# PBRM1 and BAP1 as Novel Targets for Renal Cell Carcinoma

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**Abstract:** Technological advances in genome sequencing have led to the identification of novel driver genes mutated in renal cancer. Hitherto, 1 gene was known to be frequently mutated in renal cell carcinoma of clear cell type (ccRCC), the von Hippel-Lindau (*VHL*) gene. *VHL* was identified by positional cloning as the gene responsible for a familial syndrome with renal cancer predisposition, von Hippel-Lindau. Subsequently, *VHL* was found to be inactivated in approximately 90% of sporadic ccRCC. The discovery of *VHL*, together with the elucidation of its function, transformed the treatment of ccRCC leading to the introduction of 5 new drugs into the clinic. However, no other familial ccRCC predisposing genes are frequently mutated in sporadic ccRCC. With the development of massively parallel sequencing, a plethora of somatically mutated genes has been identified. Most genes are mutated at low frequencies, but 3 genes are mutated in more than 10% of ccRCC, *PBRM1* (mutated in ~50%), *BAP1* (~15%), and *SETD2* (~15%). Like *VHL*, all 3 genes are 2-hit tumor suppressor genes. Furthermore, these 3 genes are within a 50-Mb region on the short arm of chromosome 3p that encompasses *VHL* and is deleted in ~90% of ccRCC. We discovered that *PBRM1* mutations tend to anticorrelate with *BAP1* mutations in ccRCC and that *PBRM1*- and *BAP1*-mutated tumors exhibit different biology and are associated with markedly different outcomes. This established the foundation for the first molecular genetic classification of sporadic ccRCC. Herein, I review the evidence that implicated *PBRM1* and *BAP1* as renal cancer driver genes, provide an update on the function of the gene products, and speculate on how mutations in these genes may be exploited therapeutically.

**Key Words:** epigenetic therapy, BAF180, PBAF, SWI/SNF, nucleosome remodeling complex, polycomb, HCF-1, ARID1A, SMARCC1, SMARCB1, SMARCA4, BRG1, EZH2, Calypso, H2AK119ub1, mTOR, mTORC1

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In 1993, positional cloning efforts by W. M. Linehan and colleagues in kindreds with von Hippel-Lindau (VHL) syndrome led to the identification of the *VHL* gene.[1] The following year, the same group reported frequent *VHL* mutations in sporadic renal cell carcinoma of clear cell type (ccRCC).[2] *VHL* was found to encode the substrate recognition subunit of an E3 ubiquitin ligase complex that targets for degradation an essential subunit of hypoxia-inducible factor (HIF) transcription factors.[3] As a result, HIF is constitutively activated in ccRCC and induces a gene expression program facilitating adaptation to hypoxia that includes the vascular endothelial growth factor (VEGF). This discovery, which explained why ccRCCs are exquisitely vascularized, paved the way for the development of VEGF inhibitors in ccRCC. In 2013, 5 inhibitors are in the clinic, 1 targeting the ligand (bevacizumab) and 4 targeting VEGF receptors (sorafenib, sunitinib, pazopanib, and axitinib).[4–10] A second pathway has been implicated in renal cancer that governed by mechanistic (formerly mammalian) target of rapamycin complex 1 (mTORC1).[11] mTORC1 is a major regulator of cell growth,[12] and 2 mTORC1 inhibitors (temsirolimus and everolimus) have reached the clinic.[13,14] Thus, discoveries about the molecular genetics and biology of renal cancer have transformed the care of cancer patients.[15]

Despite the discovery of other RCC predisposing genes mutated in the germline,[16] no other genes were found to be frequently implicated in sporadic ccRCC until the advent of massively parallel sequencing technologies. Technological developments have enabled the discovery of a multitude of genes somatically mutated in ccRCC, including *PBRM1* (polybromo 1), *BAP1* (BRCA1 associated protein-1), and *SETD2* (SET domain-containing protein 2).[17–20] *VHL*, *PBRM1*, *BAP1*, and *SETD2* are all on chromosome 3p in a region that is deleted in more than 90% of ccRCCs. Thus, with a single deletion, renal cells lose 1 copy of 4 tumor suppressor genes. Given the high concentration of renal cancer tumor suppressor genes in a small region, it is surprising that ccRCC is not more common. Interestingly, there appear to be both positive and negative genetic interactions among these 2-hit tumor suppressor genes. Meta-analyses show that the frequency of *SETD2* mutations in ccRCC is twice as high in tumors with *PBRM1* mutations.[21] In contrast, *PBRM1* and *BAP1* mutations tend to be mutually exclusive.[18,21] Recent advances have illustrated the complexity of renal cancer genomes, with mutations that are ubiquitous, shared, and private.[22] Mutations acquired early during the process of tumorigenesis are ubiquitous, whereas those acquired at very late stages are private, with shared mutations in between. *VHL* and *PBRM1* mutations may be acquired early, whereas *BAP1* and *SETD2* mutations may occur later. In some ccRCC, there are different mutations in *SETD2*,[22] and this convergence may be explained by cooperation between *PBRM1* and SETD2.

These discoveries are impacting the clinic. The finding that *PBRM1* and *BAP1* mutations are largely exclusive and that tumors with these mutations exhibit different biology and are associated with markedly different overall survival (OS) set the foundation for the first molecular genetic classification of sporadic ccRCC.[18,23] Incorporation of these findings into clinical practice will be facilitated by the development of immunohistochemistry (IHC) assays, which are possible because most mutations in *PBRM1* and *BAP1* lead to loss of the protein.[18] As for *VHL*, insight into the mechanism of action of *PBRM1* and *BAP1* is likely to identify targets for therapy and further consolidate a classification of ccRCC based on driver mutations.

