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Clear cell renal cell carcinoma ontogeny and mechanisms of lethality

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Abstract

There has been steady progress in dissecting the molecular features that define ccRCC initiation and progression. The recent publication of the TRACERx Renal papers and studies describing the interaction between tumor genomics and microenvironmental remodeling provide important new information for the field. This review summarizes common genomic and chromosomal copy number abnormalities in ccRCC, including 3p loss, and provides a mechanistic framework organizing these features into initiating events, drivers of progression and factors that confer lethality. The challenges researchers have faced developing animal models of ccRCC possessing these genomic features are described. The origins of DNA repair defects in ccRCC are discussed, with a focus on the role truncal mutations seen in ccRCC, including *VHL*, *SET2*, *PBRM1* and *BAP1*, may play in engendering genomic instability. Molecular subtypes in ccRCC that arise from these defects are then described, placing them into clinically and therapeutically relevant categories. These findings are then placed into the context of the tumor microenvironment, with a summary of multiple studies that describe how various mutations appear to modulate immune cell populations in ccRCC tumors. These data are then used to describe opportunities for disease prevention, early detection, prognostication and treatment are described.

1. INTRODUCTION

Renal cell carcinoma (RCC) affects over 400,000 individuals worldwide per year [1]. The age of diagnosis is approximately 60, and twice as many men are diagnosed as women [1]. There are several subvariants of RCC, and approximately 70 percent of individuals are diagnosed with clear cell RCC (ccRCC). Although ccRCC is a disease that can be caught early and successfully treated with surgical or ablative strategies, up to a third of cases will present with or develop metastases[2]. This disease state is almost uniformly lethal, and represents a critical distinction when we consider the biology of ccRCC.

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A timeline of discoveries leading to our current understanding of ccRCC ontogeny are summarized in Figure 1. These fundamental genetic and molecular features define the clear cell RCC tumors.

In brief, chromosomal features of three RCC cell lines were described in 1979 [3] and 3p loss was identified in primary tumor samples less than a decade later [4,5]. The discovery of the *VHL* gene in 1993 [6] provided the foundation of our understanding of ccRCC biology, both in the sporadic and hereditary settings. We had to wait nearly two decades, until the widespread use of next generation sequencing, to identify additional mutations in ccRCC [7–10]. Additional chromosomal copy number abnormalities were further characterized and found to be associated with prognosis [11–15]. Gene expression profiles subclassified ccRCC into prognostic risk groups [16,17]. From 2010 onwards a rapidly expanding series of publications describing genomic changes in ccRCC opened up a new chapter of our understanding of ccRCC genomics [7–10], and shortly thereafter studies of genomic heterogeneity provided both further insight into ccRCC clonal evolution [18–21] and raised important questions on how much tissue information is required to adequately characterize the genetic features of any one given tumor.

These studies raise important questions about the drivers of ccRCC ontogeny and how ccRCC evolves into a potentially lethal state. This review summarizes the consequences of VHL loss of function, the significance of 3p deletion and of additional chromosomal events on ccRCC tumor development, and mechanisms by which these changes may create the phenotypes observed in ccRCC. The review then focuses on the potential contribution of drivers of genomic instability, such as defective DNA repair, in ccRCC, an area of intense study with the potential to yield insights into the etiology and evolution of ccRCC A description of ccRCC molecular subtypes and events fostering tumor lethality then follows, and the interaction between ccRCC tumor cells and the microenvironment is discussed along with potential therapeutic implications. Figure 2 summarizes the key features of nascent and evolving ccRCC.

2. INITIATING FACTORS IN ccRCC ONTOGENY

Chromosome 3p Deletion- Initiating Factor in Nonhereditary ccRCC

The quest for additional truncal changes in RCC tumors has led to assessment of chromosomal copy number changes as a function of tumor progression and lethality [11,13]. Clear cell RCC is characterized by near-universal loss of most or all of chromosome 3p [10,13,22]. Polybromo 1 (*PBRM1*), SET domain containing 2 (*SETD2*) and BRCA associated protein 1 (*BAP1*) reside on 3p, and these genes are mutated at relatively high frequencies in ccRCC [7,9,10]. Additionally, several genes on 3p may be haploinsufficient, including the mismatch repair gene *MLH1* [23], and *SETD2* [24].

Chromosome 3p loss itself was described as early as 1987 [4] and the timing of 3p loss relative to subsequent genomic alterations was defined using array comparative genomic hybridization in combination with distance-based and branching-tree methodologies in 2000 [15]. More recently, the TRACERx Renal program prospectively collected tumor tissue samples from over 100 patients with ccRCC and collected multiple samples from each

tumor to ascertain tumor ontogeny, phylogeny and intratumoral heterogeneity [19–21]. These studies showed that loss of 3p was the earliest event in RCC ontogeny, likely occurring in adolescence [19,20]. A study in patients with VHL disease who underwent multiple ccRCC resections demonstrated that 3p loss in synchronously arising tumors occurred via different 3p breakpoint locations, or through whole chromosome 3 loss [25], suggesting that although 3p loss itself is a necessary event for carcinogenesis, the process leading to that loss is not driven by a specific chromosomal breakpoint.

The TRACERx study demonstrated that chromothripsis is a likely driver of 3p loss, in association with 5q gain [19,20]. Micronuclei form after mitosis when DNA fragments acquire a nuclear envelope, and are a caustic environment for the DNA contained within- a driver for DNA damage, mutation, and chromothripsis [58]. Underlying causes of micronuclei formation include aneugens and clastrogens, the latter generally associated with formation of acentric chromosome fragments [26], which lack a centromere and fail to attach to the mitotic spindle. The presence of chromosome bridges during telophase can also lead to DNA breakage during cytokinesis and formation of micronuclei [27]. Hypoxia and oxidative stress have been linked to micronucleus formation as well, [26] conditions that are potentially present in the proximal tubule. Other physical changes in the cell, including pressure, temperature, radiation, UV and ultrasound can increase micronuclei [26]. It is important to bear in mind that the kidney proximal tubule and nephron is a fairly harsh environment, dictated by intense gradients of oxygen, salts, and nutrients. These factors are further misregulated in renal states including renal failure as well as in polycystic kidney disease [28]. Thus, conditions around the RCC cell of origin may create a “perfect storm” that permits the development of micronuclei, chromothripsis and 3p loss.

VHL Loss in ccRCC

The loss or mutation of the von Hippel Lindau (*VHL*) gene is generally considered to be one of the obligate initiating steps in the development of ccRCC. Germline loss of *VHL* is inherited in an autosomal dominant fashion in families with VHL disease [6], and these individuals often develop multifocal, bilateral ccRCC [29,30]. Somatic loss of *VHL* is observed in the majority of patients with sporadic clear cell RCC [31], and based on the TRACERx studies, may follow 3p loss in these patients [19].

VHL and HIF regulation—The VHL protein is an E3 ubiquitin ligase whose best understood function is the ubiquitination of the prolyl hydroxylated transcription factors hypoxia inducible factors 1 and 2 alpha (HIF1α and HIF2α) [32–34] under normoxic conditions, with their subsequent proteolytic degradation [35]. HIF1α and 2α regulate transcription of a number of genes involved in angiogenesis, metabolism and chromatin remodeling [36]. Even before the functional link between VHL and HIF was made, ccRCC was shown to have increased levels of vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF) mRNA [37] and HIF mediated transcription of pro-angiogenic factors including VEGF was shown to facilitate the development of neovasculature [33].

