Multiple tumor suppressors regulate a HIF-dependent negative feedback loop via ISGF3 in human clear cell renal cancer

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Abstract

Whereas VHL inactivation is a primary event in clear cell renal cell carcinoma (ccRCC), the precise mechanism(s) of how this interacts with the secondary mutations in tumor suppressor genes, including PBRM1, KDM5C/JARID1C, SETD2, and/or BAP1, remains unclear. Gene expression analyses reveal that VHL, PBRM1, or KDM5C share a common regulation of interferon response expression signature. Loss of HIF2α, PBRM1, or KDM5C in VHL-/-cells reduces the expression of interferon stimulated gene factor 3 (ISGF3), a transcription factor that regulates the interferon signature. Moreover, loss of SETD2 or BAP1 also reduces the ISGF3 level. Finally, ISGF3 is strongly tumor-suppressive in a xenograft model as its loss significantly enhances tumor growth. Conversely, reactivation of ISGF3 retards tumor growth by PBRM1-deficient ccRCC cells. Thus after VHL inactivation, HIF induces ISGF3, which is reversed by the loss of secondary tumor suppressors, suggesting that this is a key negative feedback loop in ccRCC.
**Introduction**

Kidney cancer is among the top ten cancers, both in incidence and mortality in both men and women. Inactivation of the *VHL* tumor suppressor gene is a causal event in the pathogenesis of clear cell Renal Cell Carcinoma (ccRCC), the most frequent subtype of kidney cancer. Approximately 70-80% of ccRCC are sporadic tumors that harbor biallelic inactivation of *VHL*. In the rare disease of hereditary kidney cancer, germline *VHL* mutation leads to early-onset bilateral kidney tumors. Biochemically, the protein product of the *VHL* tumor suppressor gene pVHL acts as the substrate recognition module of an E3 ubiquitin ligase complex. This complex targets the α subunits of the heterodimeric transcription factor Hypoxia-Inducible Factor (HIF) for poly-ubiquitylation and proteosomal degradation. When HIFα is hydroxylated on either of two prolyl residues by members of the EglN family (also called PHDs or HPHs) under normal oxygen tension, it is recognized by pVHL. Without pVHL, HIFα protein accumulates and activates the hypoxia response transcriptional program. This constitutively active HIF subsequently drives ccRCC tumorigenesis and tumor growth. Interestingly, HIF targets include both tumor-promoting and tumor-suppressive genes, but its overall activity is potently oncogenic.

Restoration of pVHL in *VHL*-/ ccRCC cells suppresses their ability to form tumors in immune-compromised mice, while stabilization of HIF2α overrides the effect of pVHL. Conversely, HIF2α suppression in *VHL*-/ ccRCC cells cripples their ability to form tumors. The current standard of care for metastatic ccRCC focuses on inhibiting the VEGF receptor, yet drug resistance eventually develops in most cases. Thus it is urgent to identify new drug targets that operate in a high percentage of RCC tumors, with the
expectation that the dual actions on VEGFR and a new target might prevent drug
resistance.

Most types of solid tumors harbor multiple, sometimes dozens of mutations, in cancer
genes to establish the hallmarks of cancer\(^7,\,8\). In addition to \(\text{VHL}\), several frequently
mutated genes were identified in sporadic ccRCC. Varela et al. reported that 41% of
ccRCC tumors had inactivating mutations in the \(\text{PBRM1}\) gene. PBRM1 is a specificity
subunit of the SWI/SNF chromatin-remodeling complex\(^9\). The high mutation rate of
\(\text{PBRM1}\) in ccRCC has been confirmed by multiple studies, together with mutations in
other genes such as \(\text{BAP1}, \text{SETD2}, \text{KDM5C/JARID1C}, \text{PTEN}\) and \(\text{UTX}\)\(^{10-14}\). However,
the mutation rates of the other genes are much lower than that of \(\text{PBRM1}\)\(^{15}\).

Multiple lines of evidence suggest that \(\text{PBRM1}\) is a key tumor suppressor. Its mutations
are predominantly inactivating in both alleles. PBRM1 suppression causes changes in
pathways regulating chromosome instability and cell proliferation\(^9\). Like \(\text{VHL}\) mutations,
many \(\text{PBRM1}\) mutations occur early in tumorigenesis, unlike the other secondary
mutations\(^{16}\). Recently a \(\text{PBRM1}\) germline mutation was reported to predispose patients
to ccRCC\(^{17}\). PBRM1 was also found to amplify a HIF signature\(^{18}\) and genetic ablation of
both \(\text{Vhl}\) and \(\text{Pbrm1}\) in mouse kidneys leads to ccRCC while single loss fails to do so\(^{19,\,20}\).

\(\text{KDM5C/JARID1C}\) is a histone demethylase that removes methyl groups from tri-
methylated lysine 4 on histone H3 (H3K4Me3). H3K4Me3 is a histone mark that is tightly
linked to actively transcribed genes\(^{21}\). \(\text{KDM5C}\) mutations occur in 3-7% of ccRCC tumors
\(^9,\,10,\,13,\,14\). Its mutations are mostly subclonal and happen later during tumor
development\(^{16,\,22}\). HIF increases KDM5C levels and activity, and the overall level of
H3K4Me3 is elevated when KDM5C is suppressed in VHL-defective kidney cancer cells. In addition, evidence suggests that HIF-induced KDM5C is tumor-suppressive and this constitutes a negative feedback loop in regard to tumor growth.

SETD2 is a histone-modifying enzyme that tri-methylates histone H3 at Lysine 36 (H3K36Me3). It is mutated in 10-15% of ccRCC tumors. It is also located on chromosome 3p like VHL and PBRM1. SETD2 mutations are subclonal in ccRCC tumors, and are associated with worse patient survival. SETD2 deficiency was reported to be associated with alternative splicing and transcriptional repression. Indeed, SETD2 mutations in ccRCC tumors are associated with changes in chromatin accessibility and DNA methylation or widespread RNA processing defects. Recently, SETD2 was shown to regulate interferon signaling by methylating STAT1, and to maintain mitosis and cytokinesis through methylating α-tubulin. The relevance of these SETD2 functions to tumor suppression in ccRCC remains to be elucidated.

Like KDM5C and SETD2, BAP1 is also a histone modifying enzyme. BAP1 is inactivated in 10-15% of ccRCC. The BAP1 gene is located on chromosome 3 at band 3p21, which is very close to the PBRM1 gene. As with VHL, PBRM1 and SETD2, biallelic inactivation of BAP1 via mutation and loss of heterozygosity fit a two-hit tumor suppressor profile. Germline mutations of BAP1 predispose patients to ccRCC, uveal melanomas, mesothelioma, lung adenocarcinoma, meningioma, breast carcinoma and paraganglioma. Loss of BAP1 is associated with worse patient survival in ccRCC and seems to be mutually exclusive with PBRM1 mutations. BAP1 protein was reported to form a complex with host cell factor-1 (HCF-1) and promotes DNA double-strand break repair. It also forms a complex with ASXL1 which is essential for its...
ability to deubiquitinate histone H2A on lysine 119, and this function regulates cell proliferation \textsuperscript{39}. Recently it was shown to regulate IP3R3-mediated Ca\textsuperscript{2+} to mitochondria to suppress cell transformation \textsuperscript{40}. How these functions contribute to its tumor suppressor role in ccRCC is unclear.