## PBRM1 in Renal Cancer

Exome sequencing of 7 ccRCCs and matched normal tissues by the Sanger Institute identified truncating mutations in

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*PBRM1* in 4 tumors.[17] Subsequent analyses of 221 ccRCCs revealed truncating mutations in 88 tumors. In addition, 2 in-frame deletions and 9 missense mutations were found, giving an overall mutation rate of 45%.[17] The *PBRM1* gene is located on chromosome 3p21, and in 38 tumors examined, *PBRM1* mutations were uniformly associated with loss of heterozygosity.[17] The majority of *PBRM1* mutations lead to loss of the protein.[18] *PBRM1* mutations occur most often together with *VHL* mutations,[17,18,24,25] and nearly all of the *PBRM1*-mutant tumors that Varela et al[17] examined (36/38) exhibited a hypoxia signature, including some cases without a detectable *VHL* mutation. These data suggest that *PBRM1* mutations are often associated with loss of *VHL*. Like *VHL*, *PBRM1* is rarely mutated in other tumor types (http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/).

*PBRM1* mutation in ccRCC was initially reported to portend aggressiveness. *PBRM1* mutations were thought to correlate with invasiveness,[25] and by IHC, loss of the *PBRM1* gene product was associated with advanced stage, high Fuhrman grade, and poor OS.[26] However, more recent reports suggest that *PBRM1* mutations are found in tumors at similar rates regardless of stage, and *PBRM1* mutations appear not to adversely impact patient survival.[23,24]

## PBRM1 Encodes BAF180, a Subunit of a Nucleosome Remodeling Complex

In eukaryotes, DNA is wrapped around nucleosomes—octameric complexes containing typically doublets of the canonical histone subunits (H2A, H2B, H3, and H4).[27] One hundred forty-seven base pairs of DNA are wrapped around each nucleosome. Wrapped DNA is less accessible for transcription (and other processes), and accessibility is controlled by nucleosome remodeling complexes that move, restructure, destabilize, and eject nucleosomes.[28] Currently, there are 4 families of remodelers with different functions: SWI/SNF, ISWI, CHD, and INO80.[28]

SWI/SNF complexes are organized around an ATPase subunit that provides energy for chromatin remodeling (BRM or BRG1) (Table 1).[29] The BRM subunit nucleates a complex referred to as the BAF complex, and the BRG1 subunit can be incorporated into either BAF or PBAF complexes (Table 1). BAF and PBAF complexes differ in several subunits, including the putative targeting subunits that are thought to dock the complex to specific chromatin regions. BAF250A and BAF250B are thought to target BAF complexes, and BAF200 and BAF180 (encoded by *PBRM1*) are believed to target PBAF complexes.

The BAF180 protein contains 6 tandem bromodomains that may be implicated in chromatin binding. Bromodomains are made up of ~100 amino acids and bind lysine residues modified by acetylation.[30] Histones have extensions (referred to as tails) with lysine (and other) residues that are enzymatically modified.[27] Bromodomains recognize acetylated lysine residues on histone tails, and in vitro, each bromodomain of BAF180 has a distinct pattern of affinity for acetylated peptides.[30–33] Overall, the affinity of each bromodomain for each peptide is low, and BAF180 may bind cooperatively to a precise pattern of acetylated lysine residues in nucleosomes.[33] This process would bring the PBAF complex to a specific region of DNA where nucleosome remodeling would occur.

In addition to bromodomains, BAF180 contains 2 bromo-adjacent homology (BAH) domains, possibly implicated in protein-protein interactions, and a high-mobility group (HMG) domain, which may bind nucleosomal DNA.[33] The importance of bromodomains and BAH domains for BAF180 tumor suppressor function is highlighted by their being targets of missense mutations in renal cancer.[17,18] Although these domains are highly structured, and missense mutations may inactivate BAF180 by disrupting folding and protein stability, there is precedent for a missense mutation in a bromodomain not affecting BAF180 levels.[18] Should other such mutations be found, the data would suggest that each bromodomain may be required for BAF180 tumor suppressor function.

**TABLE 1.** SWI/SNF Complex Proteins

| Gene | Subunit | Complex |
|---|---|---|
| | **ATPase** | |
| *SMARCA2* | BRM | BAF |
| *SMARCA4* | BRG1 | BAF/PBAF |
| | **Targeting** | |
| *ARID1A* | BAF250A | BAF |
| *ARID1B* | BAF250B | BAF |
| *ARID2* | BAF200 | PBAF |
| *PBRM1* | BAF180 | PBAF |
| | **Other** | |
| *SMARCC2* | BAF170 | BAF/PBAF |
| *SMARCC1* | BAF155 | BAF/PBAF |
| *SMARCD1* | BAF60A | BAF/PBAF |
| *SMARCD2* | BAF60B | BAF/PBAF |
| *SMARCD3* | BAF60C | BAF/PBAF |
| *SMARCE1* | BAF57 | BAF/PBAF |
| *ACTL6A* | BAF53A | BAF/PBAF |
| *ACTL6B* | BAF53B | BAF/PBAF |
| *SMARCB1* | BAF47 | BAF/PBAF |

SWI/SNF complexes are made up of multiple subunits (Table 1). Other subunits are also mutated in renal cancer, but BAF180 is mutated out of proportion (10-fold more frequently than any other subunit; http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/ and http://cancergenome.nih.gov/). However, other subunits of SWI/SNF complexes clearly function as tumor suppressor genes in other tumor types.[34] In addition, *ARID1A* and *ARID1B*, which encode the putative targeting subunits of the BAF complex, are also mutated in renal cancer (http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/ and http://cancergenome.nih.gov/). These mutations are not exclusive with *PBRM1* mutations,[17] suggesting that mutations in these genes may cooperate in tumorigenesis. Thus, both PBAF and BAF complexes may be implicated in ccRCC. In addition, the catalytic subunits, and in particular BRG1, which forms part of both the BAF and PBAF complexes, are targeted by mutation in ccRCC (http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/ and http://cancergenome.nih.gov/). Other subunits mutated in ccRCC include BAF170, BAF60A, and BAF47. It is unclear, however, why BAF180 is preferentially inactivated. The mutation of other subunits suggests that SWI/SNF function is important, but it is conceivable that BAF180 may be implicated in other processes.