HIF1α and HIF2α regulate overlapping gene sets, with distinct differences between the two transcription factors [38–40]. HIF1α regulates a number of genes involved in enhancing glucose uptake and shifting cells towards glycolysis including pyruvate dehydrogenase kinase [41] and lactate dehydrogenase A [42], as well as mediating mitochondrial autophagy via BNIP3 [43] and BNIP3L [44]. Axl [45], a member of the TAM family of regulatory proteins [4647] was found to be a HIF1α driven gene [48]. *AXL* gene expression was associated with poor prognosis in ccRCC [4846] and Axl protein levels were increased after sunitinib treatment [49]. HIF2a has been shown to regulate the *EPO, CCND1* and *TGFA* genes [36,39]. Some evidence points towards HIF2α, but not HIF1α being the primary driver of RCC oncogenesis, with HIF2α both necessary and sufficient for the growth of VHL null cell lines [36,50]. A separate set of studies in animal models implicated HIF1α [51] as the primary driver as opposed to HIF2α [52,53] in renal carcinogenesis, and questions remain on the exact role of specific HIF isoforms in the development of RCC, perhaps implying a temporal dominance in which one factor plays a larger role at various states of disease. A more recent study showed that loss of either HIF1α or HIF2α abrogated tumor formation in a *Vhl/Trp53* deficient murine model [54], indicating both are required for tumor initiation in that system.

VHL and non-HIF targets—The VHL protein has been shown to regulate a number of additional molecules, pathways and processes [55]. These include AKT [56], nuclear factor kappa-light-chain-enhancer of activated B cells (NK-kB) [57–59], tank-binding kinase-1 (TBK1) [60], the extracellular matrix, the primary cilium and mitosis. A few of these are depicted in Figure 3, and will be discussed briefly below. VHL is also linked to regulation of DNA repair [61–64], which is discussed in a subsequent section.

VHL-mediated NFkB signaling (Figure 3a): Rettig and colleagues demonstrated that loss of VHL was associated with an upregulation of NF-kB activity [57] through HIF dependent induction of transforming growth factor alpha (TGFα). In addition to this HIF-transcription circuit, VHL was subsequently shown to serve as an adaptor to promote the inhibitory phosphorylation of the nuclear agonist Card9 by casein kinase 2 (CK2) and reduce NF-kB activation in RCC independently of HIF [58]. Peri and colleagues demonstrated upregulation of NFkB and IFN signaling pathways in the absence of VHL [59]. More recently, zinc fingers and homeoboxes 2 (ZHX2), an activator of NF-kB was found to be targeted and downregulated by VHL directly through a similar mechanism of ubiquitylation and proteasomal degradation [65]. Thus, loss of VHL leads to coordinated stabilization of factors which can have independent activity to promote NF-kB signaling.

VHL activity related to the extracellular matrix (Figure 3b): VHL has been reported in several lines of investigation to be involved in the regulation of intercellular junctions [66] and the extracellular matrix (ECM) [67–70]. Several investigators have shown that VHL binds to fibronectin, and this step is necessary for the promotion of a correct ECM assembly in cells [68]. All tumor causing *VHL* mutants have been reported to be defective in in fibronectin binding [71–73]. This binding is regulated by CK2, as phosphorylation of VHL by CK2 at amino acids 33, 38 and 43 was found to be necessary for fibronectin binding by VHL and the development of a normal ECM [74]. Mutation of serines 33, 38 and 43 was associated with eventual tumor growth in a xenograft model when compared to wildtype VHL [74].

Conversely, CK2 inhibition resulted in VHL stabilization due to decreased N-terminal cleavage and proteasomal degradation, suggesting regulation by CK2 is linked to VHL protein stability [75].

VHL and microtubule stability (Figure 3c): VHL has also been reported to enhance microtubule stability [76], an interesting observation given the more recent linkage of SETD2 to methylation of the cytoskeleton (see below). This has implications for several important microtubule structures in the cell, including the primary cilium and the mitotic spindle. Microtubules comprise the central core of the ciliary axoneme, and loss of VHL results in defects in ciliogenesis[77–79]. As a result, VHL disease is often classified as a "ciliopathy", along with other diseases linked to defects of the primary cilium such as polycystic kidney disease [80]. Activation of Aurora kinase A (AURKA) is known to inhibit ciliogenesis, and this kinase was recently shown to be regulated by VHL. VHL mono-, rather than poly-ubiquitinates AURKA in a PHD-independent manner, targeting AURKA for degradation, which in quiescent cells is required to maintain the primary cilium [81].

VHL and mitotic spindle function (Figure 3c): VHL has also been linked to mitotic spindle function[82–84]. Krek and colleagues were the first to show that VHL loss resulted in reduced Mad2 (mitotic arrest deficient 2) levels, which was associated with spindle misorientation and loss of normal mitotic checkpoint control in an isoform-specific manner [82]. The underlying molecular mechanism was identified when Mad2 translation was shown to be regulated by miR-28–5p in a VHL dependent fashion, with loss of VHL resulting in increased miR-28–5p, and decreased Mad2 translation. [84] Furthermore, ischemic injury in a kidney-targeted *Vhl* deficient murine model demonstrated cyst-inducing spindle misorientation and aneuploidy immediately post-injury, and longer term follow- up demonstrated the development of cysts, clear-cell type cells and dysplasia [83]. Thus, VHL may play a role in regulation of both mitotic and nonmitotic cells via microtubular homeostasis.

VHL-mediated PI3K signaling: VHL was found to downregulate AKT [56], a key component of the PI3K pathway. Upregulation of the PI3K pathway has been associated with more aggressive behavior in many cancers [85], including ccRCC [86,87]. AKT phosphorylation was associated with increased tumor aggressivity and with cytoplasmic translocation and deactivation of p27 [88].

VHL mutations and development of animal models for RCC—Based on the observation that germline *VHL* mutations in humans drove the development of pleiotropic lesions, including ccRCC, it was presumed that the loss of *VHL* was sufficient for tumorigenesis. However, a number of studies have shown that a *Vhl* knockout or mutation will not engender RCC in murine models [89–91]. The first whole animal heterozygous knockout model published in 2001 did not produce a RCC phenotype [89] and subsequent efforts at creating kidney specific biallelic *Vhl* knockouts did not produce bona fide RCC lesions despite using a variety of kidney specific promoters [92], including a *Actb-Cre* [93], a proximal tubule specific *Pepck-Cre* [94], a pan-tubular *Pax8-Cre* [53,95], and a thick ascending limb specific *Thp-Cre* [96].

In addition, studies of renal cysts that arise in VHL patients have shown that these benign lesions have inactivated both copies of VHL[97], they often do not display other characteristic hallmarks of RCC [98]. These findings indicate while VHL loss is necessary for RCC formation, it is not necessarily sufficient.

Subsequent to these single-gene knockout models, knockout of Vhl was combined with other potential RCC driver genes. Several transgenic animal models employing the proximal tubule specific *Ksp1.3* were used to drive a *Cre* recombinase and inactivate paired or multiple genes in a kidney specific manner [99,100]. The combined inactivation of *Pten* and *Vhl* generated cysts[100] whereas the combined inactivation of *Trp53* and *Vhl* generated simple cysts, atypical cysts and neoplasms which demonstrated mTORC1 activation and elevated expression of Myc [99]. Neither model developed tumors with metastatic potential. A further evolution of the latter model involved the combined *Ksp1.3*-Cre driven inactivation of *Trp53, Vhl* and *Rb*, which produced neoplastic lesions that demonstrated histological and transcriptomic features that resembled those of human ccRCC, while still not demonstrating metastatic potential [101].

More recent efforts at model creation using a polygenic approach or a focus on the recently discovered chromatin remodeling gene mutations have yielded results which are bringing us closer to producing representative model of ccRCC [102–104]. These are described in greater detail in subsequent sections.