These secondary tumor suppressors each have their own distinct biological functions and are associated with different prognoses for the patients, and yet they all regulate chromatin biology. Thus we investigated whether they share a common tumor suppressor pathway in this study.
Results:

IFN-responsive genes are upregulated by PBRM1

To determine how the suppression of PBRM1 changes the transcriptome of ccRCC cells, we used shRNA to stably knock down the expression of PBRM1 in VHL+/+ and VHL-/- 786-O cells. Then we used Illumina HumanHT-12_V4 microarrays to compare gene expression between cells expressing a control shRNA (SCR) and PBRM1 shRNA-94. PBRM1 loss in VHL-/- cells elicited a higher number of transcriptional changes than in VHL+/+ cells (Fig. 1A). In addition, in VHL-/- cells, the transcriptional responses to PBRM1 depletion or VHL re-expression shared a group of gene targets (Fig. 1B). This is consistent with the previous observation that PBRM1 loss amplifies a HIF signature. Gene Ontology (GO) pathway analysis of the shared genes revealed that type I IFN
signaling pathway and response to virus are commonly affected.

**Figure 1. IFN-responsive genes were downregulated by VHL and upregulated by PBRM1.**

A) Venn diagram showing that PBRM1 loss had greater impact on transcriptome in VHL−/− cells than that in VHL+/+ cells; B) Venn diagram showing the overlap of genes downregulated by VHL and upregulated by PBRM1 in 786-O cells. P-values were calculated with hypogeometric probability. C) Enriched gene ontology (GO) analysis of genes regulated both by VHL and PBRM1. D) GSEA analysis of genes upregulated by PBRM1. E) Heatmap showing the expression of IFN-responsive genes after PBRM1 knockdown.

(Fig. 1C). Gene Sets Enrichment Analysis (GSEA) confirmed that the expression signature of interferon alpha response was negatively correlated with PBRM1 suppression (Fig. 1D). Gene expression analysis revealed the reduced expression of IFN-responsive genes after PBRM1 loss (Fig. 1E).
IFN-responsive genes are also maintained by KDM5C

KDM5C is a histone-modifying enzyme that is frequently mutated in ccRCC. In order to learn how the loss of KDM5C impacts the transcriptome of ccRCC cells, we stably knocked down the expression of KDM5C in VHL+/+ and VHL-/− 786-O cells. Then we compared gene expression between cells expressing a control shRNA (SCR) and KDM5C shRNA-60 with the methods described above. Similar to PBRM1 suppression, KDM5C loss in VHL-/− cells elicited greater transcriptional changes than that in VHL+/+ cells (Fig. 2A). We also observed that in VHL-/− cells, transcriptional...
responses to KDM5C depletion or VHL re-expression shared a group of gene targets (Fig. 2B). This is consistent with the previous observation that HIF activates KDM5C and this constitutes a negative feedback loop. GO pathway analysis, GSEA, and gene expression data confirmed that the type I IFN signaling pathway, the expression signature of interferon alpha response, and the IFN-responsive genes were impacted by KDM5C loss (Fig. 2C-E).

Suppression of KDM5C and PBRM1 have similar impacts on gene expression

Since the suppression of either PBRM1 or KDM5C in VHL-/− 786-O cells significantly impacted the type I IFN signaling pathway (Fig. 1B and 2B) and the gene lists largely overlapped (Fig. 1E and 2E), we compared the transcriptional responses to the loss of PBRM1 or KDM5C. The vast majority of the upregulated genes and downregulated genes after suppression of PBRM1 or KDM5C were shared (Fig. 3A). The GO pathway
Figure 3. Suppression of KDM5C or PBRM1 had similar impact on gene expression in ccRCC cells.

A) Venn diagrams showing the overlaps of genes whose expression are affected by PBRM1 or KDM5C losses in 786-O. The p-value significance of the overlap in a venn diagram was calculated using hypergeometric test in R; B) Enriched gene ontology (GO) analysis of genes regulated both by PBRM1 and KDM5C; C) Heat map of shared gene targets by PBRM1 and KDM5C; D-E) Examination of mRNA expression of individual genes by RT-qPCR in 786-O D) and Ren-02 E) after PBRM1 or KDM5C was suppressed by shRNA. The p-value significance in the bar graph was calculated with two-tailed student t test. *: p<0.05; **, p<0.01; ***: p<0.001.

analysis of the shared genes that are suppressed by PBRM1 or KDM5C did not reveal any significantly impacted pathway, but the shared induced genes showed that "response to virus" was significantly enriched (Fig. 3B). The heat map shows the commonly affected genes (Fig. 3C). To confirm that the transcriptional response was not
the result of the off-target effects of a single shRNA, we suppressed the expression of PBRM1 or KDM5C with two shRNA constructs each. Then we measured the expression of select IFN-responsive genes with RT-PCR, and found that the transcriptional changes in *IL6*, *ISG20*, *IL8*, *ARPC4*, *GAS6*, and *STMN3* were consistent (Fig. 3D). To ensure that our observation is not confined to just one cell line, we performed similar experiments in Ren-02 cells, another *VHL*-/- ccRCC cell line. Again, the suppression of PBRM1 or KDM5C with different shRNA constructs produced consistent transcriptional changes (Fig. 3E), suggesting that PBRM1 loss or KDM5C loss elicits similar transcriptional responses.

**PBRM1 interacts with KDM5C**

Since loss of PBRM1 or KDM5C elicited very similar transcriptional responses, we examined whether these two proteins exist in one complex. When both proteins are expressed in HEK293T cells, immunoprecipitation of either protein pulled down the other (Fig. 4A). In addition, over-expressed Flag-PBRM1 was able to immunoprecipitate endogenous KDM5C protein and other PBAF subunits (Fig. 4B). Flag-KDM5C was able to pull down endogenous PBRM1 along with BRG1 and BAF170, the catalytic and structural subunits of PBAF complex respectively (Fig. 4C). Interestingly, it did not significantly co-IP BRD7 or BAF57, unlike that of Flag-PBRM1 (Fig.4B), suggesting that Flag-KDM5C pulled down a partial PBAF complex. Moreover, the endogenous PBRM1 also pulled down the endogenous KDM5C in human embryonic kidney (HEK) 293T cells along with the other PBAF subunits (Fig.4D), suggesting that their interaction is not an artifact due to over-expression. PBRM1 truncation analysis suggested that its C-terminus, possibly the HMG domain, is important for KDM5C interaction (Fig. 4E). These results suggest that two proteins exist in one protein complex, and this could be the mechanism that they regulate the expression of similar target genes.
Figure 4. PBRM1 interacts with KDM5C.

