRCC) (Rini et al., 2009; Voss et al., 2013).

VHL inactivation was the only known oncogenic driver in ccRCC (Gnarra et al., 1994) until recent large-scale cancer genomic projects uncovered prevalent mutations including PBRM1/BAF180 (29%–41%), SETD2 (8%–12%), BAP1 (6%–10%), and KDM5C (4%–7%) (Cancer Genome Atlas Research Network, 2013; Hakimi et al., 2013a; Peña-Llopis et al., 2012; Sato et al., 2013; Varela et al., 2011). Remarkably, these genes encode chromatin and epigenetic regulatory proteins, and most mutations are predicted to result in functional loss, favoring their roles as tumor suppressors (Hakimi et al., 2013b). PBRM1, the second most commonly mutated gene in all stages of ccRCC (Hsieh et al., 2017a), encodes BRG1-associated factor (BAF) 180, the defining subunit of the ~2 million dalton (MDa) polybromo BAF (PBAF) SWI/SNF complex (Varela et al., 2011). The SWI/SNF chromatin remodeling complexes are macromolecular machineries, which utilize ATP to mobilize...
nucleosome and thereby modulate chromatin structure (Biegel et al., 2014; Clapier and Cairns, 2009). They regulate critical cellular processes, including cell-cycle, cell fate, cell death, metabolism, and DNA repair (Hargreaves and Crabtree, 2011). Interestingly, pan-cancer genomics have uncovered epigenetic regulators including SWI/SNF proteins as a major class of cancer genes (Dawson and Kouzarides, 2012). Mutations of individual SWI/SNF subunits have been detected in ~20% of human cancers and they displayed preferential enrichment of mutations among cancer types (Heiling et al., 2014; Kadoch et al., 2013; Marquez et al., 2015). For example, PBRM1 is most highly mutated in ccRCC, SMARCB1 (BAF47) in pediatric rhabdoid tumors and ARID1A (BAF250A) in ovarian clear cell carcinoma (Biegel et al., 2014), implicating underlying tissue tropism for disarming specific tumor suppressor gene (TSG) during tumorigenesis (Wei and Hsieh, 2015).

The knowledge of PBRM1 in mammalian biology is limited. Mice with straight knockout of Pbrm1 resulted in embryonic lethality at embryonic day 11.5 due to heart defects (Huang et al., 2008; Wang et al., 2004), whereas mice with T lymphocyte-specific knockout of Pbrm1 exhibited normal thymus and peripheral T cell development (Wurster et al., 2012). In vitro studies demonstrated that PBRM1 activated p21 upon irradiation in breast cancer cell lines (Xia et al., 2009) and participated in p53-induced replicative senescence in fibroblasts (Burrows et al., 2010), and PBRM1 knockdown enhanced proliferation and migration of kidney cancer cell lines (Varela et al., 2011). However, the in vivo tumor suppressor function of PBRM1 has not been established, and how PBRM1 loss-of-function promotes tumorigenesis remains unclear.

The long latency (>30 years) for VHL germline-mutated patients to develop ccRCC (Fisher et al., 2014; Kaelin, 2007) and the inability of VHL deficiency to induce ccRCC in mice (Kapitsinou and Haase, 2008) suggest that additional genetic/epigenetic events are probably needed for the development of ccRCC (Wei and Hsieh, 2015). PBRM1 loss-of-function is one of the most likely candidates given its high mutation frequency (~40%) in human ccRCC (Hsieh et al., 2017a). Here, we created kidney-specific deletion of Pbrm1 and/or Vhl mice to study the tumor suppressor role of PBRM1 and sought to establish a physiological mouse kidney cancer model that recapitulates human ccRCC.

