

## Molecular Genetics of Clear-Cell Renal Cell Carcinoma

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### ABSTRACT

Renal cell carcinoma of clear-cell type (ccRCC) is an enigmatic tumor type, characterized by frequent inactivation of the *VHL* gene (infrequently mutated in other tumor types), responsiveness to angiogenesis inhibitors, and resistance to both chemotherapy and conventional radiation therapy. ccRCC tumors exhibit substantial mutation heterogeneity. Recent studies using massively parallel sequencing technologies have implicated several novel driver genes. In *VHL* wild-type tumors, mutations were discovered in *TCEB1*, which encodes Elongin C, a protein that binds to VHL and is required for its function. Several additional tumor suppressor genes have been identified near the *VHL* gene, within a region that is frequently deleted in ccRCC on chromosome 3p: *SETD2*, *BAP1*, and *PBRM1*. Mutations in *BAP1* and *PBRM1* are largely mutually exclusive and are associated with different tumor biology and patient outcomes. In addition, the mTORC1 pathway is deregulated by mutations in *MTOR*, *TSC1*, *PIK3CA*, and *PTEN* in approximately 20% of ccRCCs. Mutations in *TSC1*, and possibly other genes, may predict for sensitivity to mTORC1 inhibitors. These discoveries provide insight into ccRCC development and set the foundation for the first molecular genetic classification of the disease, paving the way for subtype-specific therapies.

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### INTRODUCTION

Kidney tumors are estimated to be diagnosed in more than 270,000 individuals every year worldwide.<sup>1</sup> More than 65,000 new diagnoses and approximately 13,680 patient deaths as a result of tumors of the kidney and renal pelvis were projected in the United States for 2013.<sup>2</sup> In the United States, 15% to 20% of individuals present with lymph node metastases and a similar percentage have distant involvement at the time of diagnosis.<sup>3</sup> In the metastatic setting, renal cancer remains largely incurable. The majority of malignant kidney tumors are renal cell carcinomas (RCC) and approximately 70% are RCCs of clear-cell type (ccRCC).<sup>4</sup>

### MOLECULAR GENETICS OF ccRCC

The Cancer Genome Atlas consortium analyzed over 400 tumor/normal pairs.<sup>5</sup> On average, ccRCCs exhibit less than 20 DNA copy-number alterations, fewer changes than in colon and breast cancers.<sup>5</sup> Proportionally, however, there is an overrepresentation of copy-number alterations involving whole chromosome arms.<sup>5</sup> RNA fusions (resulting from translocation events) were observed in 10% to 20% of ccRCCs and the vast majority of them were unique.<sup>5</sup> A second study by Sato et al<sup>6</sup> evaluated more than 100 ccRCCs using whole genome or exome sequencing. Overall, ccRCCs

are characterized by one to two somatically acquired single nucleotide variants or small insertions and deletions (indels) per megabase pair (approximately 3,000 to 6,000 mutations per tumor).<sup>5,6</sup> Most of these mutations occur outside coding regions. Protein-coding regions account for approximately 1% of the genome and are subject to approximately 1% of the mutations, suggesting that mutations occur randomly.<sup>6</sup> Table 1 lists genes mutated in ccRCC in both studies.<sup>5,6</sup>

There is significant mutation heterogeneity within ccRCC tumors.<sup>7</sup> According to their prevalence, somatic mutations are classified into ubiquitous, shared, and private mutations.<sup>7</sup> Ubiquitous mutations are present in every tumor cell. Shared and private mutations are found in progressively smaller subclones. Overall, mutation prevalence reflects the time of mutation acquisition, with ubiquitous mutations representing early, truncal events and shared and private representing progressively more distant subclones or branches. However, this timeline may be distorted by later mutations with a disproportionate proliferative advantage or other factors.

According to their significance, mutations are classified into drivers and passengers.<sup>8</sup> Driver mutations include those implicated in tumor initiation and progression. Ubiquitous mutations are not necessarily driver mutations. Indeed, unselected mutations acquired during the normal process of DNA replication in the cell lineage that ultimately results

**Table 1.** ccRCC-Mutated Genes

Genes	TCGA Cohort		Japanese Cohort*	
	Tumors With Mutation (%)	Passenger Probability ( <i>q</i> value)	Tumors With Mutation (%)	Passenger Probability ( <i>q</i> value)
<i>VHL</i>	52.3	< .0001	39.6†	< .0001
<i>PBRM1</i>	32.9	< .0001	26.4	< .0001
<i>SETD2</i>	11.5	< .0001	11.3	< .0001
<i>BAP1</i>	10.1	< .0001	7.5	< .0001
<i>MTOR</i>	6	< .0001	5.7	.0431
<i>TCEB1</i>	0.7	.0566	4.7‡	< .0001
<i>PIK3CA</i>	2.9	< .0001	4.7	.0268
<i>KDM5C</i>	6.7	< .0001	3.8	.12
<i>TP53</i>	2.2	< .0001	2.8	.0176
<i>PTEN</i>	4.3	< .0001	1.9	.116

Abbreviations: ccRCC, clear-cell renal cell carcinoma; TCGA, The Cancer Genome Atlas.

\*Mutations found by whole exome sequencing.

†Including complementary approaches overall *VHL* mutation rate, 66%.