A). The indicated plasmids were transfected into 293T cells. Immunoprecipitations were performed followed by immunoblots with the indicated antibodies. Flag-PBRM1 B) or Flag-KDM5C C) was transfected into HEK293T cells. Immunoprecipitations were performed followed by immunoblots with the indicated antibodies. D) HEK293T cell lysate was immunoprecipitated with indicated antibodies followed by immunoblots with the indicated antibodies. E) A schematic diagram of PBRM1 constructs used for KDM5C interaction analysis (top). The indicated plasmids were transfected into 293T cells. Immunoprecipitations were performed followed by immunoblots with the indicated antibodies (bottom).

VHL, PBRM1 and KDM5C share the regulation of ISGF3
Since loss of both PBRM1 and KDM5C triggered greater transcriptional responses in VHL-/- cells than that in VHL+/+ cells, and HIF transcriptionally interacts with PBRM1 and KDM5C, we wondered whether VHL-regulated genes overlapped with PBRM1 and KDM5C-regulated genes. Comparison of the transcriptional changes revealed that a group of 27 genes was suppressed by VHL but induced by PBRM1 or KDM5C (Fig. 5A), and these genes were the interferon-responsive genes (Fig. 5B). A GO pathway analysis showed that these shared genes belong to the type I interferon signaling pathway (Fig. 5C).

Close inspection of these genes suggested that they are the transcriptional targets of interferon stimulated gene factor 3 (ISGF3). ISGF3 is a heterotrimeric transcription factor composed of STAT1, STAT2, and IRF9, and it can be activated either by interferon-induced posttranslational modifications or increased protein levels. Since these genes are suppressed by VHL, components of ISGF3 might be HIF targets. To test this, we suppressed HIF2α expression with two shRNA constructs in 786-O cells, then examined the expression of the ISGF3 components. After HIF2α suppression, the protein levels of IRF9 and STAT2 were reduced while the STAT1 protein level did not change (Fig. 5D). Conversely, transient expression of a stable and active form of HIF2α in VHL+/+ ccRCC cell lines, Caki-1 and Ren-01, slightly increased the expression levels of ISGF3 and its targets (Fig. 5-figure supplement 1). Suppression of HIF1α did not reduce ISGF3 protein level or function in RCC4 cells (Fig. 5-figure supplement 2). To test whether PBRM1 and KDM5C also had similar effects on ISGF3, we examined its components in 786-O cells with PBRM1 and KDM5C knocked down with various shRNA constructs. In PBRM1 knockdown cells, the protein levels of IRF9 and STAT2 were lower compared to control (Fig. 5D). In KDM5C knockdown cells, the protein level of STAT2 was lower (Fig.
5D). Consistent with this, the expression of ISGF3 target genes were significantly reduced in 786-O cells after PBRM1 or KDM5C suppression (Fig. 5E).

To ensure that this observation is not confined to just one ccRCC cell line, we knocked down HIF2α, PBRM1 or KDM5C in another VHL-deficient ccRCC cell line, Ren-02. After HIF2α suppression, all three subunits of ISGF3 showed reduced expression (Fig. 5F). After PBRM1 or KDM5C suppression, the protein levels of IRF9 and STAT2 dropped (Fig. 5F). Consistent with this, the expression of ISGF3 target genes was significantly reduced in Ren-02 cells after PBRM1 or KDM5C suppression (Fig. 5-figure supplement 3).
Figure 5. HIF, PBRM1 and KDM5C are required for the expression and activity of ISGF3.
A Venn diagram showing the overlaps of genes whose expression was affected by VHL, PBRM1 or KDM5C losses in 786-O cells. The p-value significance of the overlap in a venn diagram was calculated using hypergeometric test in R; B) Heat map of shared gene targets by VHL, PBRM1 and KDM5C; C) Enriched gene ontology (GO) analysis of genes regulated both by VHL, PBRM1 and KDM5C; D) Western blots with the indicated antibodies in 786-O cell SDS-solubilized whole cell lysates with HIF2α, PBRM1 or KDM5C suppressed by shRNA; E) Examination of mRNA expression of individual genes by RT-qPCR in 786-O cells after PBRM1 or KDM5C suppression by shRNA; F) Western blots with the indicated antibodies in Ren-02 cell SDS-solubilized whole cell lysates with HIF2α, PBRM1 or KDM5C suppressed by shRNA. The p-value significance in the bar graph was calculated with two-tailed student t test. *: p<0.05; **, p<0.01; ***: p<0.001.

Figure 5-figure supplement 1. Over-expression of HIF2α in VHL+/+ ccRCC cells increases ISGF3 levels and activity.

Empty vector or HA-HIF2αdpa (a stable and constitutively active HIF2α mutant) was transiently transfected into VHL-proficient Caki-1 or Ren-01 ccRCC cells. The SDS cellular lysates were subjected to SDS-PAGE then blotted with the indicated antibodies.
Figure 5-figure supplement 2. Suppression of HIF1α does not reduce ISGF3 protein level or function in RCC4 cells. HIF1α was stably suppressed by shRNA constructs in RCC4 cells and the lysates were immunoblotted with indicated antibodies.
Figure 5-figure supplement 3. The reduced expression of ISGF3 targets in Ren-02 cells after suppression of PBRM1 or KDM5C.

Examination of mRNA expression of individual genes by RT-qPCR in Ren-02 cells after PBRM1 or KDM5C suppression by shRNA. The p-value significance in the bar graph was calculated with two-tailed student t test. *: p<0.05; **, p<0.01; ***: p<0.001.
The ISGF3 is mostly unphosphorylated in 786-O ccRCC cells.

786-O cells expressing SCR, PBRM1-sh94 A) or KDM5C-sh60 B) were mock treated or treated with interferon alpha for two hours. The SDS cellular lysates were subjected to SDS-PAGE then blotted with the indicated antibodies.

ISGF3 can be activated by either interferon-induced phosphorylation on STAT1 and STAT2 or increased unphosphorylated protein levels \(^{41, 42}\). To determine the phosphorylation status of ISGF3, we used interferon \(\alpha\) to stimulate 786-O cells with or without PBRM1 (Figure 5-figure supplement 4A) or KDM5C (Figure 5-figure supplement 4B) knocked down and found that the ISGF3 was mostly unphosphorylated. This suggests that the elevated protein levels of ISGF3 components are responsible for the enhanced activity of ISGF3.