RESULTS

Genetic Deletion of Pbrm1 in Mouse Kidney Results in Hydronephrosis

As Pbrm1 (BAF180) deletion in mice incurred embryonic lethality (Wang et al., 2004), we deleted the conditional Pbrm1F/F allele (Wurster et al., 2012) in the mouse kidney using a transgenic Cre recombinase line Ksp-Cre (Shao et al., 2002) that has been widely utilized to model kidney cancer in mice (Adam et al., 2011; Baba and Haase, 2008; Chen et al., 2008; Igarashi, 2004). The expression of Cre from the Ksp-Cre is driven by the kidney-specific Cadherin 16 promoter, which begins expression at embryonic day 14.5 in epithelial cells of the developing kidney and genitourinary tract and continues to be expressed in tubular epithelial cells in adults. Pbrm1F/F Ksp-Cre mice were born at expected Mendelian ratio. To monitor if Pbrm1 loss results in any gross kidney abnormality, serial abdominal MRI was performed on a large cohort of mice. Obstructive hydronephrosis, enlarged kidneys containing fluid-filled renal pelvis, and non-neoplastic masses at the ureteropelvic junction or proximal ureter were detected in some Pbrm1F/F Ksp-Cre mice (Figures 1A and S1A). Among 53 Pbrm1F/F Ksp-Cre and 27 Pbrm1F/F Ksp-Cre (denoted as WT thereafter) mice examined, 18 (34%) Pbrm1F/F Ksp-Cre while 0 WT mice developed hydronephrosis (Figure 1B). Observed hydronephrosis exhibited a preponderance of female over male and left over right (Figures 1C and 1D) and could be detected by MRI as early as 6 months of age (Figure S1B). At necropsy, the volume of hydronephrotic Pbrm1F/F Ksp-Cre kidneys was at two to five times that of normal appearing kidneys (Figure 1E), whereas the creatinine of aged Pbrm1F/F Ksp-Cre and WT mice was comparable (Figure 1F).

Genetic Deletion of Vhl and Pbrm1 in Mouse Kidney Results in Polycystic Kidney Disease and Increased Mortality

As neither Vhl nor Pbrm1 deletion alone caused kidney tumors, VhlF/F Pbrm1F/F Ksp-Cre mice were generated to investigate the genetic interaction between Vhl and Pbrm1 deficiency in kidney cancer pathogenesis. The survival of 325 mice (36 WT, 30 VhlF/F Ksp-Cre, 129 Pbrm1F/F Ksp-Cre, and 130 VhlF/F Pbrm1F/F Ksp-Cre) was monitored, which revealed a markedly increased mortality in VhlF/F Pbrm1F/F Ksp-Cre mice and a moderately increased mortality in VhlF/F Ksp-Cre mice (Figure 2A). Remarkably, abdominal MRI detected diffuse polycystic kidney disease (PKD) in 30% (17/56) of 6- to 9-month-old and in 67% (14/21) of 10- to 14-month-old VhlF/F Pbrm1F/F Ksp-Cre mice, whereas only 1 of 14 aged VhlF/F Ksp-Cre mice (12- to 16-month-old) developed PKD with a few scattered cysts (Figures 2B, 2C, and S2). To investigate cystic changes, we performed histological analysis on the kidneys of VhlF/F Pbrm1F/F Ksp-Cre mice at different ages (3–13 months). Both tubular and glomerular cysts were present in young mice (Figure S3). Of note, scattered cystic anomalies of kidneys have been described in hereditary VHL patients and are implicated as pre-neoplastic lesions (Mandriota et al., 2002; Neumann and Zbar, 1997; Walther et al., 1995). Consistent with a prior report, mild hydronephrosis was also observed in VhlF/F Ksp-Cre mice (Figure 2D) (Frew et al., 2008). Elevated serum creatinine was observed in the majority of aged VhlF/F Pbrm1F/F Ksp-Cre mice (Figure 2E), which could be accountable for their early demise.