Additional Mutations in ccRCC

Several other genes, all found on chromosome 3p, are mutated at a relatively high frequency in ccRCC [7–9], and are likely associated with both convergent and divergent phenotypic characteristics in ccRCC. All are involved in some way with chromatin remodeling, and are summarized in a recent review [105].

PBRM1—PBRM1 is the most commonly mutated gene after *VHL.* Human *PBRM1* generates a 1689 amino acid protein, consisting of six bromodomains, two bromo-associated homology domains, and a C-terminal high-mobility group [106]. PBRM1 is the defining component of the switch/sucrose nonfermenting (SWI/SNF) chromatin remodeling complex known as PBAF [107]. PBAF is an adenosine triphosphate (ATP)-dependent chromatin remodeler governs nucleosome structure and positioning, and gene transcription. Loss of PBRM1 and PBAF activity has potential to significantly undermine the highly organized structure of chromatin and disrupt transcription. Numerous studies have identified *PBRM1* mutations in ccRCC [7,18, 108], with an incidence of 30–40 percent, although the most aggressive sarcomatoid variants tend to lack these mutations [109]. *PBRM1* mutations precede subsequent *SETD2* mutations, suggesting these sequential mutations participate in subclonal tumor evolution[19].

Several studies are beginning provide mechanistic insight in to the consequences of PBRM1/PBAF loss in ccRCC. Gao *et a*l demonstrated that PBRM1 deficiency was associated with increased cellular growth and a HIF transcriptional signature [110]. A study by Nargund *et al* revealed that *Pbrm1* deficiency enhanced *Vhl* loss dependent upregulation of HIF1α and STAT3, and increased mammalian target of rapamycin (mTOR) signaling, thereby enabling

the development of renal lesions in a murine transgenic model. Liao *et al* showed that PBRM1 and other chromatin remodeling genes lost in RCC antagonized tumor growth in an interferon-stimulated gene factor 3 dependent fashion, and loss of PBRM1 relieved this inhibition [111]. Espana-Agusti and colleagues showed that *Pbrm1* knockout rescued *Vhl*-loss induced replication stress and enhanced tumor growth in a transgenic model [63]. Inactivation of *Pbrm1* or *Bap1* in conjunction with *Vhl* employing a *Pax8-Cre* in a murine model recapitulated the relatively low aggressivity of *Pbrm1/Vhl* knockout in a transgenic murine model [102]. Liu *et al* demonstrated that PBRM1 loss downregulated interferon gamma (IFNγ) mediated signaling [112]. Cai *et al* showed that PBRM1 acted as a lysine reader for p53, and loss of PBRM1 resulted in decreased p53 dependent CDKN1A transcriptional regulation [113]. Similar to VHL, PBRM1 also plays a role in genomic stability. PBRM1 localizes to the kinetochore [114], and in yeast, the PBRM1 homolog RSC2 is essential for chromosome arm cohesions [115,116]; loss of PBRM1/PBAF results in genomic instability and aneuploidy [117]. Thus the potential impact of PBRM1 loss includes the augmentation of hypoxia signaling, modulation of replication stress, impairment of p53 mediated cell cycle regulation, inhibition of interferon response and genomic instability. However, in RCC, in the absence of additional mutations in SETD2, these changes appear to produce tumors that are less aggressive, with high angiogenic potential, that are immunologically cold[112,118,119].

BAP1—Another 3p chromatin remodeler inactivated in RCC is the BAP1 deubiquitinase[120]. The *BAP1* gene produces a 729 AA protein containing several functional domains: a ubiquitin C-terminal hydrolase (UCH) domain, a BRCA1-associated RING domain protein 1 (BARD1) binding domain, an HCF-C1 binding motif (HBM) and a nuclear localization signal [8]. *BAP1* is mutated in 10–20 percent of ccRCC [8,121]. Subsequently histone 2a lysine 119 (H2AK119) was shown to be the target for this deubiquitinase [8,122], which is involved in Polycomb complex mediated gene repression. In a whole animal murine model, BAP1 also interacted with the cell cycle regulator host cell factor-1 (HCF-1), the signal transduction modulator O-linked N-acetylglucosamine transferase (OGT), and the Polycomb group proteins ASXL1 and ASXL2. OGT and HCF-1 levels were decreased by *Bap1* deletion, and were associated with myeloid transformation [123]. A subsequent study reported that BAP1 modulated gene expression in a cell-type specific manner, and loss of BAP1 promoted tumorigenesis in cells that did not engage an RNF2-dependent apoptotic program [124].

Similar to both VHL and PBRM1, BAP1 also plays a role in maintenance of genomic stability. BAP1 associates with the mitotic spindle regulating microspherule protein-1 (MCRS1), and loss of BAP1 resulted in increased chromosomal instability [125]. The discovery of *BAP1* mutations in ccRCC arose from the study of patient derived xenograft models. Brugarolas and colleagues used this model to increase relative tumor purity and to identify *BAP1* as a novel tumor suppressor in ccRCC[8]. They then demonstrated that heterozygous *Bap1* deletion coupled with homozygous *Vhl* loss using a SIX homeobox 2 (*Six2*)- Cre [126] (*Six2-Cre;Vhl*$^{F/F}$*;Bap1*$^{F/+}$) to drive kidney specific gene inactivation could produce neoplasms in the murine kidney, [127]. A follow up study employed a *Pax8-Cre* to generate kidney specific *Vhl/Pbrm1* and *Vhl/Bap1* knockouts [102]. *Pax8-Cre;Vhl*$^{F/F}$*;Bap1*$^{F/+}$ mice produced neoplastic renal lesions with nuclear pleomorphism, nucleolar prominence,

atypia, mitosis and elevated phospho-S6 expression. in comparison, *Pax8-Cre;Vhl*$^{F/F}$*;Pbrm1*$^{F/+}$ animals failed to develop renal lesions and *Pax8-Cre;Vhl*$^{F/F}$*;Pbrm1*$^{F/F}$ animals produced tumors possessing low-grade features, including moderate amounts of cytoplasm, lipid and glycogen accumulation, relative lack of nuclear atypia, and the absence of phospho-S6 staining. [102]. Loss of *BAP1* is strongly associated with poor risk in patients with ccRCC, even in individuals who have clinically low stage tumors [128]. In patients treated with targeted therapy, presence of a *BAP1* mutation was associated with worse outcome [129]. A rare familial syndrome at risk for ccRCC is caused by germline mutations in *BAP1* [130]. In the TRACERx study, *BAP1* mutations were associated with high weighted genome instability index (wGII), low intratumoral heterogeneity (ITH) tumors, suggesting that the driver function of *BAP1* loss is sufficient to provide ccRCC clones with a clear fitness advantage and lethal potential [19].

SETD2—*SETD2* is a chromatin remodeling gene and a tumor suppressor in ccRCC [9]. *SETD2* mutations were first discovered through early high throughput genomic sequencing efforts [9] that preceded the Cancer Genome Atlas (TCGA) project, which also validated these findings [10]. *SETD2* is mutated in 10–20 percent of cases of ccRCC [9,10] SETD2 is a 2564aa long nonredundant histone H3 lysine 36 methyltransferase containing a SET domain and a C-terminal Set2 Rpb1 interacting (SRI) domain, specifically marking nucleosomes of actively transcribed genes with H3K36me3. In yeast, this mark, along with demethylation (H3K36me2) has a repressive role to prevent cryptic initiation from alternate internal transcriptional start sites [131]. However, utilizing selective *Set2* mutants that isolate mono, di, and trimethylation states, Rathmell and colleagues determined that the isolated loss of trimethylation, as occurs in mammalian cells with *SETD2* loss, is not sufficient to promote a cryptic initiation phenotype [132]. Instead, the chromatin effects of this mark appear to play major roles in governing DNA repair, as recently reviewed [133].

