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

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

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.
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 (Margareeas 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., 2008) 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 Pbrm1 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 et al., 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. Pbrm1+/− 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 Pbrm1+/− Ksp-Cre mice (Figures 1A and S1A). Among 53 Pbrm1+/− Ksp-Cre and 27 Pbrm1−/− Ksp-Cre (denoted as WT thereafter) mice examined, 18 (34%) Pbrm1+/− 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 Pbrm1+/− Ksp-Cre kidneys was at two to five times that of normal appearing kidneys (Figure 1E), whereas the creatinine of aged Pbrm1+/− Ksp-Cre and WT mice was comparable (Figure 1F).

**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), Pbrm1+/− 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 Vhl<sup>F/F</sup> Pbrm1<sup>F/F</sup> Ksp-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 Vhl<sup>F/F</sup> Ksp-Cre, but not in Pbrm1<sup>F/F</sup> Ksp-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. Consistent with human ccRCC originating from proximal tubule, our Vhl<sup>F/F</sup> Pbrm1<sup>F/F</sup> Ksp-Cre tumors resembled 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 Vhl<sup>F/F</sup> Pbrm1<sup>F/F</sup> Ksp-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 Vhl<sup>F/F</sup> Pbrm1<sup>F/F</sup> Ksp-Cre mouse tumors were stained positive for LTL but not THP (Figure 3C). CD45 staining did not detect increased lymphocyte infiltrate of these Vhl<sup>F/F</sup> Pbrm1<sup>F/F</sup> Ksp-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 tumors (Figure S4). Furthermore, the presence of high glycogen in these tumors was confirmed by PAS-D staining (Figure S4). Of note, our Vhl<sup>F/F</sup> Pbrm1<sup>F/F</sup> Ksp-Cre mouse tumors did not directly originate from cystic lesions (Figure S5A). Higher proliferation index (Ki-67 staining) was observed in these Vhl 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. Pbrm1<sup>F/F</sup> Ksp-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 Pbrm1<sup>F/F</sup> Ksp-Cre mice. cohorts of animals at 12 months of age on average were randomly selected for MRI scanning. ***p < 0.001 (Fischer’s exact). (C) Incidence of hydronephrosis in the Pbrm1<sup>F/F</sup> Ksp-Cre mice based on gender. (D) Location distribution of hydronephrosis in Pbrm1<sup>F/F</sup> Ksp-Cre mouse kidneys. (E) Kidney volume in WT and Pbrm1<sup>F/F</sup> Ksp-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 VhlF/F Pbrm1F/F 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 VhlF/F Pbrm1F/F Ksp-Cre mice.
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 subrenal capsules of 12 kidneys of 6 immunocompromised NOD/SCID/IL2R<sup>γδ</sup>null (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 Vhl and Pbrm1, but not single 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 (Bindea 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) motifs.
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, Vhf<F/F>Ksp-Cre, Pbrm1<1>F/Ksp-Cre, and Vhf<F/F>Pbrm1<1>F/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<sup>F/F</sup>Pbrm<sup>1<sup>F/F</sup>Cre</sup> to Vhl<sup>F/F</sup>Pmr<sup>1<sup>F/F</sup>Cre</sup> 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 summary, our data suggest that PBRM1, a SWI/SNF complex protein, could function like a transcriptional resistor to prevent self-perpetuating amplification of the HIF1 and STAT3 transcriptional outputs incurred by Vhl deficiency. To further interrogate this working hypothesis, qRT-PCR was performed on NIH 3T3 cells with knockout of Vhl, Pbrm1, or both using CRISPR-Cas9. Indeed, the loss of PBRM1 further enhances HIF1 and the STAT3 signaling that was primed upon the loss of VHL (Figure S6G).