VhlF/F Pbrm1F/F Ksp-Cre Mice Develop Multifocal, Clear Cell Kidney Cancer

Serial MRI examination of kidney in VhlF/F Pbrm1F/F Ksp-Cre mice recognized patterns of imaging changes from normal through progressive cystic abnormality to increased multifocal nodularity with decreased cystic appearance (Figures S2 and S3). Gross examination of 58 kidneys (29 mice, 8- to 17-month-old) at necropsy showed diffusely cystic changes in 58.33% (21/36) of VhlF/F Pbrm1F/F Ksp-Cre kidneys and none in VhlF/F Ksp-Cre (n = 8), Pbrm1F/F Ksp-Cre (n = 10), and WT (n = 4) kidneys (Table S1). Histologic examination revealed sheets of tumor cells interspersed within a highly vascularized stroma in 33.33% (12/36) VhlF/F Pbrm1F/F Ksp-Cre kidneys, whereas no tumors were noted in 22 kidneys of the other genotypes (Table S1). Notably, all the
tumors were observed in VhF/Pbrm1F/FKsp-Cre mice after 10 months of age with a 50% tumor incidence (12/24). The tumor cells displayed central features of human ccRCC, including clear cytoplasm and positive membranous staining of carbonic anhydrase IX (CA-IX), a target of HIF1 (Mandriota et al., 2002; Semenza, 2013) (Figure 3A). Consistent with the known aberrant HIF1 activation in the absence of VHL, weak, sporadic CA-IX staining was detected in VhlF/FKsp-Cre, but not in Pbrm1F/FKsp-Cre or WT kidneys (Figure 3A). To compare these mouse tumors with human RCCs, we performed gene expression profile analysis of these mouse tumors in comparison to human TCGA clear cell RCC (KIRC), including clear cell RCC and positive membranous staining of carbonic anhydrase IX (CA-IX), a target of HIF1 (Mandriota et al., 2002; Semenza, 2013) (Figure 3A). Consistent with the known aberrant HIF1 activation in the absence of VHL, weak, sporadic CA-IX staining was detected in VhF/Pbrm1F/FKsp-Cre, but not in Pbrm1F/FKsp-Cre or WT kidneys (Figure 3A). To compare these mouse tumors with human RCCs, we performed gene expression profile analysis of these mouse tumors in comparison to human TCGA clear cell RCC (KIRC) and chromophobe RCC (KICH) kidney cancers and demonstrated that VhF/Pbrm1F/FKsp-Cre tumors resemble KIRC but not KICH (Figure 3B). Data suggest that human clear cell RCC arises from proximal tubule (Chen et al., 2016). Accordingly, we investigated the cell type origin of VhF/Pbrm1F/FKsp-Cre mouse tumors. Staining for lotus tetragonolobus lectin (LTL) that marks proximal convoluted tubule and for Tamm-Horsfall protein (THP) that marks distal convoluted tubule was performed. Consistent with human ccRCC originating from proximal tubule, our VhF/Pbrm1F/FKsp-Cre mouse tumors were stained positive for LTL but not THP (Figure 3C). CD45 staining did not detect increased lymphocyte infiltrate of these VhF/Pbrm1F/FKsp-Cre tumors (Figure 3C). Consistent with human ccRCC reports, these tumors were positive for CD31 staining that marks endothelial cells (Figure 3C). We also performed Oil Red O staining of the fresh frozen section of these mouse tumors to evaluate lipid content in our the clear cell mouse tumors (Figure S4). Furthermore, the presence of high glycogen in these tumors was confirmed by PAS-D staining (Figure S4). Of note, our VhF/Pbrm1F/FKsp-Cre mouse tumors did not directly originate from cystic lesions (Figure S5A). Higher proliferation index (Ki-67 staining) was observed in these Vh and Pbrm1 doubly deficient clear cell kidney tumors whereas no alteration in cell death was detected by immunohistochemistry for cleaved caspase-3 and TUNEL assays (Figures S5B

Figure 1. Pbrm1F/FKsp-Cre Mice Develop Obstructive Hydronephrosis

(A) Representative MRI images of unilateral or bilateral severe hydronephrosis. The non-neoplastic mass at the proximal ureter is marked by arrow.
(B) Incidence of hydronephrosis in WT and Pbrm1F/FKsp-Cre mice. Cohorts of animals at 12 months of age on average were randomly selected for MRI scanning.
***p < 0.001 (Fisher’s exact).
(C) Incidence of hydronephrosis in the Pbrm1F/FKsp-Cre mice based on gender.
(D) Location distribution of hydronephrosis in Pbrm1F/FKsp-Cre mouse kidneys.
(E) Kidney volume in WT and Pbrm1F/FKsp-Cre mice. *p < 0.05 (Mann-Whitney).
(F) Serum creatinine levels of mice in (E). ns, not statistically significant.
and S5C). qPCR with reverse transcription (qRT-PCR) demonstrated greatly reduced Vhl and Pbrm1 expression in Vhl<sup>F/F</sup> Pbrm1<sup>F/F</sup> Ksp-Cre kidney tumors (Figure S5D). Of note, we did not detect local tumor invasion into adjacent tissues or distant metastasis to lungs, livers, bones, and lymph nodes in the examined tumor-bearing Vhl<sup>F/F</sup> Pbrm1<sup>F/F</sup> Ksp-Cre mice.

Figure 2. Vhl<sup>F/F</sup> Pbrm1<sup>F/F</sup> Ksp-Cre Mice Develop Polycystic Kidney Disease and Exhibit Premature Mortality

(A) Kaplan-Meier survival curve of WT, Vhl<sup>F/F</sup> Ksp-Cre, Pbrm1<sup>F/F</sup> Ksp-Cre, and Vhl<sup>F/F</sup> Pbrm1<sup>F/F</sup> Ksp-Cre mice.

(B) Incidence of polycystic kidney disease in WT, Vhl<sup>F/F</sup> Ksp-Cre, Pbrm1<sup>F/F</sup> Ksp-Cre, and Vhl<sup>F/F</sup> Pbrm1<sup>F/F</sup> Ksp-Cre. Age and number of animals in each group are specified.

(C) Representative MRI and gross images of kidneys of the indicated genotypes.

(D) Kidney volumes of WT, Vhl<sup>F/F</sup> Ksp-Cre, and Vhl<sup>F/F</sup> Pbrm1<sup>F/F</sup> Ksp-Cre mice. Numbers of kidneys measured in each group (n) are indicated. **p = 0.0096; ***p < 0.0001 (Mann-Whitney).