How BAF180 functions as a tumor suppressor is not well understood. However, consistent with its being a component of a nucleosome remodeling complex, analyses of gene expression in ccRCC show that *PBRM1*-mutant tumors are associated with a characteristic gene expression signature.[23] A comparison of 66 *PBRM1*-mutated tumors with 242 tumors without mutations in *PBRM1* identified 2235 genes that, after a false discovery rate correction, distinguished these 2 groups.[23] When instead 66 tumors were chosen at random, fewer than 10 genes were found that distinguished this group from the rest.[23] The difference between the *PBRM1*-mutant group and 3 random groups of tumors was

highly significant ($P < 0.0001$). The PBRM1 signature was not sufficiently dominant so as to allow the distinction of *PBRM1*-mutated ccRCCs in unsupervised hierarchical clustering algorithms. Overall, these data suggest that BAF180 may act as a tumor suppressor as the targeting subunit of a nucleosome remodeling complex through its effects on DNA accessibility and gene expression.

Interestingly, *PBRM1* mutations tend to be mutually exclusive with mutations in *BAP1*.[18,21] These data suggest that loss of *PBRM1* may not be advantageous in every context. However, this context dependency is poorly understood. Conversely, mutations in *PBRM1* and *SETD2* co-occur in tumors at a frequency higher than expected by chance alone.[21] SETD2 trimethylates lysine 36 of histone H3 (H3K36me3). Interestingly, mutations in *SETD2* in tumors are associated with loss of DNA methylation at nonpromoter regions.[35] A greater understanding of the cooperativity between *PBRM1* and *SETD2* mutations may identify opportunities for therapeutic intervention.

*Pbrm1* is required for embryonic development in the mouse and *Pbrm1*−/− embryos die shortly after midgestation, between embryonic day 12.5 (E12.5) and E15.5.[36] *Pbrm1* is widely expressed, but defects in *Pbrm1*−/− embryos were appreciated only in the placenta and the heart. Cardiac defects were autonomous and not overcome by restoration of placental function.[36] *Pbrm1* is required for ventricular myometrium and coronary artery development.[37,38] The *Pbrm1* null phenotype differs from the knockout of the ATPase subunits, *Smarca2* and *Smarca4*, which are themselves very different. *Smarca2* (encoding Brm) is dispensable for embryonic development, and adult mice exhibit a modest weight increase.[39] In contrast, loss of *Smarca4* (encoding Brg1) causes peri-implantation lethality.[40] Several factors may explain the differences and specifically the mild phenotype of *Smarca2*−/− mice, including potential compensation by Brg1, which can integrate into both BAF and PBAF complexes, and which was up-regulated in *Smarca2*-deficient embryos.[39] Phenotypic differences in knockouts of different subunits suggest functional diversification.

It is unclear whether *Pbrm1*+/− mice are predisposed to tumors. *Smarca4*+/− mice develop mammary tumors, albeit at low penetrance.[41] Mice heterozygous for *Smarcb1* (encoding Baf47, also called Snf5) are also predisposed to tumorigenesis. *SMARCB1* is biallelically inactivated in human malignant rhabdoid tumors, and 15% to 30% of *Smarcb1*+/− mice develop rhabdoid tumors.[42] In addition, conditional biallelic inactivation of *Smarcb1* in lymphocytes causes lymphoma with 100% penetrance and a short latency.[42]

At a cellular level, *PBRM1* has been implicated in the regulation of the cell cycle and in replicative senescence. Reintroduction of *PBRM1* into a *PBRM1*-deficient tumor cell line induced a cell cycle arrest that was in part dependent on the p21 cyclin-dependent kinase inhibitor.[43] *PBRM1* was also recovered in a short hairpin RNA screen for genes than when knocked down extended the proliferative capacity of primary fibroblasts in culture.[44] *PBRM1* knockdown in 4/5 RCC cell lines increased proliferation, and the cell line with discrepant results was *PBRM1* deficient.[17]

## Therapeutic Implications of PBRM1 Loss in ccRCC

The development of therapies exploiting the loss of *PBRM1* in renal cancer will be facilitated by a greater understanding of the molecular mechanism of BAF180 tumor suppressor action. Interestingly however, there is a functionally antagonistic relationship between SWI/SNF and polycomb group proteins (PcG).[42] PcG proteins are implicated in maintaining lineage specification.[45] As cells divide, lineage identity is preserved through chromatin modifications that maintain a gene expression program. PcG proteins silence the expression of lineage-inappropriate genes.[45] In mammals, PcG proteins assemble into 2 main families, polycomb repressive complexes 1 (PRC1) and 2 (PRC2).[45] PRC1 is a histone H2A lysine 119 monoubiquitylase (H2AK119ub1), and PRC2 trimethylates lysine 27 of histone H3 (H3K27me3). Both modifications are repressive. A screen in *Drosophila* for suppressors of a PcG mutant identified the ortholog of BRM/BRG1 in flies, *brm*.[46,47] Heterozygous mutations (or deficiencies) in *brm* suppressed homeotic transformations (alterations in body patterning) induced by a polycomb loss-of-function mutation.[46,47] These data suggest that the derepression of gene expression induced by a polycomb gene mutation involves BRM/BRG1 and nucleosome remodeling.