**SETD2 and BAP1 also regulate the expression and function of ISGF3**

A recent report suggested that SETD2 promotes ISGF3 activity through methylating STAT1 after interferon stimulation \(^{29}\). This prompted us to examine whether the
remaining major tumor suppressors in ccRCC, SETD2 and BAP1, also regulate ISGF3 in VHL-/-ccRCC cells. We knocked down the expression of SETD2 and BAP1 with shRNA constructs in Ren-02 cells and observed significant down-regulation of IRF9 and STAT2 (Fig. 6A, and Fig. 6-figure supplement 1). In addition, we observed reduced expression of PLSCR1 and MX1, two ISGF3 targets. BAP1 re-expression in a Ren-02 BAP1 knockdown (Fig. 6B, left) or knockout clone (Fig. 6B, right) generated by CRISPR/CAS9 increased the protein levels of STAT2, IRF9 and PLSCR1, suggesting that BAP1’s impact on ISGF3 is specific. To ensure that this effect is not confined to one cell line, we knocked down the expression of BAP1 or SETD2 in RCC4 and BAP1 in A498 cells (A498 has an inactivating mutation in SETD2 so it is not suitable for SETD2 analysis), two additional VHL-deficient ccRCC cell lines. Their suppression also led to the reduced expression of IRF9, STAT2, PLSCR1 and MX1 (Fig. 6C and D). Again, the impact of BAP1 loss in A498 cells was reversed by BAP1 re-expression (Fig. 6E). In order to further examine the impact of BAP1 on ISGF3 expression and activity, we expressed GFP, wild type BAP1, or BAP1 with tumor-derived mutations (N78S or G185R) in BAP1 null ccRCC cell lines UMRC2 and UMRC6. Expression of wild type or N78S BAP1 clearly increased ISGF3 protein levels and its activity in these cells, while the G185R mutant failed to do so (Fig. 6F and G). Thus SETD2 and BAP1 also are required to maintain the expression and function of ISGF3 in multiple VHL-deficient ccRCC cell lines. As a ccRCC tumor-derived mutation in BAP1 disrupted this function in both BAP1-null cells, it suggests that the regulation of ISGF3 is important to BAP1’s tumor suppressor function.
Figure 6. BAP1 and SETD2 are required for the expression and activity of ISGF3.

SDS-solubilized whole cell lysates of Ren-02 cells expressing control shRNA or shRNA against BAP1 or SETD2 were blotted with the indicated antibodies. B) Control vector expressing GFP or BAP1 were stably transfected into Ren-02 cells expressing BAP1-sh74 (left). The BAP1 plasmid carries silent mutations against BAP1-sh74 to cancel the effect of shRNA. Control vector expressing GFP or BAP1 were transiently transfected into a Ren-02 clone with BAP1 knocked out by CRISPR/CAS9 (right). The SDS-solubilized whole cell lysates were blotted with the indicated antibodies. C-D) SDS-solubilized whole cell lysates of RCC4 cells C) or A498 cells D) expressing the indicated shRNA constructs were blotted with the indicated antibodies. The knockdown
efficiency of SETD2 was measured with RT-PCR in panels A and C. E). Control vector expressing GFP or BAP1 with silent mutations were stably transfected into A498 cells expressing BAP1-sh74, BAP1-null UMRC2 F) or UMRC6 G) ccRCC cells were stably transfected with indicated plasmids. The SDS-solubilized whole cell lysates were blotted with the indicated antibodies.

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**Figure 6-figure supplement 1. Suppression of SETD2 expression by western blot.** Lysates from Ren-02 cells stably expressed indicated shRNA constructs were immunoblotted with indicated antibodies.

**ISGF3 is tumor suppressive in a xenograft model**

ISGF3 is known to be involved in the DNA damage response, response to immunotherapies and, canonically, the response to viral infection \(^{42}\). There is no report showing its direct involvement in regulating tumorigenesis or tumor growth. Since ISGF3 is activated by HIF and suppressed after the loss of PBRM1, KDM5C, SETD2 or BAP1, it may function as the executioner of a negative feedback loop that potently opposes tumor growth. To test this, we first confirmed that IRF9-sh69 shRNA construct successfully downregulated IRF9 expression (**Fig. 7A**). Suppression of IRF9 did not significantly change cell growth in culture (**Fig. 7B**). We injected the same number of 786-O cells expressing control shRNA (SCR) or IRF-sh69 into the flanks of nude mice. The 786-O cells with IRF9 suppression produced much larger tumors than the control cells (**Fig. 7C and D**). Knockdown of IRF9 in Ren-02 cells also significantly boosted...
tumor growth in a xenograft model (Fig. 7-figure supplement 1), IRF9 suppression did not reduce the expression of other subunits of ISGF3 (Fig. 7-figure supplement 2A), and IRF9 suppression was maintained in the endpoint tumors (Fig. 7-figure supplement 2B). Furthermore, we stably suppressed the expression of either STAT1 or STAT2 in 786-O cells then performed xenograft analysis. In both cases, the tumors were significantly bigger than the ones made by the control cells (Fig. 7-figure supplement 3). These data strongly suggest that ISGF3 has potent anti-tumor activity in vivo.

In order to learn the reason for enhanced tumor growth after IRF9 knockdown, we performed H&E and immunohistochemistry (IHC) staining on five pairs of 786-O SCR and IRF9-69 tumors. The control tumors contained higher percentages of mouse stromal cells, while the IRF9 knockdown tumors were mostly comprised of cancer cells (Fig. 7E and Fig. 7-figure supplement 4). The cancer cells in tumors with IRF9 knockdown had a slight but insignificant increase in Ki67 staining, a marker for cell proliferation, over cancer cells in control tumors (Fig. 7E). However, CD45 staining, a marker for all mouse immune cells, was significantly higher in control tumors compared to IRF9 knockdown tumors (Fig. 7E, p =0.007). There were no significant differences in cleaved caspase 3 or CD31, markers for apoptosis or blood vessels, respectively (Fig. 7-figure supplement 4). Thus the lack of infiltrating host immune cells into the tumors formed by ccRCC cells depleted of IRF9, not changes in cell proliferation, apoptosis, or blood vessel growth, might be the primary reason of enhanced growth.
Figure 7. Suppression of IRF9, a key component of ISGF3, led to enhanced tumor growth and significantly less tumor-infiltrating immune cells.
A) SDS-solubilized whole cell lysates of 786-O cells expressing control shRNA or shRNA against IRF9 were blotted with indicated antibodies; B) The growth rate of the indicated cell lines measured by WST-1 assay; C) Pictures of mice and xenografted tumors and D) Quantification of tumor weights originating from same number of cancer cells described in A) in athymic nude mice. The p-value was calculated with two-tailed student t test; E) H&E or immunohistochemistry stains of slides from the representative pair 5 tumors. The percentages of CD45- or Ki67-positive cancer cells in the tumors were quantified with analytic programs in Aperio software. The p-values were calculated with Mann Whitney-Wilcoxon.
Figure 7-figure supplement 1. Suppression of IRF9 significantly enhanced tumor growth by Ren-02 cells.