In addition to DNA repair, another role for SETD2 in maintenance of genomic stability is a newly discovered role in methylation of microtubules [134,135]. SETD2 trimethylates α-tubulin on lysine 40 (αTubK40me3). *SETD2*- deficient cells demonstrated mitotic spindle defects, and in cytokinesis, and showed an increase in the formation of chromosome bridges and micronuclei. *SETD2* mutated in the SET domain and the SRI domain could not methylate microtubules, which resulted in more chromosome bridges and lagging chromosomes relative to wild-type *SETD2*. These data indicated that in addition to the catalytic domain, a functional SRI domain was also required for αTubK40me3 [134]. Monoallelic *SETD2* deficiency, as would be expected in cells with 3p loss, was also shown to abrogate spindle microtubule trimethylation on αTubK40, but not methylation of histones on H3K36, and this was sufficient to induce genomic instability [24]. It is possible that monoallelic loss of *SETD2* may further aid in the steady increase in chromosomal copy number abnormalities seen as tumors increase in size and stage [11,13]. Most recently, SETD2 was found to also methylate cytoskeletal actin, and to promote polymerization of actin filaments and cell motility [136]. As noted above, *SETD2* mutations can arise independently of, or occur in conjunction with, *PBRM1* mutations in ccRCC [19]. Interestingly, functional convergence between SETD2 and PBRM1 was revealed with the discovery that PBRM1 recognizes the αTubK40me3 SETD2 mark on microtubules [137]. In this study, PBRM1 was

shown to localize the PBAF complex to the mitotic spindle to maintain genomic stability. Consistent with a “reader” “writer” relationship, in the TRACERx study, when both *SETD2* and *PBRM1* mutations co-occur, loss of PBRM1 precedes loss of SETD2. At this point in time there is no published animal tumor model of *SETD2* inactivation in RCC.

3. POTENTIAL ETIOLOGY OF DNA REPAIR DEFECTS in ccRCC

The initiating events for ccRCC involve biallelic *VHL* loss and chromosome 3p loss [10,19,31]. In addition to these events, ccRCC demonstrate an intermediate number of mutations per megabase with a relatively limited range [10,20] and a stage dependent increase in the number of chromosomal copy number alterations [13]. The mechanisms driving these genomic events are slowly being elucidated, and although not part of current clinical practice, have the potential for providing new therapeutic approaches in ccRCC.

Evidence for a mismatch repair defect in ccRCC

Several pathways exist involved in the repair of specific types of DNA damage, and have recently been reviewed in in the context of potential therapeutic approaches [138]. Alexandrov *et al* analyzed mutational patterns in tumors with known genomic defects. Using an algorithm that considered nucleotides surrounding mutational sites, they identified 21 different patterns, which were associated with known germline deficiencies [139]. Using this approach, ccRCC was linked to a pattern associated with aging, as well as a pattern found in tumors with mismatch repair (MMR) in a subset of individuals. There are several potential mechanisms that could lead to a mismatch repair deficiency in ccRCC (Figure 4). The MutL Homolog 1 (*MLH1*) gene, an essential component of the MUTL complex, is found on chromosome 3p. Evidence for MUTL haploinsufficiency has been reported in pancreatic cancer [23]. Loss of VHL upregulates histone deacetylase 6 (HDAC6) levels [140]. HDAC6 sequentially deacetylates and ubiquitinates MutS protein homolog 2 (MSH2) leading to MSH2 degradation [141]. In addition, HDAC6 was shown to reduce sensitivity to DNA-damaging agents and cellular DNA mismatch repair activities via MSH2 downregulation [141] In addition, SETD2 mediated H3K36 trimethylation acts as an essential docking site for the MUTS/MUTL complex [142]. Specifically, the hMutSα complex is directed to actively transcribed genes through interaction with the H3K36me3 mark. Thus, in the absence of H3K36me3, although MMR is intact, microsatellite instability is present, and most importantly repair may be less efficiently targeted to the most relevant areas of the genome. This interaction implies a critical activity of H3K36me3, targeting mismatch repair the active coding regions.

Taken together, these data point to multiple potential defects in the integrity of the MMR complex in ccRCC. However, there is a very low rate of microsatellite instability seen in RCC [143,144]. Additionally, ccRCC has a relatively low absolute mutation rate, with frequencies approximately one tenth of those seen in tumors from patients with Lynch syndrome[139]. These findings suggest that if present, a nonclassical form of MMR defect is operative in ccRCC. Further study is needed to clearly elucidate the role of MMR defects in ccRCC mutagenesis.

Evidence for a Homologous Recombination Repair (HRR) Defect in ccRCC

The two main pathways involved in double-stranded DNA repair include nonhomologous end joining (NHEJ) which acts throughout the cell cycle and homologous recombination repair (HRR) which is operative in S phase and requires two DNA strands for error-free repair [145]. Defects in HRR are associated with increased mutational frequency and cancer predisposition, classically manifested in families bearing mutations in HRR related genes, including *BRCA1* [146,147], *BRCA2* [148], *PALB2* [149], and *ATM* [150].

A number of groups have assessed the integrity of HRR in ccRCC (Figure 5). Metcalf and colleagues reported that suppressor of cytokine signaling 2 (SOCS2) mediated monoubiquitination of VHL at K63 [61]. Loss of VHL, or *VHL* mutations that compromise its K63-ubiquitylation attenuated the DNA-damage response (DDR), resulting in decreased HRR and persistence of DSBs [61]. Espana-Agusti *et al* demonstrated that loss of *Vhl* enhanced replication stress in an animal model [63], suggesting a role in the maintenance of genomic integrity during mitosis. Glazer and colleagues demonstrated a close link between hypoxia and HRR defects, driven mainly via transcriptional regulation of key HRR components [151–153], and demonstrated that *VHL* loss was associated with a HRR defect [62]. VHL was found to associate with, and decrease activity of KAT5/TIP60, an acetyltransferase associated with p53 activation [154]. TIP60 was also found to be essential for H4K16 acetylation, which evicts 53BP1 from chromatin and favors use of HRR in DNA repair [155], and in ATM acetylation [156], which is a necessary step prior to autophosphorylation and activation. Recent work has further expanded this observation to demonstrate that VHL mediated regulation of TIP60 shifts DNA damage repair to HRR from error-prone nonhomologous end joining (NHEJ) in a HIF-independent fashion, and ATM activation was impaired in VHL deficient cells due to decreased ATM acetylation [64].

SETD2 is involved in regulating a number of processes associated with HRR. Most well characterized is the recruitment of DNA repair machinery to the sites of actively transcribed genes [157,158]. Since H3K36me3 is preferentially placed at sites of active transcription, it provides a convenient mechanism for readers involved in DNA repair to be preferentially recruited to high priority genomic sites for repair [159]. The loss of SETD2 was shown to specifically alter the efficiency of repair at these active sites. Further work demonstrated a more active role in mediating repair, via participation in engaging the p53 checkpoint, which was also discovered to be deficient in SETD2 knockout cells [158]. This defect in repair is not unlike mutations in *RAD51*, which produce a signature of DNA defects, one hallmark of which is microdeletions due to microhomology repair mechanisms, and a reliance in the absence of SETD2 on nonhomologous repair (as is observed also with *RAD51* deficiency) [160]. More recently, the effect of impaired HRR to induce replication stress and mutations has been considered as a source of heterogeneity and tumor evolution in ccRCC [161]. Thus, *SETD2* loss may function in providing a hallmark of cancer, which is the genomic instability sufficient to generate a broad array genetic defects, and promote cancer evolution and the acquisition of deleterious events that have metastatic and lethal consequences. SETD2 was recently found to regulate H4K16 acetylation via modulation of H3K36 methylation [162], an important molecular determinant between HRR and error-prone nonhomologous end joining

(NHEJ) [155]. As such, it is possible the combination of *SETD2* loss together with *VHL* deficiency potentiates the development of DNA double strand repair breaks.