The relationship between SWI/SNF and PRC2 appears to be bidirectional. Loss of *SMARCB1*, frequently observed in malignant rhabdoid tumors, results in PRC2-dependent repression of *p16*.[48] Reconstitution of *SMARCB1* into *SMARCB1*-mutant tumor cells leads to an SWI/SNF-dependent eviction of PRC1 and PRC2 complexes from the *p16* locus thereby restoring expression of the p16 tumor suppressor protein.[48] Furthermore, PRC2 appears to play a critical role in tumorigenesis following *Smarcb1* inactivation. Lymphoma development in mice with conditional biallelic inactivation of *Smarcb1* is suppressed by simultaneous inactivation of *Ezh2*, which encodes the catalytic subunit of PRC2.[49] Interestingly, *SMARCB1* is mutated in RCC, although at low frequency (~1%) (http://cancergenome.nih.gov/). These data raise the intriguing possibility that EZH2 inhibitors, which are in development, may be active against *SMARCB1*-mutant ccRCC. In addition, a link has been reported between EZH2 and DNA methyltransferases.[50]

Whether loss of *PBRM1* would similarly activate EZH2 and sensitize RCC to EZH2 inhibitors remains to be determined. The phenotypes of *Pbrm1* and *Smarcb1* knockout mice are very different, suggesting that the gene products exert divergent functions. In addition, the preferential mutation of *PBRM1* over *SMARCB1* in ccRCC further suggests that they are not equivalent. However, this could be explained, at least in part, by their chromosomal location; *PBRM1* is in a region on 3p that is frequently lost in ccRCC, whereas that is not the case for *SMARCB1*, which is on chromosome 22.

Of note, *EZH2* expression is up-regulated in RCC compared with paired normal renal tissues,[51] and *EZH2* is on chromosome 7q36.1, a region amplified in 13% of ccRCC.[52] Knockdown of EZH2 reduces cell proliferation[52] and causes apoptosis in a subset of RCC cell lines.[51,52] Given the low mutation frequency of *SMARCB1*, it is unlikely that *SMARCB1* was the determinant of sensitivity in these cell lines. Of note, 1 cell line that did not undergo apoptosis in response to EZH2 knockdown, 769-P,[51] is *BAP1* mutant.[18] EZH2 levels in ccRCC may have prognostic value, but these data are controversial.[53,54] In addition, it is speculated that the EZH2 mark (H3K27me3) may itself be prognostic.[55]

It is interesting that another gene encoding an SWI/SNF subunit is located on 3p, *SMARCC1*. The *SMARCC1* gene is on 3p21.31, between the *PBRM1* and *VHL* genes. Given its location, ~90% of ccRCCs would be expected to have a single *SMARCC1* allele and thus would be susceptible to the loss of the second *SMARCC1* allele. Assuming a fixed mutation rate per nucleotide, given the size of the coding sequence, by chance, *SMARCC1* would be expected to be mutated at 70% of the frequency of *PBRM1*. However, *SMARCC1* mutations in ccRCC are exceedingly rare (0 mutations in 459 ccRCCs sequenced; http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/). These data suggest that mutations

in *SMARCC1* and *PBRM1* are nonequivalent and raise the possibility that *SMARCC1* mutations would be detrimental for ccRCC. Consistent with this notion, *Smarcc1* is broadly expressed during development, and *Smarcc1* is implicated in the survival of embryonic stem cells.[56,57] In addition, BAF155 appears to be required for the stabilization of other SWI/SNF complex components,[58] and *SMARCC1* is very rarely mutated in other tumor types (http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/). The lack of mutations in *SMARCC1* suggest that BAF155 may be required for ccRCC survival. Interestingly, inactivation of another SWI/SNF gene, *Smarca4* (encoding Brg1), abrogates lymphomas arising from biallelic loss of *Smarcb1*.[59] Thus, inactivation of SWI/SNF complexes may be a viable approach for cancer therapy. SWI/SNF complexes may be broadly required for cell survival, but in the case of ccRCC with 3p deletions, the loss of 1 *SMARCC1* allele may sensitize tumor cells to this approach.

Finally, BAF180 has been reported to localize to structures linking chromosomes to the mitotic spindle during mitosis, kinetochores.[60] Although the function of BAF180 at the kinetochore is not well understood, mutations in kinetochore components may sensitize cells to microtubule-destabilizing drugs.

## BAP1 Discovery

BRCA1 associated protein-1 (BAP1) was identified in a yeast 2-hybrid screen for proteins that interacted with the RING finger of BRCA1.[61] The N-terminus had significant homology to the catalytic domain of a family of deubiquitinases, the ubiquitin C-terminal hydrolases (UCH), and BAP1 expressed in bacteria had deubiquitinase activity.[61] Two putative nuclear localization signals (NLS) were identified in the C-terminus, including one that was later validated,[62] and ectopically expressed BAP1 localized to the nucleus.[61] BAP1 was shown to interact with BRCA1 in overexpression studies,[61] but several subsequent studies have failed to identify BRCA1 among BAP1-interacting proteins. However, it is possible that BAP1 interacts with BRCA1 under a particular set of circumstances, such as in response to DNA damage.

BAP1 localizes to 3p21, a region frequently deleted in a variety of tumor types besides ccRCC. An analysis of multiple lung cancer cell lines identified a cell line with biallelic inactivation, suggesting that *BAP1* may be a 2-hit tumor suppressor gene.[61] Subsequently, BAP1 overexpression was shown to inhibit proliferation, and this effect was compromised by mutations disrupting catalytic activity or nuclear localization.[62] However, the inhibition of cell proliferation by BAP1 is cell-type specific.[63–65] In NCI-H226, where BAP1 suppresses cell proliferation, ectopic *BAP1* expression also inhibited tumor formation in xenografts.[62]

## *BAP1* Mutations in Cancer

*BAP1* mutations in tumors were first identified in a search for metastasis-related genes in uveal melanoma (UM).[66] Loss of chromosome 3 is the most important cytogenetic predictor of UM metastases, and Harbour et al[66] embarked on exome sequencing of 2 metastatic UMs with chromosome 3 loss. Interestingly, only 1 gene was mutated in both samples, *BAP1*. *BAP1* sequencing of a larger UM cohort showed *BAP1* mutations in 84% of metastasizing UM but in 4% of non-metastasizing tumors.[66] Most *BAP1* mutations were truncating mutations, and *BAP1* was biallelically inactivated. In non-metastasizing tumors, BAP1 localized to the nucleus, and BAP1 knockdown in a UM cell line caused epithelioid changes and cell rounding, reproducing features observed in metastasizing UM.[66]