VHL-deficient ccRCC cells Ren-02 were stably infected with SCR or IRF9-sh69. A) Pictures of mice and xenografted tumors and B) Quantification of tumor weights originating from same number of cancer cells in athymic nude mice.

Figure 7-figure supplement 2. Stable suppression of IRF9 in ccRCC cells and xenograft tumors. A) The IRF9 was suppressed by shRNA in 786-O or Ren-02 cells. The lysates were examined with indicated antibodies. B) Lysates were made from two pairs of tumors described in figure 7C (derived from 786-O cells) and immunoblotted with indicated antibodies.
Figure 7-figure supplement 3. Suppression of STAT1 or STAT2 promotes tumor growth.

STAT1 A) and STA2 B) were stably suppressed by shRNA in 786-O cells. Control cells and STAT1 or STAT2-suppressed cells were subjected to xenograft analysis. C) and E) Pictures of mice and xenografted tumors. D) and F) Quantification of tumor weights originating from same number of cancer cells in nude mice.
IRF9 loss does not lead to significant changes in apoptosis or blood vessel density.
Representative 786-O xenograft tumors expressing SCR or IRF9-sh69 were subjected to H&E or immunohistochemistry analyses with indicated antibodies.

Over-expression of ISGF3 in PBRM1-deficient ccRCC cells strongly suppresses tumor growth

Suppression of IRF9 in 786-O or Ren-02 cells greatly enhanced tumor growth (Fig. 7 and Fig. 7-figure supplement 1). As PBRM1 suppression led to reduced ISGF3 expression and activity (Fig. 5) and enhanced tumor growth (data not shown), we asked whether ISGF3 over-expression reverses the enhanced tumor growth elicited by PBRM1 deficiency. To this end, we stably infected 786-O PBRM1-sh94 cells with different combinations of viral vectors expressing STAT1, STAT2 or IRF9. The combination of STAT2+IRF9 or all three factors strongly induced ISGF3 targets PLSCR1 and MX1 (Fig. 8A) and we chose the cells expressing STAT2+IRF9 for further analysis. The over-expression of STAT2+IRF9 did not change cell growth in culture (Fig. 8B), however, overexpression of these ISGF3 components strongly suppressed tumor growth by PBRM1-deficient 786-O cells in a xenograft model (Fig. 8C and D). Thus depletion of ISGF3 greatly enhanced tumor growth while its activation very potently blocked it, suggesting that ISGF3 is a central player in ccRCC tumor growth that is targeted by most of the cancer genes.
Figure 8. Over-expression of IRF9 and STAT2 restored ISGF3 function and retarded tumor growth of 786-O cells with PBRM1 suppressed.

A) 786-O cells were infected with the indicated viral vectors. SDS-solubilized whole cell lysates were blotted with the indicated antibodies. B) Cellular growth of indicated cell lines measured by WST-1 assay; C) Pictures of mice and xenografted tumors and D) Quantification of tumor weights originating from same number of cancer cells expressing either an empty vector or STAT2 plus IRF9 in athymic nude mice. The p-value was calculated with two-tailed student t test.
IRF9 and STAT2 expression significantly correlated with the expression of PBRM1, SETD2, or BAP1 in human ccRCC tumors

To verify that the observations are relevant for human ccRCC, we analyzed TCGA ccRCC datasets to investigate possible connection between ISGF3 and the secondary tumor suppressors. However, we did not find consistent correlations between mRNA levels of BAP1, PBRM1, KDM5C, SETD2 and ISGF3 components in the whole tumors (data not shown). One possibility for these results is that the mRNA expression of ISGF3 components detected in TCGA is not primarily coming from cancer cells, but from other stromal cells which can distort the results. To investigate this, we analyzed the correlations between well-known immune and tumor cell markers (CD45 or PTPRC, CD3, CD8, etc and CDH1, EPCAM) with ISGF3 subunits within the TCGA dataset. We found that the mRNA expression of ISGF3 subunits in the TCGA dataset comes predominantly from immune cells as opposed to tumor cells (surrogated by EPCAM and E cadherin) (Fig. 9-figure supplement 1). We also sought to confirm this with immunohistochemistry (IHC). We found that immune cells had much higher protein expression of ISGF3 subunits STAT2 than the normal or cancerous human kidney cells. On the same slide, the immune cells in lymph node, spleen and lung tissues were stained strongly positive with antibody against STAT2, but the normal and cancerous kidney tissue stained negative or very weakly (Fig. 9-figure supplement 2A). Interestingly, in the lung tissue, the strong staining of STAT2 in the immune cells did not seem to significantly increase the staining intensity of the surrounding cells. Similar observation was made with IRF9 IHC (Fig. 9-figure supplement 2B). Thus immune cells have much higher expression of ISGF3 subunits STAT2 and IRF9 than that in kidney cancer cells, and this could disrupt the detection of ISGF3 mRNA expression in the cancer cells. Since our hypothesis is that BAP1, PBRM1, KDM5C, SETD2 are critical to maintain the expression and/or function of ISGF3 subunits in the ccRCC cancer cells,
we concluded that mRNA levels in the bulk tumors from the TCGA dataset are not suitable for our validation experiments.

To validate our hypothesis, we decided to examine the ISGF3 protein levels in ccRCC cancer cells with IHC and correlate their protein levels with the protein levels of BAP1, PBRM1, and SETD2. We have previously examined the protein levels of PBRM1 and SETD2 in a ccRCC tissue microarray (TMA) generated by our colleagues at Fox Chase Cancer Center in our previous publication\(^4\). The specificity of the BAP1 (Fig. 6A), STAT2 (Fig. 7-figure supplement 3) and IRF9 (Fig. 9-figure supplement 3A) antibodies used for IHC was validated by western blots. We probed the same TMA with antibodies against BAP1, IRF9 and STAT2. The staining of BAP1 or IRF9 mostly occurs in the nucleus, while STAT2 staining is found in both cytoplasm and nucleus (Fig. 9-figure supplement 3B). In many cases the protein expression levels of PBRM1, SETD2 or BAP1 correlated with that of STAT2 or IRF9 on the same tumor samples (Fig.9A).

After scoring the samples and performing statistical analysis, the Spearman association analysis revealed that nuclear IRF9 and cytoplasmic STAT2 both significantly correlated with the expression of PBRM1, SETD2, or BAP1(Fig.9B). The correlations were all statistically significant when all foci were considered. In individual tumor stage or grade, the associations were mostly statistically significant as well. Interestingly, the loss of nuclear staining of IRF9 was associated with worse patient survival, consistent with a tumor suppressor role of ISGF3 (Fig.9C). The loss of cytoplasmic STAT2 staining was not associated with worse patient survival (Fig.9D), suggesting that other ISGF3 components are the more dominant players in the human ccRCC. Consistent with this idea, the change of IRF9 protein in the TMA (Fig.9B) and in the cultured cells (Figs. 5 and 6) tracked better with the changes of PBRM1, SETD2 or BAP1. Taken together, our
data suggests that the links between PBRM1, SETD2 or BAP1 and ISGF3 are preserved in human ccRCC tumors.