‡Possibly higher *TCEB1* mutation rates in preselected ccRCC population. Data are obtained from Creighton et al (Table S4).<sup>5</sup> and Sato et al (Table S4).<sup>6</sup> For methodology, see Creighton et al<sup>5</sup> and Sato et al.<sup>6</sup>

### VHL COMPLEX IS BROADLY INACTIVATED IN ccRCC

The von Hippel-Lindau (*VHL*) gene is inactivated by either mutation or methylation in over 80% of ccRCC.<sup>6,14-16</sup> *VHL* was originally identified as the gene responsible for the ccRCC-predisposing syndrome, von Hippel-Lindau.<sup>17</sup> *VHL* is a two-hit tumor suppressor gene and, typically, one allele is inactivated through an intragenic mutation and the second is deleted as part of large deletion. The *VHL* gene is on chromosome 3p25.3 and deletions in this region, which often involve the whole short arm of chromosome 3, are observed in approximately 90% of ccRCC.<sup>18-20</sup> At times, a *VHL* mutation is found without a 3p deletion. However, a deletion may have occurred, accompanied by duplication of the remaining chromosomal region, resulting in copy-neutral loss of heterozygosity (LOH). Consistent with this, ccRCCs with *VHL* mutations and copy-neutral LOH exhibited *VHL* MARs that were higher than for control genes (mutated genes in diploid regions without LOH).<sup>6</sup> In this setting, both alleles of *VHL* would be inactivated by the same mutation.

The VHL protein forms a complex with Elongin B, Elongin C, Cul2, and Rbx1 that functions as an E3 ubiquitin ligase toward, most prominently, the  $\alpha$  subunits of HIF (hypoxia-inducible factor) transcription factors (Fig 1).<sup>21</sup> Many mutations in *VHL* disrupt protein expression, but missense mutations often cluster in the interface between VHL and Elongin C.<sup>22</sup> Interestingly, the Elongin C gene (called *TCEB1*) is mutated in 0.5% to 5% of ccRCCs (Table 1).<sup>6</sup> *TCEB1* mutations are uniformly associated with LOH of 8q21, where *TCEB1* is located.<sup>6</sup> As expected, these mutations are exclusive with *VHL* mutations ( $P < .0001$ ).<sup>6</sup> This is consistent with the notion that mutations in either *VHL* or *TCEB1* are sufficient to inactivate the function of the complex.

Enigmatically, *TCEB1* mutations are not typical loss-of-function mutations. All mutations reported by Sato et al<sup>6</sup> were missense mutations at two conserved residues, Tyr79 ( $n = 7$ ) and Ala100 ( $n = 1$ ). These mutations seemingly interfere with VHL binding to Elongin C and lead to the stabilization of HIF- $\alpha$  subunits. However, the pattern of mutation suggests that the situation is more complex. Perhaps other functions of elongin C need to be preserved.

Overall, Sato et al<sup>6</sup> found evidence of VHL complex inactivation in 92% of ccRCC (97 of 106 tumors). In this cohort, *VHL* mutations were found in 66% (70 of 106 tumors), *VHL* methylation in 21% (22 of 106 tumors), and *TCEB1* mutation in 5% (five of 106 tumors). Whether the VHL complex is inactivated in the remaining tumors is unclear. In the nine remaining ccRCCs, no mutations were found in other complex components.<sup>6</sup> However, immunohistochemistry (IHC) analyses showed that seven tumors expressed HIF- $\alpha$  (HIF-1 $\alpha$ , HIF-2 $\alpha$ , or both) at levels comparable to *VHL*-deficient tumors and several of these had low mutation numbers, raising the possibility that some mutations may have been missed. In addition, *VHL* may have been inactivated through mutations outside sequenced regions. The remaining two ccRCCs had no detectable HIF- $\alpha$  expression. One of these was a translocation carcinoma involving the *TFE3* gene, and translocation carcinomas may lack *VHL* mutations.<sup>5</sup> Thus, most, if not all, ccRCC may have deregulation of the VHL pathway.

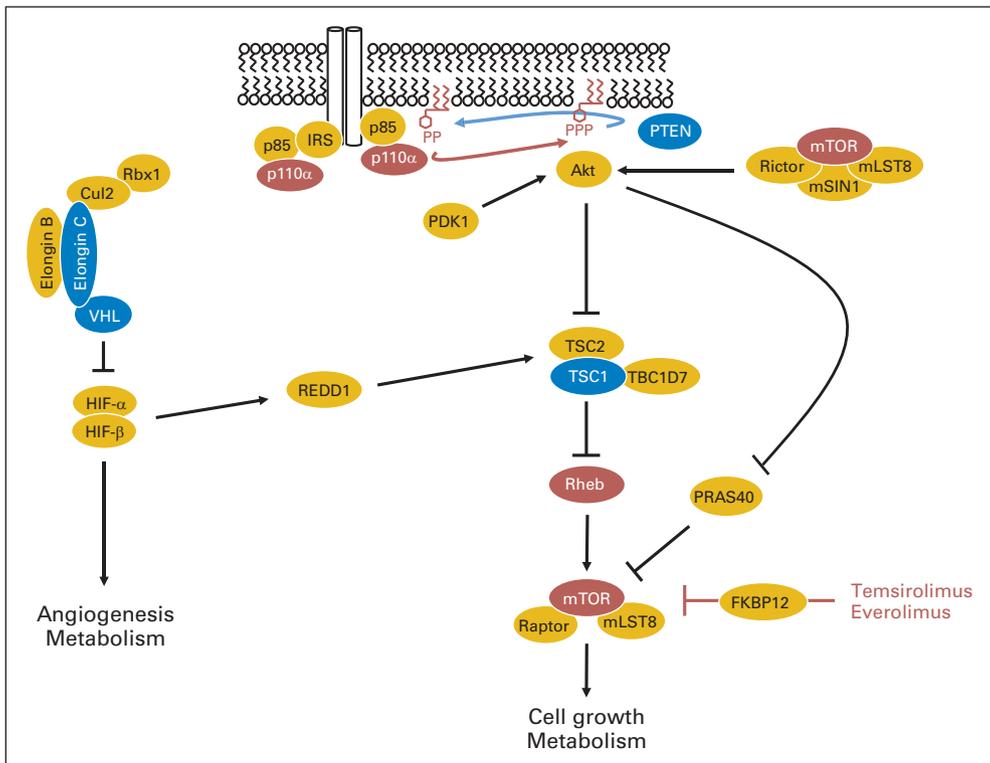
### PBRM1 IS THE SECOND MOST FREQUENTLY MUTATED GENE IN ccRCC

Polybromo 1 (*PBRM1*) is mutated in approximately 45% of ccRCC.<sup>23</sup> Lower mutation frequencies in recent studies<sup>5,6</sup> may reflect reduced

in the initial tumor clone represent ubiquitous passengers.<sup>9</sup> Only a subset of mutations (possibly fewer than 10 protein-coding gene mutations) are drivers. In addition, driver mutations may be found among shared and private mutations.