A number of studies have evaluated the effects of BAP1 loss on DNA repair. Pena-Llopis *et al* showed that loss of BAP1 sensitized RCC cell lines to radiation and PARP inhibitors [8]. BAP1 was shown to mediate poly (ADP-ribose)-dependent recruitment of the Polycomb-repressive deubiquitinase complex (PR-DUB) to sites of DNA damage, was phosphorylated by ATM, and promoted repair of DNA double-strand breaks [163]. As noted above, loss of BAP1 destabilized MCRS1 and decreased mitotic stability [125]. These data suggest BAP1 is regulator of DNA repair and helps maintain copy number integrity, and its loss may foster genomic instability in ccRCC.

A few significant caveats exist for the existence of a bona fide HRR defect in ccRCC. The first is that RCC is not typically sensitive to platinum and other DNA damaging agents. The second is that the Alexandrov paper does not identify ccRCC as possessing a DNA damage pattern typically seen in tumors from patients with stereotypical germline defects in HRR genes [139]. The possible explanation for this discrepancy is that the signature derived from BRCA mutated tumors is sufficiently specific that it does not translate into other variants of HRR deficiency. Evidence supporting this possibility include a study that generated a homologous recombination defect (HRD) signature to identify tumors which have broad defects in HRR [164]. This HRD signature identifies tumors with vulnerabilities in HRR despite not having mutations in classical HRR genes. Preliminary data suggest these signatures could help identify whether a HRD exists in ccRCC and guide therapeutic approaches [165].

4. ccRCC MOLECULAR SUBTYPES AND DRIVERS OF LETHALITY IN ccRCC

One of the key questions in ccRCC cancer evolution is whether lethality is an innate characteristic, or develops over time and through acquisition of additional genomic lesions. A number of studies in the past few years have demonstrated that both scenarios are operative [19,21].

The TRACERx study defined seven major clonal subtypes of ccRCC: VHL monodriver, PBRM1-SETD2, PBRM1-somatic copy number alteration (SCNA), PBRM1-PI3K, VHL wildtype, multiple clonal driver, and BAP1 driven [19]. These subtypes are genomically distinct and are prognostically heterogeneous. A number of determinants were assessed in these subtypes, including the weighted genomic instability index (wGII), which is a measure of the fraction of the tumor genome affected by somatic copy number alterations (SCNA); percentage of cells positive for Ki67; the intratumoral heterogeneity (ITH) index, defined as the number of subclonal drivers divided by the number of clonal drivers, where drivers include all driver mutations and driver SCNAs; clonal structure; and clinicopathological parameters [19].

The VHL monodriver subcategory was largely found in small tumors, and demonstrated low ITH, wGII and Ki67 levels, and may represent temporally earlier tumors, or tumors that

inherently lack the ability to develop additional lethal events. On the other end of the spectrum, the multiple clonal driver subtype showed high levels of wGII and Ki67 but relatively low levels of ITH, showing that simultaneous appearance of a small number of clonal drivers is sufficient to create RCC clones with a strong selective advantage [19].

Prognostic Implications of SCNAs in ccRCC

Several studies in addition to the TRACERx papers have shown that SCNAs are associated with poor clinical outcome [166,16711–14]. Partial or whole chromosomal losses or gains occur in chromosomes 5, 6, 7, 8, 9 and 14 at a relatively high frequency, and increase as a function of tumor size and grade [11,13].

Not only are higher overall SCNAs associated with more aggressive disease, specific chromosomal changes are linked to metastatic potential suggesting a driver function. A study of 703 ccRCC tumors published in 2009 revealed that loss of 9p was associated with poor outcome [12]. An analysis of chromosomal copy number changes in 111 ccRCC patients revealed that loss of 14q together with a gain in 8q was significantly associated with poor prognosis [13,14]. In the TRACERx study, 9p loss was identified as the factor most significantly associated with the formation of metastases [21], and 14q loss was significantly associated with development of metastasis prior to correction for multiple testing [21]. In addition, 9p loss in metastasizing clones was a significant predictor of OS after correction for known clinical variables [21]. Assessment of potential tumor suppressor or pro-oncogenic genes on these chromosomal regions identifies several candidates. Chromosome 14q contains *HIF1A*, which has been shown to act antagonistically to HIF2a in ccRCC progression [168]. Chromosome 9p encodes *CDKN2A*, which generates p16/INK4A, a critical G1 cell cycle regulatory protein. Chromosome 8q harbors the *MYC* gene. Copy-number alterations of these genes may be responsible for engendering a selective advantage for tumor cells, or there may be additional genes on these chromosomes whose loss or gain cooperatively enhance cell fitness [21]. The challenge for the field is to understand the mechanism underpinning SCNA, with the goal of either blocking its development, or targeting specific vulnerabilities in cells possessing these SCNAs.

ITH in ccRCC

ITH is a major point of concern in the molecular characterization of ccRCC. Clinically relevant ITH is generated by mutations in key genes and by driver SCNAs [19]. The TRACERx Renal team defined the ITH index as the number of subclonal drivers over the number of clonal drivers, where drivers included all driver mutations and driver SCNAs [19]. Future work will permit a more nuanced weighting of these driver events. Lower levels of ITH are seen at both ends of the tumor lethality spectrum. Tumors harboring only *VHL* mutations as well as tumors with multiple driver mutations have low ITH. In the latter case, the clonal genomic events likely confer a significant selective advantage permitting these cells to be the primary clone generating the tumor mass and impart aggressive tumor behavior.

Genotypic divergence is likely limited by constraints in cellular viability and fitness, and this may lead to phenotypic convergence and a manageable number of targetable pathways [169].

Further work needs to be performed to define the phenotype to be targeted and to come up with tailored therapies that address these specific tumor features.

Sarcomatoid RCC

A special comment concerns aggressive subsets of ccRCC. Sarcomatoid variation is a histologic designation assigned to highly aggressive tumors with underlying ccRCC features. Sircar *et al* performed transcriptomic and genomic analyses on sarcomatoid RCC. They found a significantly lower rate of 3p mutation overall, and an enrichment of *PTEN*, *TP53*, and *RELN* compared with ccRCC [109]. This pattern held up even when the tumors were regionally sampled in areas harboring sarcomatoid patterns as compared to regions with more traditional histology. Similar findings were observed by others [170,171]. These findings suggest that sarcomatoid ccRCC may have fundamental differences in its early molecular pathogenesis when compared to non-sarcomatoid ccRCC. Interestingly, the overall mutational burden is not broadly different between sarcomatoid and nonsarcomatoid ccRCC [170,171], suggesting either similar mechanisms of mutation or a cell-type specific threshold for mutational burden that is similar between the two entities.

5. INTERACTION BETWEEN ccRCC AND THE TUMOR MICROENVIRONMENT

Multiple groups have assessed the diversity of intratumoral immune cells in ccRCC (Table 1) [119,172–177112]. One of the key questions is whether there are identifiable interactions between tumor genomic features and the microenvironment that can inform therapeutic decisions (Figure 6). A number of studies have been performed assessing these interactions, and are summarized in Table 1.