Figure 9. IRF9 and STAT2 expression significantly correlated with the expression of PBRM1, SETD2, or BAP1 in ccRCC tumors. A) 20x IHC images of representative ccRCC foci stained with indicated antibodies. B) Spearman correlation coefficient, rho, and p-value of nuclear (nuc) IRF9 or cytoplasmic (cyto) IHC signals with those of PBRM1, SETD2 or BAP1. The analysis was performed with either all the foci or the foci within each tumor stage or tumor grade. C) Kaplan-Meier curves of patient overall survival based on the positive or negative IHC signal of nuclear IRF9 and D) cytoplasmic STAT2. Statistical significance calculated using the log-rank test. E) A schematic diagram showing how HIF and secondary tumor suppressors converge on ISGF3 to regulate tumor growth in ccRCC. HIF activates ISGF3 that suppresses tumor growth. PBRM1, KDM5C, SETD2 and BAP1 all support the function of ISGF3. Mutations in any of them relieve the tumor suppressive function of HIF2α.
**Figure 9-figure supplement 1. ISGF3 subunits correlate positively with immune cell markers and negatively with cancer cell markers in the TCGA dataset.**

Correlations between IRF9, STAT1 or STAT2 with immune and tumor cell markers within the TCGA dataset. N = 534; PTPRC, CD45, CD247, T-Cell Receptor T3 Zeta Chain; CD3G, T-Cell Receptor T3 Gamma Chain; CDH1, E-cadherin.
Figure 9-figure supplement 2. STAT2 and IRF9 protein levels are significantly higher in immune cells than that in kidney cells. IHC images of STAT2 A) and IRF9 B) stained human tissue samples from kidney, renal cell carcinoma, lymph node, spleen, and lung. For each antibody, the top is the macroscopic image of the slide, and the bottom shows the 20x image of each tissue.
Figure 9-figure supplement 3. IHC staining of ccRCC tumors with BAP1, IRF9 or STAT2 antibodies. A) 786-O cells were infected with the indicated shRNA constructs and the lysates were immunoblotted with indicated antibodies. B) Representative images of foci from the TMA that were stained with indicated antibodies. Scoring: 2: >=50% of cancer cells stained positive; 1: 5-49% of cancer cells stained positive; 0: <5% of cancer cells stained positive.
**Discussion**

Although the overall activity of HIF is strongly oncogenic, our previous results indicate that it also triggers a significant anti-tumor response. For instance, KDM5C is induced by HIF, and its demethylase activity against global H4K4Me3 marks is dependent on HIF in VHL-/-ccRCC cells, and yet it clearly plays an anti-tumor role in ccRCC\(^2^3\). In addition, some other HIF downstream targets, such as VEGF or Cyclin D1, are strongly pro-tumor growth, whereas other HIF downstream targets such as GLUT1 or IGFBP3, are obviously tumor suppressive \(^4\). Consistent with this, VHL conditional knockout in mouse kidneys alone is not sufficient to generate ccRCC despite fully activated HIF. Only after combination with PBRM1 or BAP1 conditional knockout can VHL loss lead to kidney tumors\(^1^9,^2^0\). Thus HIF could activate ISGF3, which constitutes a negative feedback loop in ccRCC. Its inhibitive effect on tumor growth can be relieved by the loss of PBRM1, KDM5C, SETD2 or BAP1 to promote robust tumor growth (Fig. 9E).

ISGF3 is traditionally activated by interferon stimulation through phosphorylation of STAT1 and STAT2\(^4^2\). However, days after the initial activation, the posttranslational modifications (PTMs) disappear, yet ISGF3 can still maintain its activity as unphosphorylated ISGF3(U-ISGF3) with greatly enhanced protein levels \(^4^1\). U-ISGF3 activates fewer targets than phosphorylated ISGF3, but it still potently antagonizes viral infection and DNA damage. In oncology, ISGF3 is known to be a predictive biomarker for chemotherapy and radiation in breast cancer \(^4^4\), and it is also associated with drug-resistance to immune checkpoint blockade\(^4^5\). Recently, immune checkpoint inhibitors, specifically those that target Programmed Death 1 (PD-1), Programmed Death Ligand 1 (PD-L1), and Cytotoxic T-Lymphocyte Associated 4 (CTLA-4) have gained traction in metastatic RCC (mRCC). The FDA has granted priority review for ipilimumab/nivolumab...
in intermediate and poor-risk mRCC\textsuperscript{46}, with RCC experts declaring this combination the new standard of care in the treatment of mRCC\textsuperscript{47, 48}. Loss-of-function mutations in PBRM1 were discovered to confer clinical benefit to ccRCC patients treated with anti-PD-1 alone or in combination with anti-CTLA-4 therapies \textsuperscript{49}. In addition, PBRM1 mutations were found to enhance T-cell-mediated killing in an unbiased, high-throughput screen in a melanoma model\textsuperscript{50}. As PBRM1 and other ccRCC TSGs converge on ISGF3, it will be very interesting to examine whether ISGF3 status is a predictive biomarker for response to immunotherapy. The fact that PBRM1 mutations, but not those of BAP1, SETD2, and KDM5C, are a predictive biomarker to response to immunotherapy is likely because PBRM1 and BAP1 mutations are truncal (root change) in many cases while the mutations of the other two are subclonal\textsuperscript{16, 22}. BAP1 mutations might also elicit an additional change that cancels their immunotherapy-sensitizing effect.

cCRCC is known to harbor rampant intra-tumoral heterogeneity \textsuperscript{16, 22, 43, 51}. Inactivating mutations in PBRM1, SETD2, KDM5C and BAP1 tend to be focal in the tumors, so therapies that are designed to exploit the weakness induced by mutation of an individual gene could be problematic since it cannot treat the remaining cancer cells free of that mutation. Since the suppression of PBRM1, KDM5C, SETD2 or BAP1 all lead to the reduction of ISGF3, a potent tumor suppressor, it is possible that any effective therapeutic intervention that restores ISGF3 function could benefit ccRCC patients with a wide variety of mutations.