Mutation heterogeneity may be advantageously exploited. The best therapeutic targets may be found in pathways deregulated by ubiquitous driver mutations present in every tumor cell. These mutations may be more easily identified by exploiting mutation heterogeneity. Furthermore, tumors likely develop as a set of conditional dependencies in which new mutations build on the confines imposed by pre-existing mutations,<sup>10</sup> and the degree of dependency of a tumor on a pathway may be related to how early the corresponding mutation occurred. This conditional or contextual nature of oncogenic mutations fits well with the empiric observation that mutations exert their protumorigenic effect in a tissue-dependent manner.<sup>11</sup> For example, in dominantly inherited familial cancer-prone syndromes, tumors develop in a subset of tissues despite the presence of the mutation in every diploid cell.

Experimentally, whether a mutation is ubiquitous can be inferred from sampling multiple areas of the tumor.<sup>7</sup> In addition, mutant-allele ratios (MAR), referring to the fraction of mutant over mutant plus wild-type alleles for each mutation, may also help determine the prevalence of a mutation. Ubiquitous heterozygous mutations have MARs of approximately 0.5. However, if the mutation arose later and is only present in 50% of the tumor cells, the MAR would be 0.25. Similar MARs may be found in mutations arising around the same time, and this approach was used by Sato et al<sup>6</sup> to define subclonal populations. However, MARs are confounded by DNA copy-number alterations as well as by contamination with normal DNA (from stroma or inflammatory cells). While cumbersome, the problem of contamination may be resolved by implanting the tumors in mice, which results in the selective expansion of tumor cells while the stroma is replaced by the host.<sup>12</sup> Although the focus of this article is on genetic events, epigenetic alterations most likely contribute to cancer development.<sup>13</sup>



**Fig 1.** Interplay between VHL and mTORC1 pathways. In the presence of growth factors, transphosphorylation by the intracellular domains of receptor tyrosine kinases leads to recruitment of the regulatory subunit of class IA PI3K, p85 (either directly or through adaptor proteins like IRS) and releases its inhibition of the catalytic subunit (p110 $\alpha$ ; encoded by the *PIK3CA* gene), which phosphorylates phosphatidylinositol-4,5-trisphosphate to generate phosphatidylinositol-3,4,5-trisphosphate (PIP<sub>3</sub>). PIP<sub>3</sub> recruits interacting proteins to the plasma membrane, such as Akt, which is phosphorylated and activated by PDK1 and mTORC2 (mTOR complex 2). Akt phosphorylates TSC2, which is in a complex with TSC1 and TBC1D7, releasing its inhibition on Rheb. Activated Rheb binds to and activates mTORC1. mTORC1 is inhibited by PRAS40, and this inhibition is also released by AKT activation. mTORC1 is also inhibited by REDD1 in a manner that requires TSC1/TSC2. REDD1 is transcriptionally induced by both HIF-1 and HIF-2, which are activated following the inactivation of the VHL complex through mutations in either *VHL* or *TCEB1* (encoding Elongin C). mTORC1 is inhibited by temsirolimus and everolimus, which interact with FKBP12 and subsequently bind to mTORC1. Brown ovals, oncoproteins activated by mutation in ccRCC; blue ovals, tumor-suppressor proteins inactivated by mutation in ccRCC.

sensitivity, and other studies have shown comparable mutation frequencies.<sup>12</sup> The majority of mutations are truncating, and *PBRM1* functions as a two-hit tumor suppressor gene.<sup>23</sup> Furthermore, *PBRM1* is on the same chromosome arm as *VHL* and the second allele is frequently codeleted with *VHL*.<sup>23</sup> As expected, most *PBRM1* mutations are accompanied by loss of the protein.<sup>12</sup> Analyses of MARs (as well as tumor sampling studies) show that *PBRM1* mutations may be ubiquitous.<sup>6,7</sup>

*PBRM1* encodes BAF180, a component of a nucleosome-remodeling complex. Nucleosomes are histone octamers composed, typically, of two copies of each of four canonical histone proteins (H2A, H2B, H3, and H4), around which 147 bp of DNA are wrapped.<sup>24</sup> DNA binding to histones limits its accessibility to transcription factors and RNA polymerases. DNA accessibility is regulated by remodelers, which unwrap, reposition, and eject nucleosomes.<sup>25</sup>

There are currently four different families of remodeler complexes, including the switching defective/sucrose nonfermenting (SWI/SNF) family.<sup>25</sup> These families differ in subunit composition and biologic function.<sup>25</sup> SWI/SNF complexes are organized around an ATPase that provides energy to break DNA/histone contacts, brahma homolog (BRM), and brahma-related gene 1 (BRG1).<sup>26,27</sup> According to the subunit composition, SWI/SNF complexes are subdivided into BRG1-associated factor (BAF) and polybromo BRG1-associated factor (PBAF) complexes (Table 2). Both contain approximately 15 subunits, but whereas BAF complexes contain either a BRM or BRG1 subunit, only a BRG1 is in PBAF complexes.<sup>27,28</sup> BAF complexes are thought to be targeted to chromatin by BAF250 proteins, whereas targeting of PBAF complexes involves BAF180 and BAF200.<sup>27,29</sup>

BAF180 (encoded by the *PBRM1* gene) contains six tandem bromodomains, two bromo-adjacent homology (BAH) domains and a high-mobility group (HMG) box.<sup>29</sup> Bromodomains bind

acetylated lysine residues in histone tails and may target PBAF to chromatin (Fig 2A). Different BAF180 bromodomains show different affinities for acetylated lysine residues in vitro, and BAF180 may target PBAF to a specific pattern of acetylated lysines.<sup>29,31</sup> Disruption of a single bromodomain may suffice to abrogate tumor suppressor function.<sup>12,31</sup>

Other genes encoding subunits of the SWI/SNF complex are mutated in ccRCC but at much lower frequencies (Table 2).<sup>5,6,32</sup> However, these mutations are not exclusive with *PBRM1*,<sup>6,23</sup> and how they cooperate in tumor development is unclear.