(E) Serum creatinine levels in WT, Vhl<sup>F/F</sup> Ksp-Cre, and Vhl<sup>F/F</sup> Pbrm1<sup>F/F</sup> Ksp-Cre mice (the same as Figure 1D). ***p < 0.001 (Mann-Whitney).
Vhl and Pbrm1 Doubly Deficient Clear Cell Kidney Tumors Are Transplantable

To assess the tumor initiating capacity of the de novo Vhl and Pbrm1 doubly deficient kidney tumors, we transplanted 12 tumor fragments from two Vhl<sup>F/F</sup> Pbrm1<sup>F/F</sup> Ksp-Cre kidneys into the sub-renal capsules of 12 kidneys of 6 immunocompromised NOD/SCID/IL2RG<sup>nu</sup> (NSG) mice. All the recipient kidneys, except for the one animal that died prematurely of known cause, showed large visible kidney tumors upon dissection at 10–12 months after transplantation (Figure 4A). In two of the transplanted cases, we also observed tumor invasion into organs such as liver (Figure 4B). Furthermore, tumors that developed in the transplanted NSG mouse could be further successfully propagated into a NSG mouse (Figure 4A). Together, these data demonstrated the malignant potential of Vhl<sup>F/F</sup> Pbrm1<sup>F/F</sup> Ksp-Cre kidney tumors. Histology of these tumor allografts showed the same histological features, positive CA-IX staining as donor tumors, and genotypes (Figure 4). Importantly, this stepwise, morphological progression observed in Vhl<sup>F/F</sup> Pbrm1<sup>F/F</sup> Ksp-Cre kidneys from normal appearance through cystic changes (~6 months) to ccRCC formation (~10 months) offered an opportunity to temporally dissect the mechanisms by which PBRM1 loss cooperates with VHL loss to initiate the development of ccRCC.

Gene Expression Profiling of WT, Vhl-Deficient, Pbrm1-Deficient, or Vhl and Pbrm1 Doubly Deficient Mouse Renal Cortices Identifies Distinct Clusters

To determine why double deficiency of either gene, resulted in ccRCC, we performed gene expression profiling of RNA isolated from renal cortices of 12-week-old WT, Vhl<sup>F/F</sup> Ksp-Cre, Pbrm1<sup>F/F</sup> Ksp-Cre, and Vhl<sup>F/F</sup> Pbrm1<sup>F/F</sup> Ksp-Cre mice. Kidneys from 12-week-old mice were chosen to avoid potentially confounding transcriptional changes secondary to cystic anomalies that normally manifest after 6 months of age. Of note, none of the 12-week-old mouse kidneys displayed discernible macroscopic or microscopic cystic abnormalities at necropsy regardless of genotypes. Genes differentially expressed in at least one genotype were identified (Table S2A) and subjected to unsupervised hierarchical
clustering analysis, which revealed three distinct clusters (Figure 5A). Cluster I showed enrichment of genes that were upregulated in Vhl-deficient renal cortices and further upregulated in Vhl and Pbrm1 doubly deficient renal cortices. In contrast, cluster III showed enrichment of genes that were downregulated in Vhl and Pbrm1 doubly deficient renal cortices. Each cluster was then analyzed and visualized using ClueGO (Binde et al., 2009) to interrogate functionally grouped gene ontology and pathway networks (Figure 5B; Table S2B). Pathways over-represented that are highly pertinent to known ccRCC pathogenesis were HIF1 and JAK-STAT pathway genes in cluster I and oxidative phosphorylation (OXPHOS) genes in cluster III (Figure 5B), whereas no pathway was significantly enriched in cluster II.

Figure 4. Vhl<sup>F/F</sup>Pbrm1<sup>F/F</sup>Ksp-Cre Mice Tumors Are Transplantable and Invasive

(A) Representative gross images (column 1), histopathological images (column 2), and immunohistochemistry of CA-IX (column 3) of donor Vhl<sup>F/F</sup>Pbrm1<sup>F/F</sup>Ksp-Cre kidney tumors (row 1), primary allograft kidney tumors (row 2), and secondary allograft kidney tumors (row 3). (B) Representative histopathological image (top) and immunohistochemistry of CA-IX (bottom) of the transplanted invasive tumors. (C) PCR genotyping of WT kidney and donor and allograft Vhl<sup>F/F</sup>Pbrm1<sup>F/F</sup>Ksp-Cre tumors.