Chevrier and colleagues performed mass cytometric high-dimensional single cell analysis of ccRCC samples from 73 individuals with varying stages of disease with a focus on T-cell and tumor associated macrophages (TAM) [172]. This analysis demonstrated diverse populations of immune and macrophage populations, with significant intra- and inter- cell population and patient heterogeneity. Specific patterns of T-cell and macrophage co-expression were reported, with exhausted/regulatory T-cells associated with a specific TAM subgroup expressing high levels of HLA-DR, CD68, and CD64, as well as CD204 and CD38. Other TAM subgroups expressed both pro- (CD163, CD204, CD206) - and antitumoral (CD169) TAM markers, although all of these subgroups were associated with more advanced (as defined as higher than stage 1) tumors, prevalence of additional TAM subgroups not specifically associated with a T-cell subpopulation were associated with worse progression free survival. This study illustrates the tremendous diversity of T-cell and TAM populations, and the challenges in identifying driver populations and therapeutic strategies. This study did link these cell populations to tumor genomic features. Doing so will help our understanding of the interplay between tumor-cell specific features and the immune microenvironment.

Wang *et al* used a comparison between uninvolved kidney, bulk tumor and tumorgraft transcriptomes to identify stromal and immune contributions to the overall expressed gene

set in ccRCC and in other RCC tumor subtypes. Employing an in-house algorithm based on a Bayesian hierarchical model they named DisHet, they identified an immune/stromal gene set (eTME) specific to ccRCC, with 2080 genes expressed 3-fold higher and 904 genes expressed 20-fold or higher in the immune/stroma component than in the tumor [175]. Using the eTME gene sets, the authors found that 65, 22 and 9 percent of immune signature genes in the Immunome[178], ESTIMATE[179] and Winslow[180] sets were not abundantly expressed in the ccRCC immune/stromal compartment. They applied the eTME gene set to TCGA RCC datasets, including those representing ccRCC, papillary and chromophobe RCC. They identified an eTME high subgroup, which was associated with increased immune cell and complement transcripts, and a noninflamed group. There was a relatively low prevalence of inflamed tumors in type I papillary RCC. In ccRCC, the inflamed group was enriched for *BAP1* mutations, and, using an annotated institution-specific patient cohort, demonstrated the inflamed subgroup manifested IMDC poor-risk features, including anemia and thrombocytosis, and worse prognosis [181].

A study assessing the immune microenvironment in 409 patients treated with sunitinib or pazopanib applied unsupervised consensus nonnegative matrix factorization (cNMF) clustering on expression microarray data and identified four biologically distinct clusters[119]. These clusters demonstrated varying response rates and OS. Cluster 4 was associated with worse OS, IMDC poor risk categorization, elevated PD-L1 levels and *BAP1* and *TP53* mutations, and anticorrelated with *PBRM1* mutations. Tumors harboring *PBRM1* mutations were associated with higher angiogenesis gene expression, and *BAP1* mutated tumors with lower angiogenesis gene expression. Pathway analysis demonstrated elevated inflammatory signatures, including IFN gamma response in Cluster 4, and the highest immune score using the ESTIMATE[179] algorithm. Higher macrophage infiltration was associated with worse response to antiangiogenic therapy and with worse OS. Scoring based on angiogenesis (Angio) and macrophage (Macrophage) status demonstrated that patients in this antiangiogenic therapy treated group that had $Angio^{lo}Macrophage^{hi}$ tumors demonstrated the worse outcomes compared to the $Angio^{hi}Macrophage^{lo}$ group.

McDermott *et al* reported on the clinical outcomes of 301 patients with advanced ccRCC treated with the programmed death-ligand 1 (PD-L1) inhibitor atezolizumab, the combination of atezolizumab plus the anti VEGF antibody bevacizumab or the small molecule VEGFR inhibitor sunitinib. They also performed hypothesis generating analyses on the genomic and transcriptomic tumor features in the primary tumors from these patients[182]. These analyses identified angiogenesis, T-effector, and myeloid signatures, which were used to group patients into clinically relevant categories. The angiogenesis high subgroup was more likely to respond to sunitinib therapy whereas the T-effector high group was more likely to respond to the combination of atezolizumab plus bevacizumab. Further subdivision demonstrated that in the absence of a significant myeloid population, there was a trend towards improved response to atezolizumab monotherapy relative to sunitinib, a trend that was reversed if the myeloid population was present [182] An additional analysis demonstrated that *PBRM1* mutations were significantly associated with response to sunitinib, and not with response to either atezolizumab or the combination of atezolizumab plus bevacizumab [182].

Clark *et al* performed a multiparametric analysis of 103 treatment naïve ccRCC and 84 matched uninvolved tissue samples, including genomic, transcriptomic, proteomic and phosphoproteomic assays [118]. The authors identified a subset of ccRCC with chromosome-level genomic instability potentially associated with worse prognosis. At a protein level, upregulation of glycolysis and corresponding downregulation of oxidative phosphorylation (OXPHOS) was noted, with the changes in OXPHOS observed at a protein, but not at an mRNA level, with later stage tumors once again showing increased OXPHOS. Immune classification divided tumors into CD8+ immune inflamed, CD8- immune inflamed, metabolic immune desert, and VEGF immune desert tumors. Notably, CD8+ immune inflamed tumors were enriched for *BAP1* mutations, whereas the VEGF immune desert tumors had a higher proportion of *PBRM1* mutations.

Liu *et al* recently developed an isogenic murine Renca based model assessing the impact of PBRM1 deficiency on immune response [112]. Transcriptomic mapping of immune gene expression in murine tumors closely paralleled that seen in the KIRC TCGA dataset. They found that loss of PBRM1 in both animal models and in human samples decreased immune infiltration, and *Pbrm1* knockout tumors were more resistant to anti-PD-1 antibody. They determined that loss of PBRM1 in both murine and human tumors resulting in lower IFN gamma mediated transcription of the chemoattractive chemokines, which was at least partially due to loss of PBRM1 mediated upregulation of interferon receptor gamma 2 (*IFNGR2*) gene expression.

In contrast to these prior studies, an assessment of primary tumor genomics from 63 metastatic ccRCC patients treated with checkpoint blocking antibodies indicated that *PBRM1* mutated tumors were more likely to respond to checkpoint antibody therapy [176]. A follow up study by the same group demonstrated similar outcomes [183]. The results of these studies may be due to patient selection or to other confounding factors including prior therapies.

6. TREATMENT IMPLICATIONS

Prevention and Early Detection

At this point in time the mechanism of 3p loss in renal precursor cells has not been formally defined. A better understanding of the etiology of this driver of ccRCC tumor initiation could lead to prevention strategies. Due to the relatively low incidence of ccRCC at a population level, this may pose challenges, but would be of greater use in high-risk populations, like those with germline losses in the VHL gene.

Early detection may be possible through either blood or urine-based detection systems with sufficient sensitivity to detect a small amount of genomic material harboring 3p loss. It may be possible to develop circulating tumor DNA (ctDNA) assays that capture specific SCNAs which identify these changes. If such a technology were successfully developed, it would encourage the development of a treatment strategy that selectively targets cells harboring 3p loss, thereby eradicating the foundational clones responsible for ccRCC development.