How *PBRM1* loss promotes tumorigenesis is poorly understood. In keeping with its role in nucleosome remodeling, ccRCCs deficient in *PBRM1* are associated with a distinct gene-expression signature.<sup>30</sup> *PBRM1*-mutant ccRCCs are enriched for genes in pathways implicated in the cytoskeleton and cell motility.<sup>30</sup> In addition, reintroduction of *PBRM1* into *PBRM1*-deficient cells induces the expression of the cyclin-dependent kinase inhibitor *p21*.<sup>33</sup> This is accompanied by a reduction in cell proliferation.<sup>33</sup> Finally, *PBRM1* was identified in a small-hairpin RNA (shRNA) screen for genes whose inactivation would extend the proliferative capacity of primary fibroblasts in culture.<sup>34</sup> Thus, *PBRM1* appears to regulate cell proliferation. Studies in insect cells and mice suggest that SWI/SNF complexes are in a functionally antagonistic relationship with polycomb group proteins.<sup>26</sup> However, whether this will offer opportunities for therapeutic intervention remains to be determined.<sup>35</sup>

## SETD2 GENE

The gene encoding SET domain containing protein 2 (SETD2) is somatically mutated in approximately 10% to 15% of ccRCCs (Table

**Table 2.** SWI/SNF Genes and Proteins

Location	Gene	Subunit	Complex	Mutated in ccRCC
<b>ATPase</b>				
9p22.3	<i>SMARCA2</i>	BRM	BAF	+
19p13.2	<i>SMARCA4</i>	BRG1	BAF/PBAF	++
<b>Targeting</b>				
1p35.3	<i>ARID1A</i>	BAF250A	BAF	+++
6q25.1	<i>ARID1B</i>	BAF250B	BAF	++
12q12	<i>ARID2</i>	BAF200	PBAF	
3p21	<i>PBRM1</i>	BAF180	PBAF	(+++++) <sub>2</sub>
<b>Other</b>				
12q13.2	<i>SMARCC2</i>	BAF170	BAF/PBAF	++
3p21.31	<i>SMARCC1</i>	BAF155	BAF/PBAF	
12q13-q14	<i>SMARCD1</i>	BAF60A	BAF/PBAF	++
17q23-q24	<i>SMARCD2</i>	BAF60B	BAF/PBAF	
7q35-q36	<i>SMARCD3</i>	BAF60C	BAF/PBAF	
17q21.2	<i>SMARCE1</i>	BAF57	BAF/PBAF	
3q26.33	<i>ACTL6A</i>	BAF53A	BAF/PBAF	
7q22	<i>ACTL6B</i>	BAF53B	BAF/PBAF	
22q11	<i>SMARCB1</i>	BAF47	BAF/PBAF	+

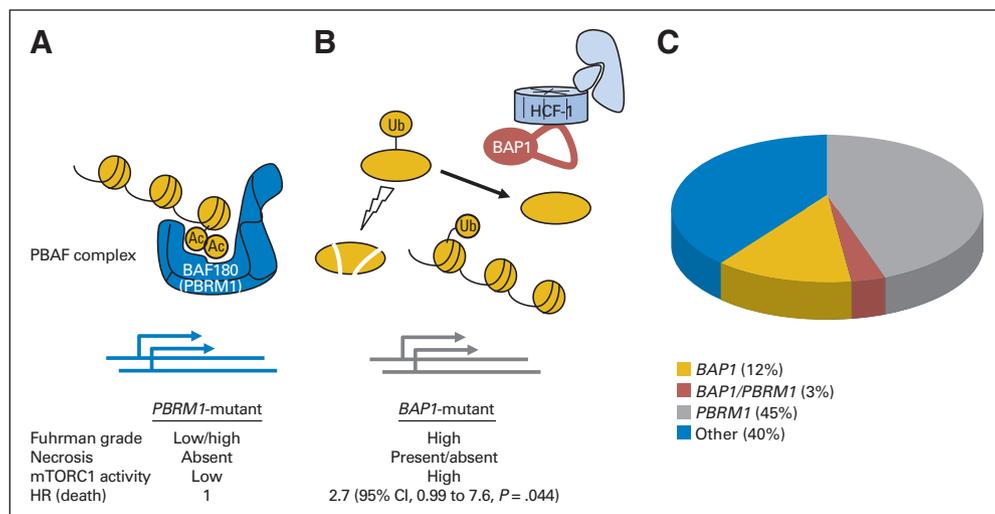
1).<sup>5,6,36,37</sup> Like *VHL* and *PBRM1*, *SETD2* is a two-hit tumor suppressor gene and is located on chromosome 3p. *SETD2* mutations tend to be in the shared group.<sup>6,7</sup> Analyses of data provided by Sato et al<sup>6</sup> show that *SETD2* MARs are lower than *VHL* MARs in one third of ccRCCs, suggesting that in these tumors *SETD2* mutations are subclonal. In addition, sampling studies have shown different *SETD2* mutations in different samples of the same tumor.<sup>7</sup> This mutation convergence suggests a high selective pressure to mutate *SETD2* in some contexts; a meta-analysis suggests that *SETD2* mutations cooperate with mutations in *PBRM1*.<sup>11</sup> Though the molecular basis remains unclear, both BAF180 and SETD2 converge on histones, one as a reader (BAF180) and the other as a writer (SETD2).