PBRM1 Loss Amplifies the Transcriptional Outputs of HIF1 and STAT3 Incurred by VHL Loss

HIF1α is stabilized upon VHL loss and plays a central role in the pathogenesis of ccRCC. It was reported that HIF1 induces PKM2 to activate STAT3, which in turn induces HIF1α expression (Demaria and Poli, 2012; Luo and Semenza, 2012). Furthermore, it is known that HIF1 and STAT3 cooperate to activate the expression of HIF1 targets including genes involved in angiogenesis (Jung et al., 2005). Consequently, the intricate interplay between HIF1 and STAT3 establishes a feed-forward amplification loop to maximize target gene expression. Our discovery that HIF1 and JAK-STAT pathway genes were enriched in Vhl-deficient renal cortices and further enriched in Vhl and Pbrm1 doubly deficient renal cortices raises a hypothesis in which PBRM1 prevents the amplification of the HIF1 and STAT3 transcriptional outputs that are initiated upon VHL loss.

To test this, we identified HIF1 and STAT3 motifs from differentially expressed genes to determine the strength of regulation of the targets by individual transcription factor binding motifs (TFBMs). RNA expression data were analyzed using Integrated System for Motif Activity Response Analysis (ISMARA) (Balwierz et al., 2014), which produced an output denoting the inferred activity of HIF1 and STAT3 motifs in every sample. An increase in the HIF1 (p = 0.08) and STAT3 (p = 0.017) motif activities was detected when comparing Vhl<sup>F/F</sup>Ksp-Cre to WT kidneys, and a marked increase in both HIF1 (p = 0.00076) and STAT3 (p = 0.00018) outputs was identified when comparing Vhl<sup>F/F</sup>Pbrm1<sup>F/F</sup>Ksp-Cre to WT kidneys. In contrast, no changes in HIF1 (p = 0.49) or STAT3 (p = 0.98) motif activity were observed when comparing Pbrm1<sup>F/F</sup>Ksp-Cre to WT kidneys. Remarkably, the effects of Pbrm1 deletion on HIF1 and STAT3 targets manifested only under the premise of Vhl loss, resulting in a further increase of the HIF1 (p = 0.035) and STAT3 (p = 0.0022) motif
Figure 5. PBRM1 Loss Amplifies the Transcriptional Outputs of HIF1 and STAT3 Incurred by VHL Loss

(A) Heatmap of genes with significantly different expression in the renal cortices of WT, Vhl<sup>F/F</sup> Ksp-Cre, Pbrm1<sup>F/F</sup> Ksp-Cre, and Vhl<sup>F/F</sup> Pbrm1<sup>F/F</sup> Ksp-Cre mice at 12 weeks of age. Unsupervised hierarchical agglomerative clustering identified three distinct clusters using Pearson correlation and average linkage as similarity measures for pairs of genes and pairs of inchoate clusters, respectively.

(B) Clusters I and III were tested for pathway enrichment and presented using ClueGO.

(C) Inferred HIF and STAT motif activities across the indicated genotypes. *p = 0.035; **p = 0.0022 (one-sided t test).

(D) The mRNA levels of the indicated genes from the indicated genotypes were assessed by qRT-PCR. Data were normalized against GAPDH (mean ± SD, n = 3 independent experiments). *p < 0.05; **p < 0.005 (Student’s t test).
activities when comparing Vhl+/−Pbrm1F/F Ksp-Cre to Vhl+/−Ksp-Cre kidneys (Figure 5C). To further validate these findings, we performed qRT-PCR analyses on Hif1α, Stat3, and representative HIF1 target genes (Pdk1 and Egln3) as well as STAT3 target genes (Socs3, Il4r, and Il6) (Figures 5D and S6A–S6D), which demonstrated consistent results among different gene expression assays. Of note, we did not see any transcriptional upregulation of HIF2α. In contrast to cluster I that encompasses upregulated genes in Vhl+/−Pbrm1F/F Ksp-Cre kidneys and subjected to unsupervised hierarchical clustering analysis on common human kidney cancer types, including ccRCC, including the known therapeutic benefit of administering mTORC1 inhibitors in treating metastatic ccRCC (Voss et al., 2014; Wei and Hsieh, 2015) and the observed prevalent mTORC1 pathway activation in human ccRCC (Lienhan et al., 2010; Robb et al., 2007). Induction of REDD1 by HIF1α may activate a tumor suppressor checkpoint that restrains the oncogenic potential of HIF1α. Hence, activation of mTORC1 activity through additional genetic/epigenetic events may be required for the initiation of ccRCC in Vhl and Pbrm1 doubly deficient renal epithelial cells. In fact, significant downregulation of Tsc1 and Tsc2 was demonstrated in Vhl and Pbrm1 doubly deficient kidney tumors (Figure 6F), which would activate mTORC1 even when REDD1 was upregulated. In summary, the emergent mTORC1 activation detected in the Vhl and Pbrm1 doubly deficient ccRCC may represent a prerequisite oncogenic driver event in the pathogenesis of ccRCC once kidney epithelial cells lost VHL and PBRM1.