A year later, *BAP1* mutations were reported in 23% of malignant pleural mesotheliomas (MPMs).[67] Despite no appreciable *BAP1* mutation, an additional 25% of MPMs exhibited BAP1 loss by IHC.[67] Correlative studies with other genes mutated in mesothelioma (*p16* and *NF2*) failed to reveal a relationship. However, other investigators have postulated an inverse relationship between *BAP1* and *NF2* mutations.[68] Interestingly, *BAP1* mutations appear to occur more frequently in the epithelioid subtype.[69] Epithelioid MPMs tend to have better outcomes than sarcomatoid and biphasic MPMs, but no correlation was observed between *BAP1* mutation and OS in MPM.[67]

## BAP1 in Renal Cancer

Guo et al[20] performed whole-exome sequencing in 10 ccRCCs and subsequently analyzed more than 1000 genes across 88 matched tumor/normal pairs. They reported a list of genes mutated in ccRCC at higher-than-expected frequencies that included *BAP1* (9/98 tumors). Duns et al[70] similarly performed exome sequencing in 10 ccRCCs and identified 1 mutation in *BAP1*. In addition, *BAP1* was found to be mutated in 1 of 10 ccRCC cell lines examined.[70]

We performed exome sequencing in 7 ccRCCs largely of high grade.[18] Two tumors had mutations in *BAP1*, and subsequent analyses of *BAP1* in 168 matched ccRCC/normal pairs identified 22 tumors with additional mutations. The majority of the mutations were truncating and abrogated protein expression by IHC.[18] Immunohistochemistry had a sensitivity and specificity of greater than 98%.[18] Three tumors without detectable mutations were negative for BAP1 by IHC, suggesting that, as in MPM,[67] other mechanisms exist for BAP1 inactivation. Overall, BAP1 loss (either by mutation or IHC) was observed in 15% of ccRCCs (25/168). This frequency is higher than that reported in other studies,[20,25] but (as determined by *VHL* mutation rates) is in keeping with a higher sensitivity for mutation detection. *BAP1* mutation rates increase as a function of stage, suggesting that *BAP1* may be implicated in ccRCC progression.[24]

We observed an association between *BAP1* mutation and high Fuhrman grade.[18] These results were independently confirmed.[25] In addition, greater than 50% of *BAP1*-mutated tumors exhibited coagulative necrosis, which is a predictor of poor outcomes.[23]

Interestingly, mutations in *BAP1* and *PBRM1* in ccRCC tend to be mutually exclusive.[18] These findings are supported by meta-analyses.[21] Although mutation exclusivity is often interpreted to suggest that the genes function in the same pathway, multiple lines of evidence suggest that *BAP1* and *PBRM1* function in 2 different processes. First, whereas *BAP1* mutations in tumors are associated with high Fuhrman grade and mTORC1 activation, *PBRM1* mutations are associated with low grade and a lack of mTORC1 activation.[18,23] In addition, *BAP1* and *PBRM1* mutations in ccRCC are associated with characteristic and nonoverlapping gene expression signatures.[23] Finally, the outcomes of patients with ccRCCs mutated for *BAP1* and *PBRM1* are quite different.[23] Thus, these data suggest that *BAP1* and *PBRM1* mutations define 2 different molecular subtypes of ccRCC, with different biology and outcomes.

Kaplan-Meier analyses of patients with *BAP1*- and *PBRM1*-mutated tumors showed that *BAP1* mutation was associated with a significantly worse OS than *PBRM1* mutation (median OS of 4.6 years; 95% confidence interval [CI], 2.1–7.2 vs 10.6 years; 95% CI, 9.8–11.5) corresponding to a hazard ratio of 2.7 (95% CI, 0.99–7.6; $P = 0.044$). A similar hazard ratio was observed in a second independent cohort from the TCGA (2.8; 95% CI, 1.4–5.9; $P = 0.004$). Another series from Memorial Sloan-Kettering showed similar results.[24]

Interestingly, a small subset of patients (2%–3%) has tumors simultaneously deficient for both *BAP1* and *PBRM1*.[18] Although given the mutation heterogeneity observed in renal cancer this could reflect 2 different cell populations individually mutated for *BAP1* and *PBRM1*, studies of pure populations of human tumor cells in tumorgrafts strongly suggest that these 2 mutations co-occur in tumor cells. Furthermore, simultaneous loss of *BAP1* and *PBRM1* gene products is observed by IHC in tumor cells. These tumors tend to exhibit rhabdoid features and are associated with dismal outcomes.[23,24]

Somatically acquired mutations in *BAP1* have been identified at low frequencies (<2%) in tumors from other sites including breast, lung, uterus, large bowel, ovary, and prostate (http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/).

## BAP1 Is Mutated in the Germline

During their studies, Harbour et al[66] discovered a *BAP1* frameshift mutation in the germline of a patient with UM, suggesting that *BAP1* mutations may also predispose to familial UM. *BAP1* was subsequently found to be mutated in affected individuals of 2 families with a syndrome of autosomal dominant inheritance characterized by UMs and cutaneous melanomas.[71,72] The estimated frequency of germline *BAP1* mutations in patients with familial cutaneous melanoma is less than 1%, but this percentage is higher for families with both cutaneous melanoma and UM.[71] In keeping with *BAP1* function as a 2-hit tumor suppressor gene, the majority of melanomas show loss of the remaining wild-type allele.[72] The second allele was lost through a variety of mechanisms including deletions and focal mutations.[72] In all tumors, BAP1 expression was lost by IHC.[72] Interestingly, cutaneous tumors exhibited characteristic histologic features, including epithelioid morphology, and are referred to as "atypical Spitz tumors."[72]

Germline *BAP1* mutations were also discovered in families with a high incidence of mesothelioma.[73] Families with germline *BAP1* mutations exhibit a variety of other tumors including lung and breast cancers.[73–76] Other less frequent tumor types include meningioma, cholangiocarcinoma, leimyosarcoma, and ovarian cancer. Renal cancer was reported in 2 unrelated individuals.[73,77] Interestingly, the age at onset of tumors appears to be relatively late compared with that of other familial cancer syndromes.[76]