Pbrm1 loss in mice and PBRM1\textsuperscript{-/-} vs PBRM1 re-expressed human A704 cells showed that PBRM1 deficiency was linked to the activation of the JAK/STAT3 pathway \textsuperscript{19, 49}. STAT3 and ISGF3 are two different, often times opposing, pathways: ISGF3 is activated by type I IFN \textsuperscript{41}, while STAT3 is activated by the IL6 family, oncogenes, and growth
factors. They have very different gene targets. STAT3 is often considered an oncogene, while STAT1 is usually considered a TSG, and thus they appear to play opposing biological roles in tumorigenesis. In human T lymphoma cells or mouse cells, loss of STAT3 leads to prolonged activation of STAT1. In addition, STAT3 negatively regulates the type I IFN signaling pathway by inhibiting expression of IRF7, IRF9, STAT1 in diffuse large B cell lymphoma. Thus STAT3 and ISGF3 are distinct and activated STAT3 might suppress ISGF3 in ccRCC cells, and this deserves further investigation.

In a recent publication, SETD2 was shown to methylate STAT1 to promote ISGF3 function. However, this requirement of SETD2 is not observed unless IFN is added to activate ISGF3. In our system, no exogenous IFN was present, and HIF activation seemed to be sufficient to increase the protein levels of STAT2 and IRF9, thus it might activate U-ISGF3 (Fig. 5-figure supplement 1). In 786-O cells with or without PBRM1 or KDM5C suppression, the mRNA levels of STAT1, STAT2 and IRF9 mirror that of the proteins (data not shown), suggesting that the regulation of U-ISFG3 occurred at the transcriptional level. Interestingly, BAP1 loss reduced the protein levels of STAT2 and IRF9 independent of PBRM1 status, since RCC4 cells express no endogenous PBRM1 (Fig. 6C).

Why ISGF3 is a potent tumor suppressor in ccRCC tumors is currently not well understood. One possibility is that interferon stimulated genes (ISG) have anti-proliferative and pro-apoptotic function in ccRCC cancer cells. ISGs activated by IFN do have such activity to curb the spread of virus in the infected cells, and the U-ISGF3 was thought to be weaker in this regard with a much smaller target pool. However, the U-ISGF3 may still possess sufficient anti-proliferative/pro-apoptotic activity to impair the
fitness of the cancer cells. Our current evidence suggests that this does not apply in our model. Alternatively, U-ISGF3 may change how the cancer cells interact with the immune system. The nude mice used in our study do not have adaptive immune system but the innate immune response remains intact. The heightened U-ISGF3 may attract more innate immune cells such as macrophages and NK cells to attack the tumors and curb tumor growth, as a previous report showed that over-expression of IFN-β recruited macrophages which suppressed tumor growth and metastasis in nude mice 60. However, further studies to closely monitor the cancer and host cells are needed to provide a definitive answer to this question. It will be important to identify the mechanism of how ISGF3 curbs tumor growth in ccRCC with human tumor samples and immune-competent mouse models so we can exploit this pathway to treat ccRCC patients.

With our current preliminary data we think that PBRM1 and KDM5C regulate ISGF3 subunits at transcriptional level, while SETD2 and BAP1 regulate ISGF3 at post-translational modification level. They might use quite different mechanisms to achieve the same goal: maintaining the ISGF3 activity, so loss of any of them could promote tumor development. Further investigation into these mechanisms is needed.

It is highly surprising that so many major TSGs in ccRCC share the same target in ISGF3. Undoubtedly each TSG has its own unique tumor suppressing functions, as each carries different prognoses for the patients. KDM5C mutations were reported to occur in the cells with PBRM1 mutations61. It is possible that their unique tumor suppressor functions are synergistic so their mutations are selected in the same cells during tumor development. However, the fact that they all converge on ISGF3 warrants further investigation into how this pathway impacts drug response, patient survival, animal modeling and drug development.
### Materials and Methods

#### Key Resources Table

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### Gene Expression Analysis

Genome-wide gene transcription was profiled using Illumina HumanHT-12_V4 Expression Bead Chip. The raw gene expression data were preprocessed in Genome Studio (https://www.illumina.com/techniques/microarrays/array-data-analysis-experimental-design/genomestudio.html), and further processed using R limma package for force-positive background correction, log₂ transformation and quantile normalization. Data quality control was performed by selecting present probes using the
detection threshold of 0.05. Outlier samples were examined using hierarchical clustering and no samples were removed. The average value across the probesets of an annotated gene was used as the gene-level expression and the data matrix was exported for downstream analysis. Differential gene expression analyses were performed using limma package controlling for batch effects in a linear model. Gene set enrichment was performed using a preranked GSEA algorithm based on log2 fold change. GO analyses were performed using the DAVID online tools. For the GO analyses, Venn diagrams, and heatmaps, the cutoff of adjusted p-value less than 0.05 and fold change greater than 2 were applied to select genes. The statistical significance of the overlaps in the Venn diagrams was calculated based on hypergeometric distribution function – phyper implemented in R (https://www.r-project.org/). The gene expression values used in the heatmaps were preprocessed by ComBat to remove batch effects, and then centered by the mean values of each row. Source file: GEO accession GSE108229:


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**Analysis of TCGA dataset**

Gene expression for the TCGA dataset, Kidney renal cell carcinoma (KIRC), were obtained through the "cgdsr" R package. RSEM expression data were transformed into log2(RSEM+1). Transformed data were used for Pearson correlation and plotted with the "ggplot2" R package. Scripts available upon request.

**Analysis of TMA**

Association between different IHC targets were determined using Spearman correlation. Kaplan-Meier survival analyses were performed and plotted using "survival" and
“survminer” R packages\textsuperscript{69-71}. Statistical significance of survival curves was calculated using the log-rank test.

**Mutagenesis, subcloning and primers for RT-PCR:**

Mutagenesis was performed via the QuikChange method using phusion DNA polymerase (ThermoFisher). Primers were designed using Agilent’s QuikChange Primer Design program. The sequences are as follows: BAP1-N78S-F:

CTGGTGCGGAACAGACTGTTCACACTACATCATCATCAAT, BAP1-N78S-R:

GTGATTGATGATATTGAGTACATGTTCTTTGCCCACCAG, BAP1-G185R-F:

GACCTTCAGCCTATCCAGCTCAAAGAGCAG, BAP1-G185R-R:

CGGCTCTTTGAGCTGATGCTGAAGGTC. A temperature gradient was performed to ensure primer annealing during PCR cycling. Following DpnI digestion, DNA was transformed into XL10 Gold Ultracompetent Cells (Agilent). Plasmids were sequenced to confirm the point mutations.