How biallelic *SETD2* inactivation leads to ccRCC is unclear. The SETD2 protein is a nonredundant histone H3 lysine 36 trimethylating (H3K36me3) enzyme.<sup>38</sup> Though H3K36 methylation is generally linked to active transcription, it is also associated with alternative

splicing and transcriptional repression.<sup>39</sup> Interestingly, a recent study has linked SETD2 and H3K36me3 to DNA mismatch repair,<sup>40</sup> and microsatellite instability was found in a subset of ccRCC.<sup>41</sup> In addition, a link has been reported in ccRCC between *SETD2* mutation and DNA methylation.<sup>5</sup>

**BAP1 IS A DRIVER OF TUMOR AGGRESSIVENESS**

The BRCA1 associated protein-1 (*BAP1*) gene is mutated in 10% to 15% of patients with ccRCC.<sup>12,42</sup> *BAP1* was originally identified in a yeast two-hybrid screen for BRCA1-interacting proteins,<sup>43</sup> but endogenous BAP1 seems not to bind BRCA1 in mammalian cells. Guo et al<sup>42</sup> performed exome sequencing in a small number of ccRCCs with targeted sequencing of selected genes in an expansion cohort. They reported a list of 12 genes that mutated in ccRCC at frequencies higher



**Fig 2.** *PBRM1*- and *BAP1*-mutant tumors are associated with different biology, pathologic features, and outcomes, setting the foundation for a molecular genetic classification of clear-cell renal cell carcinoma (ccRCC). (A) BAF180 (encoded by the *PBRM1* gene) contains six tandem bromodomains that bind to acetylated lysine residues in histone tails, thereby localizing the PBAF chromatin remodeling complex to specific chromatin regions and regulating gene expression. (B) BAP1 interacts with HCF-1 and functions to deubiquitinate proteins, including histone H2AK119ub1. By deubiquitinating its substrates, BAP1 may inhibit protein degradation or, in the case of H2A, alter gene expression. *PBRM1*- and *BAP1*-mutant tumors are associated with different gene expression signatures, pathologic features, mTORC1 activation, and outcomes (Kapur et al<sup>30</sup>). (C) Pie chart representation of ccRCC subtypes and their approximate frequencies. HR, hazard ratio; other, tumors without detectable mutations in *PBRM1* and *BAP1*.

than expected by chance alone. This list included the *TSC1* gene, which was reported previously to be mutated in ccRCC,<sup>44</sup> *BAP1*, and several other genes not identified in other studies.<sup>5,6,42</sup> A similar approach focusing on high-grade ccRCC led to the identification of *BAP1* mutations in ccRCC by our group.<sup>12</sup>

*BAP1* is a two-hit tumor suppressor gene located on chromosome 3p between the *VHL* and *PBRM1* genes. The *BAP1* protein interacts with host cell factor-1 (HCF-1), a protein that serves as a scaffold for several chromatin remodeling complexes.<sup>45-47</sup> *BAP1* mutations are typically associated with loss of the protein.<sup>12</sup> As determined by analyses of results provided by Sato et al,<sup>6</sup> MARs for *BAP1* are similar to *VHL* in 70% to 80% of ccRCCs and substantially lower in the rest. These data suggest that in a subset of tumors, *BAP1* mutations are subclonal. Recent studies using a validated IHC test in approximately 1,400 ccRCCs identified focal loss of *BAP1* in 2% to 3% of ccRCCs.<sup>48</sup> However, the number of tumors with focal loss of *BAP1* is likely to be much larger, as a single section per tumor was examined.

Frequent somatic mutation of *BAP1* was first described in metastasizing uveal melanoma and subsequently in malignant pleural mesothelioma.<sup>49,50</sup> Notably, *BAP1* is also mutated in the germline.<sup>51,52</sup> Germline *BAP1* mutations are associated with a syndrome characterized by uveal and cutaneous melanoma, mesothelioma, and RCC.<sup>51-54</sup> The presence of different tumors in individual families<sup>51,53</sup> suggest that the specific mutation alone does not dictate the tumor spectrum. In two families, ccRCC was the dominant feature.<sup>53,54</sup> The finding that germline mutations in *BAP1* predispose to ccRCC suggest that *BAP1* loss can initiate RCC development.

*BAP1* is a deubiquitinase of the ubiquitin C-terminal hydrolase (UCH) family.<sup>43</sup> *BAP1* localizes to the nucleus, and nuclear localization is required for *BAP1* tumor-suppressor function.<sup>55</sup> *BAP1* contains an N-terminal catalytic domain, an HCF-1 binding motif (HBM), and a C-terminal UCH37-like domain (ULD).<sup>56</sup> The catalytic domain is often targeted by missense mutations in ccRCC.<sup>12</sup>

In *Drosophila*, *BAP1* (Calypso) functions as an H2A deubiquitinase.<sup>57</sup> Similarly, mammalian *BAP1* is able to deubiquitinate H2AK119ub1.<sup>57</sup> *Drosophila* Calypso is a polycomb repressive deubiquitinase that silences genes implicated in body planning and patterning.<sup>57</sup> Polycomb complexes regulate different gene expression programs in different lineages.<sup>58,59</sup> This cell-context dependency also characterizes *BAP1*. Furthermore, *BAP1* deubiquitinates different proteins in different cell types,<sup>60</sup> and *BAP1* can both promote and suppress cell proliferation in a cell-type dependent manner.<sup>61</sup>

An important difference between Calypso and mammalian *BAP1* is that Calypso lacks the HBM motif implicated in binding to HCF-1 (Fig 2B). This motif may be important as most *BAP1* in cells seems to be bound to HCF-1.<sup>46</sup> In addition, mutation of the motif impairs the growth suppressive function of *BAP1* in renal cancer cells.<sup>12</sup> HCF-1 serves as a chromatin scaffold protein for multiple histone modifying enzymes.<sup>62</sup> HCF-1 is also a substrate for *BAP1*,<sup>45,56,60</sup> but this seems to be cell-type specific and its relevance in renal cancer is unclear.<sup>12</sup>