Identifying lethal versus nonlethal primary tumors

In the nonmetastatic setting, identifying patients who are at higher risk of developing metastatic disease would help to develop targeted adjuvant therapy strategies. A number of nomograms and algorithms have previously been generated which use a combination of clinicopathological data to risk-stratify patients [184–186]. Applying our broader understanding of the impact tumor genomic features on patient outcome may help refine these algorithms. As an example, the seven TRACERx groups were divided into three broad groups of clinical context: linear evolution which include the VHL monodriver patients; branched evolution, which include the PBRM1-SCNA, PBRM1-SETD2 and the PBRM1-PI3K groups; and punctuated evolution which include the BAP-driven, multiple clonal driver and the VHL wildtype groups [21]. Tumors with linear evolution patterns are less likely to metastasize, are more likely to be cured after nephrectomy, and are unlikely to need adjuvant therapy. The patients with branched evolution are potentially associated with intermediate prognosis. Surgical removal of the primary tumor would reduce the pool of potential metastatic subclones in these patients, and the genomic changes seen in these tumors may provide opportunities for neoadjuvant or adjuvant therapy development. The tumors with punctuated evolution/multiple clonal drivers are likely high risk for metastatic disease, and if metastatic, for progression. These individuals could be considered for adjuvant therapy trials, and if discovered with synchronous metastases, are most likely to benefit from upfront systemic therapy as opposed to cytoreductive nephrectomy.

The further evolution and validation of molecular subclassifiers as proposed by the TRACERx project will improve prognostication of ccRCC. As these tumor subtypes become more robustly defined, the measurement of circulating tumor DNA (ctDNA) could potentially provide an aggregate readout of tumor genomic features, and identify clones associated with tumor aggressivity and metastatic potential [187,188]. As a whole this field is in a state of evolution, with significant unanswered questions on how best to develop clinically impactful ctDNA platforms [189]. As of now, there are very few reported studies assessing ctDNA in ccRCC [190], and despite its clear potential utility, additional work is required to bring this approach into routine practice for patients with ccRCC.

Targeting genomically and metabolically defined vulnerabilities in ccRCC- tumor cell-centric approach

The process of developing a molecularly defined taxonomy of ccRCC provides a parallel opportunity to develop subtype-tailored therapeutic options (Table 2). At this point in time the treatment of ccRCC has not reached the granularity seen in other tumor types like breast and lung carcinomas[191,192]. Nonetheless, an understanding of ccRCC biology has already yielded significant advances in treatment, and further work in the next few years will undoubtedly continue this trend.

The near-obligate loss of *VHL* in these tumors has spurred efforts to develop synthetic lethal targeting strategies[193] and to rescue function of point-mutated VHL protein[75,194,195]. Metabolic consequences of *VHL* loss have been elegantly defined by Iliopoulos and colleagues[196] and this research has led to the testing of glutaminase inhibitors in ccRCC (NCT03428217, NCT03163667). Upregulated HIF[35] also provides a fairly unique target for

ccRCC, and HIF2a inhibitors are showing promising results in preclinical and clinical studies [197,198]. Ongoing trials are testing HIF2a inhibitors both in combination with tyrosine kinase inhibitor (TKIs) in advanced ccRCC (NCT03634540) and as monotherapy in patients with hereditary VHL disease (NCT03401788). Preliminary data from these studies show that the small molecule HIF2a inhibitor MK6482 is active both in advanced ccRCC [199] and in VHL disease related ccRCC [200].

The mechanistic consequences of *BAP1*, *SETD2* and *PBRM1* mutations on ccRCC are under active investigation. While prognostic differences exist between tumors harboring one or another of these mutations, distinct and targetable molecular features of these subtypes remain a work in progress.

Loss of *BAP1* was shown to increase enhancer of zeste homolog 2 (EZH2) levels in a mesothelioma model [201], and to increase sensitivity to EZH2 inhibition. Similar efforts in uveal melanoma appeared less promising [202]. An EZH2 inhibitor, tazemetostat, is currently in clinical trials. At this point in time the applicability of this approach to ccRCC harboring *BAP1* mutations is not known.

Preclinical studies showed that a *SETD2* deficiency increased sensitivity to WEE1 inhibitors in cell line experiments via nucleotide depletion, due to decreased expression and increased degradation of a ribonuclease reductase subunit, RRM2[203]. The WEE1 inhibitor adavosertib (AZD1775, MK-1775) demonstrated preclinical efficacy in H3K36me3 deficient tumor xenografts [203]. A number of clinical trials are now testing this agent[138], including a study focused on *SETD2* deficient tumors (NCT03284385). Additionally, a preclinical study has shown that synthetic lethality exists between *SETD2* deficiency and PI3Kβ inhibition in a ccRCC model [204].

Agents that target TORC1 signaling are approved for treatment of patients with advanced RCC, but have shown modest efficacy[205,206], as have agents that target AKT [207]. More recently, the PI3K pathway was found to modulate elements of HRR, and inhibition of PI3K pathway signaling could sensitize various tumor subtypes to PARP inhibition [208209]. In a similar manner, the RCC 786–0 cell line, known to have elevated PI3K pathway signaling, and relatively resistant to PARP inhibition, could be sensitized through concomitant blockade of PI3K pathway signaling [164]. These observations provide a potential new direction in modulating ccRCC signaling in a way to render it more susceptible to DNA repair targeted therapy.

Integrating treatment of tumor cells and microenvironment

A broader understanding the proangiogenic consequences of VHL mutation in ccRCC spawned a panoply of agents targeting either vascular endothelial growth factors[210,211] or their receptors[212–217] in ccRCC. These agents moved the treatment of ccRCC from relatively toxic and inconsistently effective cytokines firmly to predominantly orally bioavailable agents with consistent efficacy and manageable side effects. In hereditary VHL disease, use of these agents has resulted in a salutary effect in patients harboring ccRCC [218,219], as well as showing some benefit in other affected organ systems.

As the various phenotypic and genotypic subvariants of ccRCC become defined, it is essential to understand how their specific molecular features influence the tumor immune microenvironment, and how this information can be used to add precision to therapy selection. As an example, in sarcomatoid RCC, programmed death- ligand 1 (PD-L1) is upregulated relative to nonsarcomatoid ccRCC, and an increased number of T-cells is present in the tumor microenvironment [220,221]. Emerging data suggest that patients with sarcomatoid RCC demonstrate superior response to checkpoint antibody therapy[222], with larger confirmatory analyses being performed (NCT02420821).

Recent studies have assessed correlations between specific molecular signatures, the tumor microenvironment and clinical outcome. Multiple studies have showed that *PBRM1* mutation was associated with upregulated angiogenesis and downregulated immune cell infiltration. Congruent with the preclinical data summarized above [103,110], data from the RECORD-3 study indicated that patients with *PBRM1* mutated tumors responded well to antiangiogenic therapy [129]. Results from the IMmotion 150 study demonstrated that patients with *PBRM1* mutated tumors responded better to antiangiogenic agents than those with wildtype tumors, and the directionality was reversed with PD-L1 blocking therapy- patients with PBRM1 mutated tumors showed a trend towards worse outcome after atezolizumab treatment[177]. This is in contradistinction to data reported by a different group, who indicated that *PBRM1* mutations were associated with a better response to checkpoint antibody therapy [176183]. Clearly more work needs to be done to provide clarity on how this commonly mutated gene impacts immune response in ccRCC.

B*AP1* mutated tumors demonstrate a more inflamed immune microenvironment [118,175], suggesting that some form of immune-targeting strategy may benefit these patients as well. Less is known about the impact of *SETD2* mutations on the immune microenvironment and treatment response, with ongoing efforts to better characterize their effect.

The expression of Axl on both ccRCC tumor cells[49] and on immune suppressive cell populations in the tumor microenvironment [223,224] provide additional opportunities for targeted, integrated therapeutic approaches. Clinical trials testing this hypothesis are underway using the combination of cabozantinib and nivolumab (NCT03141177), and there are clear opportunities to test agents that more precisely target TAM family members in combination with immunotherapy.