During our search for somatically acquired *BAP1* mutations in ccRCC, a germline *BAP1* missense variant was discovered in 1 patient who was found to have familial RCC, suggesting that *BAP1* mutations may predispose to renal cancer as well.[18] A study of 83 families with an unexplained predisposition to renal cancer revealed a novel missense mutation in *BAP1* that cosegregated with the ccRCC phenotype in 1 family.[78] Loss of heterozygosity was observed in tumors, and there was uniform loss of BAP1 protein by IHC. Another study of familial nonsyndromic RCC led to the identification of a second *BAP1* germline mutation that cosegregated with the cancer phenotype.[79] As in our family, IHC revealed loss of BAP1 protein in the renal tumors examined. These tumors could be of low or high Fuhrman grade. In neither family was UM diagnosed, but the penetrance of UM, even within families with UM, is low.[80] In additional studies of 32 unrelated individuals with familial RCC, no more *BAP1* mutations were found.[79] In contrast, an evaluation of 60 unrelated individuals from kindreds with a predisposition to uveal melanoma, cutaneous melanoma, or MPM identified 11 probands with deleterious *BAP1* germline mutations. In 6 of the families, RCC was present. The risk of RCC was markedly increased compared with the general population, suggesting that *BAP1* was an RCC-predisposing gene and that RCC should be added to the list of tumors associated with germline mutations in *BAP1*.[79]

## BAP1 Mechanism of Action

At a functional level, BAP1 is a deubiquitinating enzyme (DUB).[61] Deubiquitinating enzymes are proteases that cleave ubiquitin (or ubiquitin-like proteins) from substrates. Most DUBs, including BAP1, are cysteine proteases and rely on a thiol group in the active site.[81–83] There are 4 families of cysteine proteases with DUB activity, including the UCH family. The UCH family shares a common ~230-residue catalytic domain. In mammals, there are 4 members: UCH-L1, UCH-L3, UCH37, and BAP1. Whereas UCH-L1 and UCH-L3 are largely made up of the UCH domain, UCH37 and BAP1 have C-terminal extensions. The catalytic domain of BAP1 most closely resembles that of UCH37, and it shares an additional region of homology in the C-terminus, the UCH37-like domain (ULD).[84] In addition, BAP1 contains a motif implicated in binding host cell factor 1 (HCF-1) and a NLS.[62,84]

*BAP1* mutations target residues across the protein, although the UCH domain appears to be targeted most frequently (http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/). Many mutations (insertion, deletions, and splice site mutations) alter the reading frame and abrogate protein expression.[18] Protein expression may also be disrupted by missense mutations.[18] Mapping of missense mutations not affecting protein levels to a BAP1 structure model suggests that BAP1 tumor suppressor function requires not only ubiquitin binding, but also an intramolecular interaction between the UCH and ULD domains.[18]

The BAP1 ortholog, Calypso, has been implicated in cell fate preservation in *Drosophila*.[85] As cells divide, the cell differentiation state is maintained through enforcing a particular gene expression program. Two groups of genes are broadly implicated in preserving gene expression patterns established during early development, the PcG and Trithorax group. Trithorax and polycomb have antagonistic effects on gene expression. Trithorax group proteins are broadly classified into histone modifiers and nucleosome remodelers, and they are implicated in activating gene expression.[86] In contrast, PcG proteins, including PRC1 and PRC2, silence gene expression.[87] Often, both polycomb complexes are required to maintain gene silencing.[87] Several other polycomb repressive complexes have been found in *Drosophila*, including a polycomb repressive deubiquitinase (PR-DUB).[85] PR-DUB deubiquitinates H2AK119ub1 (K118 in *Drosophila*), and despite that, at face value, this would antagonize PRC1, PR-DUB seemingly synergizes with PRC1.[88] PR-DUB includes the BAP1 ortholog, Calypso, as well as the PcG protein ASX, which is required for Calypso stability.[85] *Calypso* was originally identified in a forward genetic screen in *Drosophila* for mutants unable to repress HOX genes.[89] *Calypso* mutant fly embryos failed to suppress HOX genes, leading to homeotic transformations characteristic of mutations in PcG genes.[89] As expected, Calypso/ASX complexes bound to HOX genes by ChIP, and *calypso* and *Asx* mutant embryos exhibited global increases in H2Aub1 levels.[85] Complicating things though, in the brain there was instead a partial loss of HOX gene expression.[85]

As *Drosophila* Calypso, human BAP1 deubiquitinates H2AK119ub1.[85] Furthermore, depletion of BAP1 in UM cells increases global H2AK119ub1 levels.[90] However, whereas ASX is required for Calypso deubiquitinase activity in *Drosophila*,[85] the activity of recombinant BAP1 protein is undistinguishable from that of BAP1 complexes.[91]

Knockdown of BAP1 in UM[66,90] and mesothelioma cells[67] altered gene expression, and in mesothelioma cells, there was an enrichment for polycomb target genes. However, this accounted for a small percentage of BAP1-deregulated genes. In addition, whereas Calypso has been shown to suppress *HOX* gene expression in *Drosophila*, the expression of *Hox* genes was not increased in

*Bap1*-mutant bone marrow–derived cells.[92] Thus, whether mammalian BAP1 functions as part of a PRC that controls *Hox* gene expression remains to be determined.