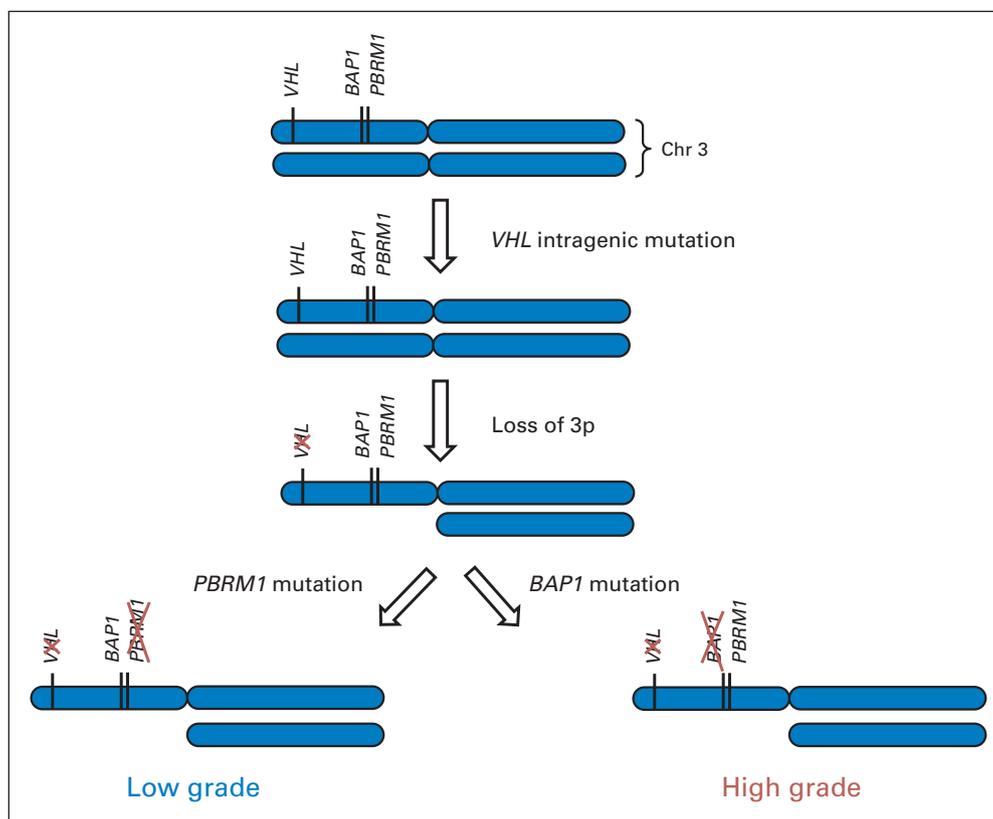
*BAP1*-deficient ccRCCs are characterized by a specific gene-expression signature.<sup>30</sup> This signature is enriched for pathways implicated in growth factor and phosphatidylinositol 3-kinase (PI3K) signaling.<sup>30</sup> Consistent with this, *BAP1*-deficient tumors exhibit increased mammalian (or mechanistic) target of rapamycin (mTOR) complex 1 (mTORC1) activation.<sup>12</sup> In addition, *BAP1* mutations in

tumors seemingly correlate with methylation changes of polycomb repressive complex 2 (PRC2) target genes.<sup>6</sup>

Interestingly, *BAP1* and *PBRM1* mutations in ccRCC are largely mutually exclusive (Fig 2C).<sup>12</sup> As determined by a meta-analysis, the odds of having mutations in *BAP1* are reduced by 70% in *PBRM1*-mutated tumors.<sup>11</sup> The molecular basis of this relationship is unknown. Mutation exclusivity is often interpreted to mean that genes are in the same pathway (such as *VHL* and *TCEB1*). However, *BAP1*- and *PBRM1*-mutant tumors are associated with different histologic features, biology, and outcomes (Fig 2). Whereas *BAP1*-mutant tumors tend to be of high grade and may show coagulative necrosis, *PBRM1*-mutant tumors may be of high or low grade and less frequently exhibit necrosis.<sup>12,30,37</sup> *PBRM1*- and *BAP1*-mutant tumors are associated with characteristic, but independent, gene-expression signatures.<sup>30</sup> In addition, *BAP1*-mutant tumors tend to be associated with mTORC1 activation, but *PBRM1*-mutant tumors are not.<sup>12,30</sup> Finally, *BAP1*- and *PBRM1*-mutant tumors are associated with markedly different outcomes in patients. In the localized or locoregional setting, patients with *BAP1*-mutant tumors have a 2.5- to three-fold higher hazard ratio for death than those with *PBRM1*-mutant tumors.<sup>30</sup> Similar results have been obtained in several other studies.<sup>5,6,37,63,64</sup> An IHC test has been developed with high sensitivity and specificity for *BAP1* loss.<sup>12,48,64</sup> Using this test, *BAP1* loss seems to be an independent predictor of outcome.<sup>48</sup> In a retrospective analysis of approximately 1,400 patients with nonmetastatic ccRCC, loss of *BAP1* in the tumor was associated with an increased risk of ccRCC-associated death (hazard ratio, 3.06; 95% CI, 2.28 to 4.10;  $P = 6.77 \times 10^{-14}$ ). *BAP1* loss remained an independent predictor after adjusting for University of California, Los Angeles-integrated staging system (UISS) variables and in patients with low stage, size, grade, and necrosis (SSIGN) scores.<sup>48</sup> The higher aggressiveness of *BAP1*-mutant tumors is reminiscent of its role in uveal melanoma, in which *BAP1* mutation correlated with metastasizing potential.<sup>65</sup> However, whether *BAP1* and *PBRM1* predict for outcomes in patients with metastatic disease remains to be determined. The largely exclusive nature of *BAP1* and *PBRM1* mutations in ccRCC coupled with associated differences in tumor biology and outcomes establishes a foundation for a molecular genetic classification of ccRCC (Fig 2C).