**The Expression of OXPHOS Genes Is Markedly Downregulated in Vhl and Pbrm1 Doubly Deficient Mouse Kidneys**

In contrast to cluster I that encompasses upregulated genes in the Vhl<sup>F/F</sup>Pbrm<sup>1<sup>F/F</sup>Cre</sup> kidneys, cluster III mainly consists of significantly downregulated genes (Figure 5A) within which most enriched are OXPHOS genes (Figure 5B). The significant downregulation of nuclear-encoded OXPHOS genes was further confirmed by qRT-PCR of genes involved in different electrontransport chain complexes, including Ndufa2 (complex I), Sdhd (complex II), Cox5a (complex IV), and Atp4a (complex V) (Figures 5D, S6E, and S6F). HOMER analysis was performed to determine if specific transcription factors might have directly mediated such repression. However, we did not detect enrichment of any pertinent TFGBMs within the cluster III genes. It has been shown that HIF1 inhibits mitochondrial biogenesis and respiration through downregulation of PGC1β transcription in RCC4, a VHL-deficient human kidney cancer cell line (Zhang et al., 2007). However, neither gene expression profiling nor qRT-PCR detected significant downregulation of Pgc1b in Vhl<sup>F/F</sup>Pbrm<sup>1<sup>F/F</sup>Cre</sup> kidneys (Figure S6F). TCGA pan-cancer kidney cancer analysis on common human kidney cancer types, including Ndufa2 (complex I), Sdhd (complex II), Cox5a (complex IV), and Atp4a (complex V) (Figures 5D, S6E, and S6F). HOMER analysis was performed to determine if specific transcription factors might have directly mediated such repression. However, we did not detect enrichment of any pertinent TFGBMs within the cluster III genes. It has been shown that HIF1 inhibits mitochondrial biogenesis and respiration through downregulation of PGC1β transcription in RCC4, a VHL-deficient human kidney cancer cell line (Zhang et al., 2007). However, neither gene expression profiling nor qRT-PCR detected significant downregulation of Pgc1b in Vhl<sup>F/F</sup>Pbrm<sup>1<sup>F/F</sup>Cre</sup> kidneys (Figure S6F). TCGA pan-cancer kidney cancer analysis on common human kidney cancer types, including Ndufa2 (complex I), Sdhd (complex II), Cox5a (complex IV), and Atp4a (complex V) (Figures 5D, S6E, and S6F).

**Vhl and Pbrm1 Doubly Deficient Clear Cell Kidney Tumors Display Hyperactive mTORC1 Signaling**

The observation that clear cell kidney tumors occurred in Vhl<sup>F/F</sup>Pbrm<sup>1<sup>F/F</sup>Cre</sup> mice after a long latency period suggests the involvement of additional genetic and/or epigenetic events. To investigate whether transcriptional aberrations might be responsible, RNA sequencing (RNA-seq) was performed on Vhl<sup>F/F</sup>Pbrm<sup>1<sup>F/F</sup>Cre</sup> kidney tumors and age-matched WT renal cortices. The complex pathological changes observed in the aged Vhl<sup>F/F</sup>Pbrm<sup>1<sup>F/F</sup>Cre</sup> mouse kidneys precluded the isolation of adjacent normal renal cortices for comparison. Differentially expressed genes were identified (Table S3) and subjected to unsupervised hierarchical clustering analysis, which identified two clusters denoting tumors and normal controls (Figure 6A). These genes were analyzed and visualized with ClueGO to interrogate functionally grouped gene ontology and pathway networks (Figure 6B; Table S4). Gene Set Enrichment Analysis (GSEA) revealed upregulation of HIF1 and STAT3 pathways and downregulation of OXPHOS pathway in Vhl<sup>F/F</sup>Pbrm<sup>1<sup>F/F</sup>Cre</sup> tumors (Figure 6C), consistent with the findings observed in the 12-week-old Vhl<sup>F/F</sup>Pbrm<sup>1<sup>F/F</sup>Cre</sup> renal cortices (Figure 5B). Significantly, downregulation of the mTOR signaling pathway was shown in the Vhl and Pbrm1 doubly deficient tumors but not in the 12-week-old Vhl<sup>F/F</sup>Pbrm<sup>1<sup>F/F</sup>Cre</sup> renal cortices (Figures 5B, 6B, and 6C; Table S4). To validate RNA-sequencing findings, qRT-PCR focused on the HIF1 and mTOR pathway genes was performed (Figures 6E and 6F), which demonstrated consistent findings among gene expression analysis platforms. We also performed immunohistochemistry on Vhl<sup>F/F</sup>Pbrm<sup>1<sup>F/F</sup>Cre</sup> mouse tumors to assess HIF1 and mTOR signaling. A significant nuclear accumulation of HIF1, a weak nuclear accumulation of HIF2, and an expression of GLUT1, an HIF target, were detected in these tumors (Figure S7A). Furthermore, increased phosphorylation of 4E-BP1 and S6K, two well-established mTORC1 substrates, were detected in Vhl<sup>F/F</sup>Pbrm<sup>1<sup>F/F</sup>Cre</sup> tumors but not adjacent normal-looking kidney tissues (Figures 6D and S7B). In contrast, the phosphorylation of ERK was not increased in the Vhl and Pbrm1 doubly deficient kidney tumors (Figure 6D).