An important difference between *Drosophila* Calypso and mammalian BAP1 is that Calypso lacks an HCF-1–binding motif (HBM).[84] This may be significant as most mammalian BAP1 protein appears to be bound to HCF-1,[91] and this interaction is preserved in cell types in which BAP1 functions as a tumor suppressor, including MPM and ccRCC cell lines.[18,67] BAP1 interacts with the β-propeller of HCF-1 through a tetrapeptide motif (NHNY) similar to that found in other HCF-1–binding proteins including the canonical HSV protein VP16.[84] Mutation in the Kelch domain of HCF-1 (P134S) disrupts binding to HBM-containing proteins and also markedly impairs BAP1 binding. Notably, the prototypical HBM ([D/E] HxY, where "x" is any residue) is also present in E2F transcription factors, which are implicated in cell cycle regulation, and HCF-1 binding plays an important role in the activation of E2F target genes.[93] HCF-1 serves as a transcriptional scaffold protein and participates in a variety of histone-modifying complexes. HCF-1 associates with histone methyltransferases (Set1, MLL1),[94,95] acetyltransferases (MOF),[96] and deacetylases (mSin3 complex).[97] Importantly, HCF-1 binding to VP16 is essential for expression of viral immediate early genes and viral infection.[93]

BAP1 forms complexes 1.3 to 1.8 MDa in size by gel filtration chromatography that include HCF-1.[91] Affinity purification experiments have led to the identification of multiple proteins that complex with BAP1 besides HCF-1, including ASXL1 and ASXL2, O-linked *N*-acetylglucosaminetransferase (OGT), HAT1, UBE2O, KDM1B, ANKRD17, and the transcription factors FOXK1 and FOXK2.[63,91,98] Several of these interactions have been subsequently validated in mouse tissues.[92] In addition, the transcriptional repressor protein YY1, which functions as a PcG protein in *Drosophila*,[99] also interacts with BAP1.[91] The overall organization of these complexes and their function are not well understood.

BAP1 complexes are associated with open chromatin.[91] ChIP-seq maps of BAP1 in bone marrow–derived macrophages showed that 65% of BAP1 complexes are within 2 kb upstream or downstream of transcription start sites.[92] DNA sequence analyses revealed an enrichment for SP1 and Ets family–binding sites.[92] Interestingly, 85% of the DNA sequences that associated with BAP1 were also identified with HCF-1 ChIP, and a lower percentage (27%) immunoprecipitated with OGT.[92]

Several substrates of BAP1 have been identified besides H2AK119ub1. BAP1 regulates HCF-1 ubiquitylation and protein levels in several cell types, including spleenocytes.[63,84,92] BAP1 also deubiquitinates OGT and regulates its levels.[92] Reducing OGT levels decreases global O-GlcNAcylation levels.[92] Since O-GlcNAcylation is required for HCF-1 proteolytic maturation, HCF-1 is also indirectly affected. However, OGT levels are not affected by BAP1 loss in all cell types, and in mouse embryo fibroblasts, OGT is unaffected.[92] In addition, HCF-1 levels are not affected by BAP1 in several other cell types.[18,63,84] BAP1 has also been proposed to control the levels of PGC-1α,[100] a transcriptional coactivator for steroid receptors implicated in metabolism, but this effect is also cell type–specific (Y. Gu and J. Brugarolas, unpublished observations). Thus, BAP1 regulates different proteins in different cell types.

*Bap1*−/− mice exhibit developmental retardation at E8.5 and are not detected past E9.5.[92] Postnatal systemic Bap1 inactivation (using an inducible *Cre* from a ubiquitously expressed locus) led to the development of myelodysplasia with thrombocytopenia and anemia.[92] Thrombocytopenia developed as early as 1 week after Bap1 inactivation. Bone marrow transplantation experiments showed that the phenotype was cell autonomous, and Bap1 was required for hematopoietic reconstitution. Monocytes and neutrophils were increased resembling chronic myelomonocytic leukemia, and chronic myelomonocytic leukemia developed in recipient mice from BAP1-deficient bone marrows.[92]

BAP1 has been implicated in cell cycle regulation. BAP1 suppresses the proliferation of some cell types.[18,61,62] However, this is a cell type–dependent effect, and in other cell types, BAP1 is actually required for cell proliferation.[63–65,84] In MPM, BAP1 depletion in 3 different cell lines reduced cell proliferation.[67] It is possible that not all cell types are amenable to transformation by BAP1 loss, even cell types where BAP1 is known to function as a tumor suppressor. Reintroduction of *BAP1* into BAP1-deficient mesothelioma cells either had no effect or modestly increased cell proliferation.[67] Thus, the effects of BAP1 on cell proliferation are complex and seem to be cell type dependent.

BAP1 may play a role in the DNA damage response. BAP1 is phosphorylated in response to DNA damage.[101,102] Both γ-radiation, which causes double-strand DNA breaks, as well as ultraviolet radiation, which causes DNA adducts, induces BAP1 phosphorlation.[101,102] Although the role of BAP1 in the DNA damage response is unclear, several ubiquitin ligases (including BRCA1 and PRC1), and DUBs, accumulate at sites of DNA breaks, and gene expression is suppressed.[103,104] In addition, BAP1 depletion has been postulated to increase sensitivity to ionizing radiation.[64]

Some data suggest that BAP1 loss cooperates with $BRAF^{V600E}$ mutations. In atypical Spitz tumors, which uncommonly have mutations in *BRAF*, $BRAF^{V600E}$ mutations tend to co-occur with *BAP1* mutations. Among 32 tumors examined, 9 had lost BAP1 expression, and 8 of them had mutations in *BRAF*. In contrast, only 1 of 23 tumors with BAP1 expression had a *BRAF* mutation ($P < 0.0001$).

## BAP1 Function in Renal Cancer

BAP1 binds to HCF-1 in renal cancer cell lines.[18] BAP1 binding was demonstrated through reciprocal immunoprecipitation experiments, and most BAP1 protein cofractionated with HCF-1 by gel filtration chromatography.[18] BAP1 binding to and cofractionation with HCF-1 were also observed in orthotopic tumorgrafts in mice.[18] Consistent with previous observations,[91] immunoprecipitations of BAP1 and HCF-1 deplete BAP1 protein to a similar extent, suggesting that most BAP1 is bound to HCF-1.[18] Binding to HCF-1 is important for BAP1 suppression of cell proliferation.[18] BAP1 reintroduction into 2 different BAP1-deficient ccRCC cell lines reduced cell growth.[18] The inhibition of cell proliferation by BAP1 was compromised by disruption of the HBM.[18] Taken together, these data suggest that BAP1 binding to HCF-1 is important for its tumor suppressor function in renal cancer.