Full-length PBRM1 was subcloned into p3xFlag-CMV10 with primers PBRM1-F:

CCCAAGCTTATGGGTTCCAAGAGAAGAAG and PBRM1-R:

GGAATTCTTAACATTTTCTAGGTGT. For 1-1297 and 1-1184 PBRM1 constructs, the following primers were used to introduce nonsense mutants through mutagenesis:

1297(3892C-T): 3892-F: CCAATTGTTCCTTAGAAGGAGCCAT; 3892-R:

ATGGCTCTTCTAAGGAACATTTG

1184 (3553C-T):3553-F: AAAGTAGGGGTTGAGATGAGAAGGT; 3553-R:

CAGCTCCATCTCAAACCCATTTT

Primers for real-time PCR

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Cell culture and viral infection

Kidney cancer cell lines 786-O and A498 were purchased from ATCC. Ren-02, RCC4 and HEK293T were described previously\textsuperscript{23,72}. UMRC2 and UMRC6 cells were a gift from Dr. Qing Zhang from University of North Carolina at Chapel Hill. All cell lines were maintained in 37°C incubator with 5% CO\textsubscript{2} in glutamine-containing DMEM medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin. Mycoplasma contamination is tested with a PCR-based kit. The cells will be treated with antibiotics to eradicate the mycoplasma if it is detected. The cell proliferation rate was measured with a WST-1 kit from Sigma Aldrich according to the manufacturer’s instruction.

The packaging of shRNA viral particles, the infection and selection, and the expression
of shRNA resistant plasmid were the same as described before.

The generation of Ren-02 BAP1 knockout (KO) clone

Ren-02 BAP1 knockout (KO) cells were generated using an inducible CRISPR-Cas9 system previously described. Briefly, HEK293T cells were transfected with 2μg pLenti-iCas9-Neo, 2μgΔR8.9 packaging vector, and 200ng VSV-G envelope vector using lipofectamine 2000. Lentiviral supernatants were filtered and used to infect Ren-02 cells with polybrene, and infected cells were selected with 1.0 mg/ml G418. The cells were then treated with 1.0 μg/ml doxycycline to induce Cas9 expression, and sorted by GFP expression using flow cytometry. Short guide RNA (sgRNA) sequences were designed to target BAP1 exon 5 using Optimized Crispr Design (crispr.mit.edu), using the following oligonucleotides: BAP1-exon5-F: CACCGACCACCTGAGTCGCATGA; BAP1-exon5-R: AAACTCATGCAGTCAGGGGTC. The sgRNAs were cloned into pLentiGuide-Puro as previously described. The pLentiGuide-Puro-sgRNA plasmids were transfected into HEK293T cells to generate lentivirus, which was used to infect Ren-02 iCas9 cells. Following selection with 2.0 μg/ml puromycin, the Ren-02 iCas9-sgRNA cells were treated with doxycycline, diluted, and screened to identify individual clones with BAP1 KO.

Western blot and immunoprecipitation

Cells were washed in PBS buffer, then lysed with EBC buffer (50 mM Tris, pH 8.0; 120 mM NaCl; 0.5% NP-40)supplemented with a protease inhibitor cocktail. In some cases 1% SDS solution was added to the cells in the plates instead of EBC buffer, then the solution was sonicated at 20 A for 3x15-second pulses and mixed with sample buffer. The same total amount of total protein was loaded and resolved by SDS–PAGE and analyzed by immunoblotting. The blots were developed with Super Signal Pico substrate
(Pierce Biotechnology, Rockford, IL) or Immobilon Western substrate (Millipore, Billerica, MA). Immunoprecipitations were performed with EBC lysates as previously described. The antibodies used in this manuscript are: HA (Covance MMS-101P-1000 HA.11 (16B12 for IB, Santa Cruz Biotech sc-805 for IP), Flag (Sigma F3165-1mg (M2)), KDM5C (Bethyl A301-034A and A301-035A), BAP1 (Santa Cruz Biotech sc-28383), α-Actin (Santa Cruz Biotech sc-8432), PBRM1 (Bethyl A301-591A and A301-590A), STAT1 (Cell signaling 9172S), phospho-STAT1 (Cell signaling 9171L), STAT2 (Bethyl A303-512A-T), phospho-STAT2 (Cell signaling 88410), IRF9 (Cell signaling 76684S), MX1 (R&D AF7946), PLSCR1 (Santa Cruz Biotech sc-59645), Vinculin (Santa Cruz Biotech sc-73614), GAPDH (Santa Cruz Biotech sc-59540), HIF2α (Novus NB100-122), OAS1 (Cell signaling 14498S).

Short hairpin RNAs (shRNAs)

All the shRNA constructs except for SCR were obtained from Sigma (St Louis, MO). The sequences were: SCR: GCGCGCUUUGUAGGAUUCGTT; HIF2a-17: CGACCTGAAGATTGAAGTGAT; HIF2a-20: CCATGAGGAGATTCGTGAGAA; PBRM1-94: CCGGAGTCTTTGATCTACAAA; PBRM1-890: CCGGAATGCCAGGCACTATA ; BAP1-71: CGTCCGTGATTTGATGATGGA, BAP1-74: CCACAATCGATGAGTTCAT; SETD2-30: CCTGAAGAATGATGAGATAAT; SETD2-32: GCCCTATGACTCTCTGGTTA; IRF9-69: GCCATACTCCACAGAATCTTA.

Nude mice xenograft analysis

All animal experiments were conducted in accordance with protocol 01462-935A approved by the IACUC of Thomas Jefferson University and protocol 2015-11286 approved by the IACUC of Yale University. The subcutaneous nude-mice xenograft
assay was performed as described. For each cell line, $10^7$ cells were injected subcutaneously into the flanks of immunocompromised male nu/nu nude mice of four weeks old purchased from Taconic or Jackson Laboratory. All mice were sacrificed by CO$_2$ inhalation 8 to 10 weeks after injection of cells, and tumors were excised and weighed. Results are reported as mean ± se. of the mean. Nine to ten pairs of tumors were compared. Results were statistically evaluated with Mann–Whitney U statistic analysis.

**Immunohistochemistry staining and image analysis**

4μm paraffin slides were deparaffinized in Shandon Varistain Gemini ES Autostainer. Antigen retrieval was performed with DAKO PTLink using Citrate Buffer (pH6.0) at 98°C for a total time of 20 minutes. Primary immunostaining was performed using antibodies against Ki67 (abcam, Cat#: ab16667, 1:200), Cleaved Caspase 3 (Cell-Signaling, cat#:9661, 1:500) and CD45 (BD Pharmingen, Cat#: 550539, 1:200), BAP1 (Santa Cruz Biotech, sc-28383, 1:50), STAT2 (Bethyl A303-512A, 1:25), IRF9 (Sigma, HPA001862, 1:50). Antibodies were incubated at room temperature for 30 minutes. Biotinylated anti-Rabbit or anti-Rat (Vector Laboratories, cat#: BA-1000 and BA-4001) secondary antibodies and ABC-HRP complexes (Vector Laboratories, Cat#: PK6100) were applied following the primary antibodies with 30 minutes incubation of each reagent at room temperature. Three TBST washes were performed between each step above. The signals were visualized using DAB substrate (DAKO, Cat#: K3468). Slides were then washed with DI water and further processed with Hematoxylin counter stain, dehydration and clearing in Shandon Varistain Gemini ES Autostainer and coverslipped with Permount Mounting Medium.
References


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