A small percentage of tumors harbor mutations in both *BAP1* and *PBRM1*, and these tumors seem to be the most aggressive.<sup>30</sup> Though mutation heterogeneity in tumors is well documented, evidence from analyses of tumorgrafts and IHC studies suggests that these mutations co-occur in the same tumor cells.<sup>12</sup> However, double-mutant tumors should be distinguished from tumors harboring subclones individually mutated for one or the other gene, and their outcomes may be different.

Though *BAP1* mutations are not always ubiquitous,<sup>6,48</sup> because of their association with tumor aggressiveness and poor outcomes, *BAP1*-regulated pathways may be appropriate therapeutic targets. As a tumor suppressor, *BAP1* itself is not a suitable target. There is a correlation between *BAP1* loss and mTORC1 activation, but this effect does not seem to be direct.<sup>12</sup> However, whether *BAP1* loss sensitizes to mTORC1 inhibitors remains to be determined. In addition, loss of *BAP1* may sensitize ccRCCs to radiation, but this effect is modest.<sup>12</sup> Therapeutic targets may arise from a greater understanding of the mechanism of *BAP1* action and, in particular, identifying the enzyme responsible for ubiquitinating relevant *BAP1* substrates.



**Fig 3.** Model for clear-cell renal cell carcinoma (ccRCC) development. The genes *VHL*, *BAP1*, and *PBRM1* are all located on chromosome (Chr) 3p (*SETD2* is also in this region; not shown). Following an intragenic mutation in *VHL*, loss of 3p, which is observed in the majority of ccRCCs, inactivates the remaining *VHL* allele along with one allele of *BAP1* and *PBRM1*. Subsequent mutation in the remaining *PBRM1* or *BAP1* allele results in ccRCC with different pathologic features and outcomes.

### MODEL OF ccRCC DEVELOPMENT

*VHL*, *PBRM1*, *SETD2*, and *BAP1* are within a 50 Mb stretch on chromosome 3p, in a region that is lost in approximately 90% of sporadic ccRCCs.<sup>11</sup> Deletion of this region simultaneously inactivates one allele of four ccRCC tumor-suppressor genes, leaving cells vulnerable to the loss of the remaining allele.<sup>11</sup>

The available data support the following model of ccRCC development (Fig 3). ccRCC may be initiated by an intragenic mutation of *VHL*, followed by the loss of chromosome 3p. *VHL* mutations are an initiating event and *VHL* inactivation has been observed in isolated cells lining tubules and in single-layered cysts.<sup>66,67</sup> Mutations in the remaining *PBRM1* allele would contribute to transformation and may synergize with subsequent mutations in *SETD2*. A second path involves mutation of the remaining *BAP1* allele, which may confer greater aggressiveness. The frequency of tumors simultaneously mutated for *BAP1* and *PBRM1* is lower than expected<sup>11,12</sup>; simultaneous inactivation of these two tumor-suppressor genes in the same tumor cell may reduce fitness. However, because simultaneous mutations do occur in some tumors, there may be a context-dependent advantage.

In a fraction of ccRCCs, there are no deletions of 3p; instead, there is copy-neutral LOH.<sup>6</sup> Analyses of data provided<sup>6</sup> reveal that these tumors also exhibit mutations in *PBRM1*, *SETD2*, and *BAP1*. Overall, MARS for these genes are similar to those observed in *VHL*, suggesting that, as for *VHL*, mutations in these genes preceded the chromosome 3p duplication event.

It is noteworthy that *SMARCC1* (encoding BAF155, a subunit of both BAF and PBAF complexes) is located on 3p21.31, between the

*VHL* and *PBRM1* genes (Table 2). Because of its location, one copy of *SMARCC1* is lost in most ccRCCs. This would make inactivating the second allele as accessible to the tumor cell as the inactivation of *PBRM1*. However, whereas *PBRM1* is mutated in 45% of ccRCCs, mutations in *SMARCC1* have not been detected among 459 kidney tumors with information in COSMIC.<sup>32</sup> This difference may be biologically significant and suggests that, in contrast to BAF180, BAF155 is not a ccRCC-tumor suppressor.<sup>35</sup> Furthermore, BAF155 function may be required for cell fitness.<sup>68-70</sup> Because of the selective loss of one allele in ccRCC, these tumors may be particularly sensitive to strategies inhibiting BAF155-dependent BAF/PBAF complexes.

The evolution of ccRCCs with mutations in *TCEB1* may be different from those with mutations in *VHL*, as *TCEB1* is on chromosome 8. Sato et al<sup>6</sup> provided extensive data on five tumors with *TCEB1* mutations. Mutations in *PBRM1*, *SETD2*, and *BAP1* were found in only one tumor (which had a *BAP1* mutation). The absence of *PBRM1* mutations potentially highlights the importance of the physical location of tumor-suppressor genes in tumor evolution.

### MUTATIONS IN mTORC1 PATHWAY GENES

Growth factor signaling pathways are frequently deregulated in cancer.<sup>71</sup> In ccRCC, however, receptor tyrosine kinases are rarely mutated.<sup>5,6</sup> Receptor activation leads to the recruitment of adaptor proteins, as well as class IA PI3K, to the plasma membrane.<sup>72</sup> Class IA PI3Ks are made up of a catalytic subunit (p110) and a regulatory subunit (p85; Fig 1). Among the different catalytic subunits, p110 $\alpha$  (encoded by the *PIK3CA* gene) is the most frequently mutated in