Mammalian BAP1 deubiquitinates H2AK119ub1,[85] and BAP1 reintroduction into BAP1-deficient ccRCC cell lines affected global levels of H2AK119ub1.[18] Despite that restoration of BAP1 into BAP-deficient ccRCC cell lines reduced H2AK119ub1 levels, no correlation was found between H2AK119ub1 levels and *BAP1* mutation in ccRCC tumors.[18] These data suggest that other factors regulate H2AK119ub1 levels in tumors. It is also possible that BAP1 affects H2AK119ub1 levels in tumors regionally (not globally). Notably, in cell line experiments, reintroduction of a BAP1 mutant defective in HCF-1 binding had a similar effect on H2AK119ub1 levels as wild-type BAP1.[18] These data suggest that the role of BAP1 in HCF-1 binding and in H2A deubiquitination can be separated and that BAP1-mediated deubiquitination of

H2AK119ub1 is largely independent of HCF-1 binding. Given that most BAP1 appears to be bound to HCF-1 and that this interaction is important for BAP1-mediated suppression of RCC cell proliferation,[18] the role of H2AK119ub1 deubiquitination is unclear.

We observed a correlation between BAP1 status in tumors and markers of mTORC1 activation.[18,23] *BAP1*-mutant ccRCC tended to exhibit phosphorylation of S6 and 4E-BP1. It is noteworthy that mTORC1 regulates cell size, and BAP1 loss has been associated with epithelioid cells, which tend to be large. It is intriguing that BAP1 is associated with epithelioid MPM, and epithelioid features may also characterize BAP1-deficient uveal and cutaneous melanoma.[105] However, the effect of BAP1 on mTORC1 appears to be indirect, and BAP1 reconstitution into BAP1-deficient ccRCC cell lines did not seemingly affect mTORC1 activity.[18] Furthermore, the sensitivity of BAP1-deficient tumorgrafts in mice to mTORC1 inhibitors is similar to that of tumorgrafts wild-type for BAP1 (A. Pavia-Jimenez and J. Brugarolas, unpublished results).

Although the molecular mechanism of tumor suppression by BAP1 remains unclear, data from multiple systems converge on a role in the regulation of gene expression.[66,67,90,92] BAP1 does not have a DNA-binding domain and is likely to be recruited to chromatin through other proteins. We asked whether *BAP1*-mutant ccRCCs could be distinguished from other ccRCC on the basis of gene expression. We used RNA-seq data obtained by the TCGA from 308 ccRCCs including 20 *BAP1*-mutant tumors. As a control, we did the same experiment, using random groups of tumors. There were 3250 genes that distinguished the *BAP1*-mutant group, but fewer than 150 genes that distinguished the random groups ($P < 0.0001$).[23] These data show that *BAP1*-mutant tumors are associated with a characteristic gene expression signature and further support the notion that BAP1 affects gene expression. The effect, however, did not dominate sufficiently so as to be able to separate *BAP1*-mutant tumors from the rest using unsupervised hierarchical clustering algorithms. Pathway analyses revealed an enrichment for mitogenic pathways including epidermal growth factor, nerve growth factor, and insulin pathways.

## BAP1 and Therapy

In experiments performed with BAP1-deficient and reconstituted ccRCC cell lines, BAP1 loss was associated with increased sensitivity of 2 different cell lines to radiation and the PARP inhibitor olaparib.[18] However, the magnitude of the difference was modest. In mesothelioma cell lines, no difference was observed between *BAP1*-mutant and wild-type cells in sensitivity to PARP inhibitors.[67]

Interestingly, atypical Spitz tumors with *BAP1* mutations commonly exhibit a lymphocytic infiltrate.[105,106] Although this has not been reported in other tumor types, these data raise the possibility that *BAP1*-deficient tumors might be particularly immunogenic. Should something similar occur in ccRCC, BAP1 may influence responsiveness to immunomodulators such as interleukin-2.

The role of HDAC inhibitors was evaluated in UM cell lines.[90] Valproic acid seemingly counteracted the effects of BAP1 knockdown on H2AK119ub1. As determined by the development of dendrites, valproic acid also induced differentiation of tumor cells with *BAP1* mutations and shifted the gene expression signature to a more differentiated type. However, the effects on differentiation and gene expression were undistinguishable from those observed in a cell line that was wild type for *BAP1*. Thus, although valproic acid appears to differentiate UM cells, these effects may be independent of BAP1. In addition, BAP1 loss does not affect the sensitivity of mesothelioma cell lines to HDAC inhibitors in vitro, and HDAC inhibitors have failed to show activity in mesothelioma.[68] The effect of HDAC inhibitors on wild-type and *BAP1*-mutant ccRCC cell lines was undistinguishable (S. Wang and J. Brugarolas, unpublished results).

Emerging data are consistent with a model where BAP1 is recruited to discrete sites in the genome where, through the interaction with HCF-1 and histone modifiers, it controls gene expression. A greater understanding of the precise molecular mechanism of BAP1 action is required for the identification of targets for drug development. BAP1, itself a tumor suppressor whose expression is frequently lost in tumors, is not a good target. A better target might be the ubiquitin ligase responsible for the ubiquitylation of substrates that BAP1 acts on. Although at present most molecularly targeted therapies are directed toward protein kinases, enzymes involved in the ubiquitin system may represent the next frontier.[107] Empirical approaches may also be undertaken exploiting synthetic lethality. The development and evaluation of candidate compounds may be facilitated by the availability of tumorgraft models of renal cancer that reproduce the treatment responsiveness of human RCC bearing tumors with *BAP1* and *PBRM1* mutations.[108]

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