It is noteworthy that Ddit4 or Redd1, a transcriptional target of HIF1 (Brugarolas et al., 2004), was significantly upregulated in Vhl and Pbrm1 doubly deficient kidney tumors (Figure 6E). REDD1 is known to suppress mTORC1 activity by releasing 14-3-3 (Brugarolas et al., 2004; DeYoung et al., 2008). Several lines of clinical evidence support the importance of mTORC1 activation in the pathobiology of human 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 (Linehan et al., 2010; Robb et al., 2007). Induction of REDD1 by HIF1 may activate a tumor suppressor checkpoint that restrains the oncogenic potential of HIF1. 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<sup>α<sup>M3</sup></sup> transgenic model (Hif1α<sup>α<sup>M3</sup></sup> TRACK) where kidney-specific overexpression of a non-degradable as well as transcriptionally active mutant HIF1α<sup>α<sup>M3</sup></sup> (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<sup>F/F</sup>Pbrm1<sup>F/F</sup>Ksp-Cre tumors (n = 5) based on unsupervised hierarchical agglomerative clustering.

(B) The genes that were significantly, differentially expressed in Vhl<sup>F/F</sup>Pbrm1<sup>F/F</sup>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<sup>F/F</sup>Pbrm1<sup>F/F</sup>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<sup>F/F</sup>Pbrm1<sup>F/F</sup>Ksp-Cre tumors. T, tumor; N, adjacent normal. Scale bars are at 100 μm or 200 μm 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<sup>F/F</sup>Pbrm1<sup>F/F</sup>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).
Indeed, GSEA revealed upregulation of the mTOR signaling pathway during the pathogenesis of ccRCC, which further supports 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 (VhlF/F Pbrm1F/F 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.

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 (VhlF/F Pbrm1F/F 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 VhlF/F Pbrm1F/F Ksp-Cre mice developed proneoplastic 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 VhlF/F Pbrm1F/F 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 VhlF/F Pbrm1F/F 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. Amples evidences 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 Vhf^F/F Pbrm1^F/F 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 MLL-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 tumorogenesis. 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 (Pena-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 Vhf^F/F Pbrm1^F/F 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 standard of care in metastatic ccRCC, e.g., mTORC1 pathway activation is prevalent in human ccRCC (Voss and Hsieh, 2016). However, a wide range of clinical outcomes has been observed. Interestingly, genomic study of RECORD-3, a large clinical trial that randomized kidney cancer patients to either VEGFR or mTORC1 inhibitors, demonstrated that ccRCC with mutant PBRM1 associates with longer progression free survival (PFS) on everolimus, an mTORC1 inhibitor, at 12.8 months than those with wild-type PBRM1 at 5.5 months (Hsieh et al., 2015, 2017a). In parallel, the Vhf^F/F Pbrm1^F/F Ksp-Cre ccRCC and human VHL- and PBRM1-mutated ccRCC shared mTORC1 pathway aberration. Both clinical data and this study support a model in which mTORC1 activation constitutes the preferred third driver event during ccRCC tumorigenesis immediately after genetic inactivation of VHL and PBRM1 (Voss and Hsieh, 2016) (Figure 7F), which serves as an example of preferential pathway 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^F/F 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 64 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 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 affy 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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