tumors.<sup>72</sup> *PIK3CA* is mutated in 2% to 5% of ccRCCs.<sup>5,6</sup> *PIK3CA* mutations tend to be missense mutations<sup>5,6</sup> and include mutations reported previously in other tumor types to increase PI3K activity in vitro. PI3K catalyzes the formation of the second messenger phosphatidylinositol-3,4,5-trisphosphate (PIP3) at the plasma membrane, which is downregulated by the tumor suppressor phosphatase, phosphatase and tensin homolog (PTEN). *PTEN* was previously shown to be mutated in ccRCC.<sup>73</sup> *PTEN* mutations are loss-of-function mutations and occur in 1% to 5% of ccRCCs (Table 1).<sup>5,6</sup> Activating mutations in *PIK3CA* or inactivating mutations in *PTEN* should increase PIP3 levels, leading to recruitment to the plasma membrane of proteins with PIP3-binding domains, such as AKT isoforms. AKT phosphorylates multiple substrates, including tuberous sclerosis complex 2 (TSC2), which forms a complex with TSC1.<sup>74</sup> The TSC1/TSC2 complex functions as a tumor suppressor. TSC1 stabilizes TSC2,<sup>75</sup> and TSC1 was previously reported to be mutated in approximately 4% of ccRCCs.<sup>44</sup> Although our group did not find mutations in *TSC2*,<sup>76</sup> *TSC2* may also be mutated in ccRCC.<sup>5,32</sup>

The TSC1/TSC2 complex functions as a GTPase-activating protein (GAP) leading to reduced levels of the active GTP-bound form of Ras homolog enriched in brain (Rheb). *RHEB* mutations were identified in four ccRCCs from the Cancer Genome Atlas.<sup>5,32</sup> Three of these mutations affected the same amino acid (Tyr35). Tyr35 assists in TSC2-mediated GTP hydrolysis,<sup>77</sup> and by reducing the activity of TSC2, mutations at Tyr35 may increase Rheb-GTP levels (L. Kinch and J. Brugarolas, unpublished data, July 2013). GTP-bound Rheb binds to and activates mTORC1.<sup>78</sup> Thus, mutations in *RHEB* represent another potential mechanism to activate mTORC1.

*MTOR* is mutated in approximately 5% of ccRCCs (Table 1).<sup>5,6</sup> mTOR nucleates two different complexes (mTORC1 and mTORC2). mTORC1 is implicated in cell growth control and may be the relevant target in tumorigenesis. mTOR is a serine/threonine kinase composed of HEAT repeats (approximately 40 amino acids each), which make up the N-terminal half of the protein, and a kinase domain that is flanked by two domains: FAT and FATC.<sup>79</sup> Recent structural studies revealed that the kinase domain adopts a bilobed structure with a central cleft that binds ATP. The kinase domain contains several insertions, including an approximately 100 amino acid insertion corresponding to the FKBP12/rapamycin-binding (FRB) domain, a domain that binds to rapamycin (also called sirolimus). The majority of *MTOR* mutations found in renal cancer are missense mutations.<sup>5,6,32</sup> However, unlike activating mutations in other oncogenes, *MTOR* mutations affect an extensive number of residues. Seventy percent of *MTOR* mutations in renal cancer converge on two domains, the kinase and FAT domains. Several mutations map to regions implicated in restricting substrate accessibility.<sup>80</sup> A few mutations (p.L1460P, p.S2215Y, and p.R2505P)<sup>32</sup> have been evaluated in vitro and increase mTORC1 activity.<sup>81</sup> These mutations did not appear to

increase mTORC2 activity, suggesting that mTORC1 is the relevant oncogenic complex.<sup>81</sup> Importantly, in two mutations examined (p.L1460P and p.S2215Y), sensitivity to sirolimus was preserved.<sup>81</sup> *MTOR* mutations have been hypothesized to sensitize to sirolimus analogs such as temsirolimus and everolimus.<sup>82</sup> However, mutations mapping to the FRB domain may affect binding to sirolimus (as well as temsirolimus and everolimus) and could confer resistance.

Though mutation frequencies in many genes that encode components of this pathway fail to reach statistical significance, as a whole, the mTORC1 pathway seems to be activated by somatic mutation in approximately 20% of ccRCCs.<sup>5,6</sup> Mutations in proximal mTORC1 regulators may predict responsiveness to mTORC1 inhibitors clinically. The first *TSC1* mutation reported in a ccRCC was found in a patient that remained on everolimus in the second line for 13 months after progressing on sunitinib after 3 months of treatment.<sup>44</sup> This led us to hypothesize that *TSC1* mutations clinically predicted for responsiveness to mTORC1 inhibitors.<sup>44,83</sup> This concept is supported by emerging data in renal cancer and other tumor types.<sup>82,84</sup> As for *TSC1*, mutations in *TSC2* and *RHEB* may predict for responsiveness to mTORC1 inhibitors clinically. However, whether mutations in genes encoding proteins more distant to mTORC1, such as *PIK3CA* and *PTEN*, predict for responsiveness to mTORC1 inhibitors is less certain.

A negative feedback loop links VHL and mTORC1 pathways (Fig 1).<sup>83</sup> mTORC1 is downregulated in response to a variety of stresses including hypoxia<sup>85,86</sup> and this is mediated, at least in part,<sup>87</sup> by regulated in development and DNA damage response 1 (*REDD1*).<sup>88</sup> *REDD1* expression is directly induced by both HIF-1 and HIF-2 in ccRCC,<sup>44</sup> and *REDD1* induction is sufficient to inhibit mTORC1.<sup>88</sup> Like many other HIF-target genes, *REDD1* is consistently upregulated in most ccRCCs.<sup>44</sup> However, mTORC1 is often activated in ccRCC.<sup>89</sup> This may be accomplished by mutations inactivating *TSC1* (which is required for *REDD1* signaling)<sup>44,88</sup> or *PTEN*.<sup>44</sup> However, this accounts for only a small percentage of tumors and how mTORC1 is reactivated in the rest despite *REDD1* induction, remains unknown.

## CONCLUSION AND FUTURE DIRECTIONS

Discoveries about the molecular genetics of ccRCC have shed light on tumor development, have led to the identification of previously unknown subtypes with different biology and outcomes, and may help with more accurate prognostication. These discoveries set the foundation for the next generation of molecularly targeted therapies.

## AUTHOR'S DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author indicated no potential conflicts of interest.

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