**Analyses of Mouse and Human ccRCC Reveal Convergence on the mTOR Pathway Activation**

Contrary to the inability of Vhl deficiency to initiate ccRCC in mice, the Hif1α−/−M3 transgenic model (Hif1α−/−M3 TRACK) where kidney-specific overexpression of a non-degradable as well as transcriptionally active mutant HIF1α−/−M3 (P402A, F564A, N803A) resulted in renal cysts and small clear cell tumors in aged (14–22 months) mice (Fu et al., 2011, 2015). Of note,
Figure 6. Vhl and Pbrm1 Doubly Deficient Clear Cell Kidney Tumors Display Hyperactive mTORC1 Signaling

(A) Heatmap of genes with significantly different expression in age-matched WT kidneys (n = 4) and Vhl\textsuperscript{F/F} Pbrm1\textsuperscript{F/F} Ksp-Cre tumors (n = 5) based on unsupervised hierarchical agglomerative clustering.

(B) The genes that were significantly, differentially expressed in Vhl\textsuperscript{F/F} Pbrm1\textsuperscript{F/F} Ksp-Cre T/N (false discovery rate [FDR] <0.05) were tested for enrichment and represented using ClueGO.

(C) GSEA plots of the ranked list of differentially expressed genes in Vhl\textsuperscript{F/F} Pbrm1\textsuperscript{F/F} Ksp-Cre kidney tumors (T) and WT normal kidneys (N) generated using three gene sets: curated HIF targets, KEGG JAK STAT signaling pathway, KEGG oxidative phosphorylation pathway, and KEGG mTOR Pathway enrichment.

(D) Immunohistochemistry of phosphorylated-4E-BP1 (p4E-BP1) at threonine 37/46 (column 1), phosphorylated S6K (pS6K) at serine 240/244 (column 2), and phosphorylated ERK1/2 (pERK) at threonine 202/tyrosine 204 in Vhl\textsuperscript{F/F} Pbrm1\textsuperscript{F/F} Ksp-Cre tumors. T, tumor; N, adjacent normal. Scale bars are at 100 m or 200 m as indicated.

(E and F) The mRNA levels of Ddit4 (E, top left), Ldha (E, top right), Hk2 (E, bottom left), Glut1 (E, bottom right), Tsc1 (F, left), and Tsc2 (F, right) in Vhl\textsuperscript{F/F} Pbrm1\textsuperscript{F/F} Ksp-Cre tumors (n = 6) and WT kidneys (n = 4) were assessed by qRT-PCR. Data were normalized against GAPDH (mean ± SD). *p < 0.05; **p < 0.005; ***p < 0.0005 (Student’s t test).
**A** HIF Targets, JAK/STAT Signaling, Oxidative Phosphorylation, mTOR Signaling

**B**
- VHL
- PBRM1
- Ksp-Cre T/N
- HIF1α-M3 TRACK
- 038 (C)
- 1772 (E)
- 2252 (F)
- 11850 (G)

**C**
- Focal Adhesion (p = 2.2E-8)
- HIF1 Signaling (p = 2.6E-5)
- mTOR Signaling (p = 4.2E-4)

**D**
- HIF Targets
- JAK/STAT Signaling
- Oxidative Phosphorylation
- mTOR Signaling

**E**
- HIF1/VEGF Signaling (p = 1.6E-4)
- mTOR Signaling (p = 1.3E-2)

**F**
- #2 PBRM1
- #1 VHL
- #3 mTORC1 activation

Legend:
- p > 0.1
- p = 0.05-0.1
- p = 0.005-0.05
- p = 0.0005-0.005
- p < 0.0005

(legend on next page)
Indeed, GSEA revealed upregulation of the mTOR signaling pathway were also seen in the JAK/STAT signaling pathways and downregulation of the transcriptomic data of the Hif1α mechanistically preferred node after the hyperactivation of efficiency alone is insufficient for kidney tumor initiation. The stabilization of HIF1α-mediated asparaginyl hydroxylation of HIFs prevents the recruitment of p300/CBP, which helps explain why VHL deficiency alone is insufficient for kidney tumor initiation.

To further determine whether mTORC1 activation might be a mechanistically preferred node after the hyperactivation of HIF1 during the pathogenesis of ccRCC, we resorted to the transcriptomic data of the Hif1α-M3 TRACK mouse model. Indeed, GSEA revealed upregulation of the mTOR signaling pathway (Figure 7A). As expected, upregulation of HIF1 and JAK/STAT signaling pathways and downregulation of the OXPHOS pathway were also seen in the Hif1α-M3 TRACK mouse model (Figure 7A). We further compared the transcriptomics of the Vhl<sup>F/F</sup>Pbrm1<sup>F/F</sup>Ksp-Cre tumors to those of the Hif1α-M3 TRACK mouse model (Figure 7B). Within the 2,430 differentially expressed genes shared between these two models, enrichment in the mTOR and HIF1 pathways was evident (Figures 7B and 7C; Table S5). The shared mTOR pathway aberration between these two different mouse ccRCC models supports the convergence on mTORC1 activation once HIF1 becomes hyperactive (Figures 7A–7C).

To determine whether this observation could be extended into human ccRCC bearing both VHL and PBRM1 mutations, we first compiled differentially expressed genes in human VHL- and PBRM1-mutated ccRCC from the TCGA-KIRC dataset. Consistent with the findings observed in mouse ccRCC, GSEA of these differentially expressed genes revealed upregulation of HIF1, JAK/STAT3, and mTOR pathways and the downregulation of OXPHOS pathway (Figure 7D). Next, we compared the differentially expressed genes identified in human VHL- and PBRM1-mutated ccRCC to those shared between the two mouse models, which resulted in the identification of 1,772 genes that were shared among these three ccRCC models (Figure 7B). Within this shared gene set, HIF1 and mTOR pathway genes were statistically enriched again (Figure 7E; Table S6). Taken together, our study favors a scenario in which a sequence of at least three distinct genetic/epigenetic events including the loss of VHL, the loss of PBRM1, and the subsequent activation of mTORC1 are required for the development of ccRCC (Figure 7F).

**DISCUSSION**

VHL is the most commonly mutated gene in human ccRCC and its mutation serves as the initial driver event in the pathogenesis of ccRCC (Linehan et al., 1995). However, genetic deletion of VHL in mice is insufficient to initiate kidney tumors (Haase et al., 2001; Kapitsinou and Haase, 2008), favoring the involvement of additional genetic/epigenetic events. Such events remained elusive till the discovery of additional 3p21 tumor suppressor genes commonly mutated in human ccRCC, i.e., PBRM1, SETD2, and BAP1 (Hakimi et al., 2013b). Although PBRM1 is the second most commonly mutated gene in human ccRCC, whether and how PBRM1 loss contributes to the pathogenesis of ccRCC are unknown. Through tissue-specific deletion of both Vhl and Pbrm1 (Vhl<sup>F/F</sup>Pbrm1<sup>F/F</sup>Ksp-Cre), we created a clear cell kidney cancer mouse model that recapitulates histopathological and molecular features of human ccRCC and elucidated how PBRM1 functions as a tumor suppressor in ccRCC.

The Vhl<sup>F/F</sup>Pbrm1<sup>F/F</sup>Ksp-Cre mice developed preneoplastic polycystic kidney lesions at ~6 months and multifocal ccRCC at ~10 months, suggesting that loss of Vhl and Pbrm1 in kidney predisposes to ccRCC. The human pan-cancer genomics identified SWI/SNF complexes as commonly mutated genes (~20%) across cancer types with preferential enrichment of individual mutations in specific cancer types (Helming et al., 2014; Kadoch et al., 2013; Marquez et al., 2015), which presents challenges and opportunities in broadening our knowledge on how chromatin remodeling ATPase complexes function as tumor suppressors. Among the SWI/SNF complexes, SMARCB1 (BAF47) is the best-characterized tumor suppressor that regulates cell cycle and antagonizes PRC2 complex (Helming et al., 2014). Expression profiling of 12-week-old Vhl<sup>F/F</sup>Pbrm1<sup>F/F</sup>Ksp-Cre renal cortices revealed the tumor suppressor role of PBRM1 in preventing the self-perpetuating over-amplification of the HIF1 pathway through limiting the intricate feed-forward interplay between HIF1 and STAT3 upon VHL loss (Demaria and Poli, 2012; Jung et al., 2005; Luo and Semenza, 2012). The unexpected tumor suppressor function of PBRM1 is analogous to an electrical resistor in preventing power overdrive, in which PBRM1 restrains the HIF1 and STAT3 transcription outputs from over-amplification upon the loss of VHL (Figure 7F).

The observation that clear cell kidney tumors occurred in Vhl<sup>F/F</sup>Pbrm1<sup>F/F</sup>Ksp-Cre mice after a long latency period...
suggests the involvement of additional signaling aberrations. Immunohistochemical and transcriptomic analyses demonstrated that activation of mTORC1 rather than the ERK pathway is the preferred third event. Ampole analyses support the importance of mTORC1 activation in the pathobiology of human ccRCC, e.g., mTORC1 pathway activation is prevalent in human ccRCC (Linehan et al., 2010; Robb et al., 2007) and mTOR inhibitors are standard of care in treating metastatic ccRCC (Voss et al., 2014; Wei and Hsieh, 2015). As REDD1, a key transcriptional target of HIF1, negatively regulates mTORC1 through activation of TSC1/TSC2 (Brugarolas et al., 2004; DeYoung et al., 2008), it is foreseeable that mTORC1 activation could be a bottleneck for tumors originating from a hyperactive HIF1 signaling. Consistent with this working hypothesis, both the Vhlf+/−Pbrm1+/−Ksp-Cre and the published HIF1α−M3 TRACK mouse models (Fu et al., 2011) developed ccRCC after a long latency period and showed convergence on mTORC1 activation (Figure 7F). Moreover, mTORC1 pathway activation was also observed in human ccRCC carrying mutations of VHL and PBRM1 (Figure 6E). Of note, multi-regional sequencing of a hereditary VHL syndrome patient also detected the mutations of PBRM1 and the convergence of mTORC1 pathway activation (Fisher et al., 2014). These findings are consistent with a recurrent oncogenic theme in which many oncogenes, such as c-MYC, BRAF, and DLL-fusions, activate both oncogenic signaling and intrinsic tumor-suppressor checkpoints (Liu et al., 2014; Lowe et al., 2004; Maertens et al., 2013). Therefore, abrogating the built-in intrinsic tumor suppressor activities of individual oncogenes is essential for tumorigenesis. It was recently reported that homozygous deletion of Vhl and Bap1 in mouse kidney resulted in early lethality (<1 month), and some mice (within a cohort of 7) carrying homozygous deletion of Vhl and heterozygous deletion of Bap1 developed tumor micronodules (0.25–1.8 mm) with unknown tumor incidence, transplantability, and molecular characteristics (Wang et al., 2014). Notably, complete BAP1 inactivation is observed in human ccRCC (Peña-Llopis et al., 2012). How heterozygous loss of Bap1 cooperates with Vhl loss to initiate kidney tumorigenesis in mice remains intriguing (Wang et al., 2014).

The suppression of OXPHOS genes observed in the Vhlf+/−Pbrm1+/−Ksp-Cre ccRCC model is consistent with the global metabolomics reported on ccRCC (Hakimi et al., 2016), which lends further support for the notion that kidney cancer is a metabolic disease that manifests with massive metabolic reprogramming (Hakimi et al., 2016; Linehan et al., 2010). Inhibitors of mTORC1, the key cellular complex integrating nutrient and growth factor signaling to promote anabolic metabolism, are convergent evolution of a given cancer type (Voss and Hsieh, 2016; Wei and Hsieh, 2015) that could have predictive values for selecting patients of a given cancer genotype with matched targeted therapies.

**EXPERIMENTAL PROCEDURES**

More detailed information is available in the Supplemental Experimental Procedures.

**Mice**

*Baf180−/−* mice were obtained from Dr. Wang Zhong (Wurster et al., 2012). Animal experiments were performed in accordance to the Institutional Animal Care and Use Committee (IACUC) at MSKCC.

**Mouse MRI**

Mouse MRI scans were carried out on either 200 or 300 MHz Bruker 4.7 T or 7 T Biospec scanners (Bruker Biospin MRI GmbH) equipped with 640 mT/m ID 115 mm and 300 mT/m ID 200 mm gradients, respectively (Resonance Research).

**RNA Isolation and Microarray Analysis**

Total RNA was isolated using TRIzol (Life Technologies) and cleaned up using Qiagen column DNase digestion. RNA samples were prepared from 3-month-old Ksp-Cre, Vhl−/−Ksp-Cre, Pbrm1+/−Ksp-Cre, and Vhl−/−Pbrm1−/− Ksp-Cre mice. Microarray was performed by Integrated Genomics Operation (IGO) at MSKCC.

**RNA-Seq and Analysis**

Total RNA was process by the IGO using TruSeq RNA Sample Prep kit according to the manufacturer’s recommendation. Gene ontology (GO) analysis of mouse microarray and RNA-seq data were performed with ClueGO.

**Motif Activity Analysis**

To analyze activities of transcription factor binding motifs (TFBM) using RNA-seq data, we used the Integrated System for Motif Activity Response Analysis (ISMARA).

**ACCESSION NUMBERS**

The accession numbers for the RNA-seq and array data reported in this paper are GEO: GSE83688 and GSE83597, respectively.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and seven tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2017.02.074.

**AUTHOR CONTRIBUTIONS**

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