Can we target or circumvent the “engines” of tumor heterogeneity?

Tumor heterogeneity may influence the ability to fully characterize the genomic drivers of ccRCC [19], and can make it more difficult to choose molecularly targeted agents against key driver mutations. If sufficiently sensitive, ctDNA may help in identifying key drivers of tumor biology and in therapy selection.

Similarly, tumor heterogeneity may decrease the impact of targeted therapy in the treatment of ccRCC, especially if the molecules being targeted are fairly downstream from the driver events. By targeting the drivers of heterogeneity themselves, it may be possible to decrease or prevent further clonal and subclonal evolution, and the very presence of this aberrant programming may confer specific vulnerabilities to the tumor cell.

Immunotherapy response may actually be enhanced by tumor heterogeneity [169]. The diverse repertoire of tumor neoantigens may enhance immune response by providing a larger number of opportunities for cytotoxic T-cells to interact with and kill tumor cells[225]. MSI high tumors are particularly responsive to checkpoint antibody therapy [226,227]. Despite the intermediate level of tumor heterogeneity observed in ccRCC [139] ccRCC is quite responsive to checkpoint inhibitory antibodies[228,229], and this response appears unrelated to qualitative characteristics of the ccRCC mutational repertoire, including insertions and deletions, and associated frameshift mutational burden [177]. The reasons why ccRCC responds to immunotherapy are not fully elucidated, and future research investigating the STING pathway[230] and other mechanisms of immunogenicity may provide clues.

7. CONCLUDING STATEMENTS

In the past decade our understanding of the determinants of ccRCC ontogeny and the drivers of heterogeneity and lethality has reached the point where are beginning to develop cogent mechanistic pathways outlining tumor ontogeny and progression and are creating clinically relevant subcategories of ccRCC that may benefit from subtype-specific therapeutic interventions. Robust ongoing work to elucidate the impact driver gene mutations have on ccRCC biology will help create the necessary framework to begin developing more targeted therapeutic approaches for patients with ccRCC. As our understanding of the molecular biology of ccRCC expands, there is a parallel evolution of diagnostic, imaging and therapeutic tools that will enable us to translate these advances into substantive improvements in the prevention, early detection, and treatment of ccRCC.

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KEY POINTS

1. Chromosome 3p loss is an almost universal finding in both hereditary and sporadic ccRCC
2. The near ubiquitous loss of a second copy of VHL appears to provide a selective advantage for cells, as well as enabling defects in DNA repair and an increase in genomic instability.
3. Secondarily mutated genes in ccRCC, including PBRM1, SETD2 and BAP1, as well as copy number changes in chromosomes 9p and 14q are associated with prognostically important molecular and phenotypic characteristics that can be used to create specific subgroups.
4. Tumor genomic features are associated with distinct immune phenotypes. As an example, *PBRM1* mutations are associated with decreased T-cell infiltration.
5. Efforts are underway to link genomic features to specific therapeutic strategies and agents for patients with ccRCC

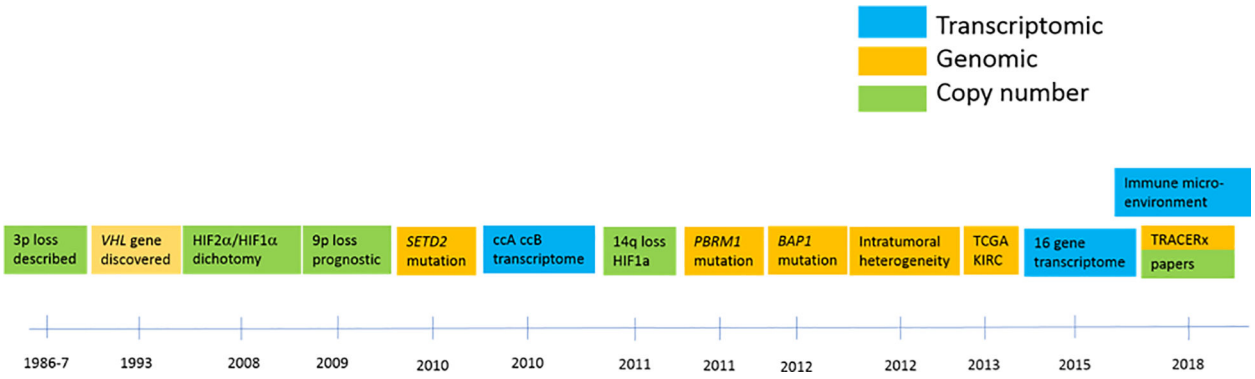


Figure 1: Timeline of key discoveries in ccRCC genomics.

Green boxes denote events related to copy number changes; orange boxes indicate key genomic findings, and blue boxes represent key transcriptomic contributions.

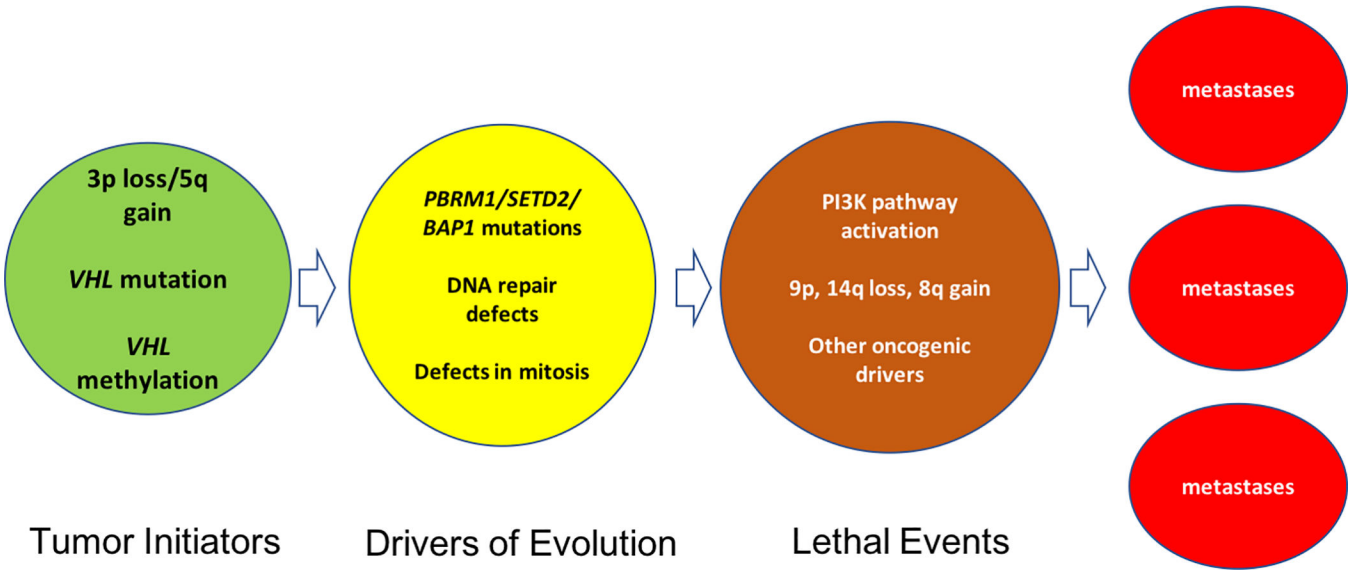


Figure 2: Key events in ccRCC progression.

Tumor initiators include 3p loss and VHL mutations. Evolution is driven by DNA repair defects and errors in mitosis that create additional aneuploidy. The acquisition of key chromosomal gains and losses, mutations in PI3K pathway elements and other oncogenic driver mutations confer lethal potential to intratumoral clones, and enhance the probability of developing metastases.

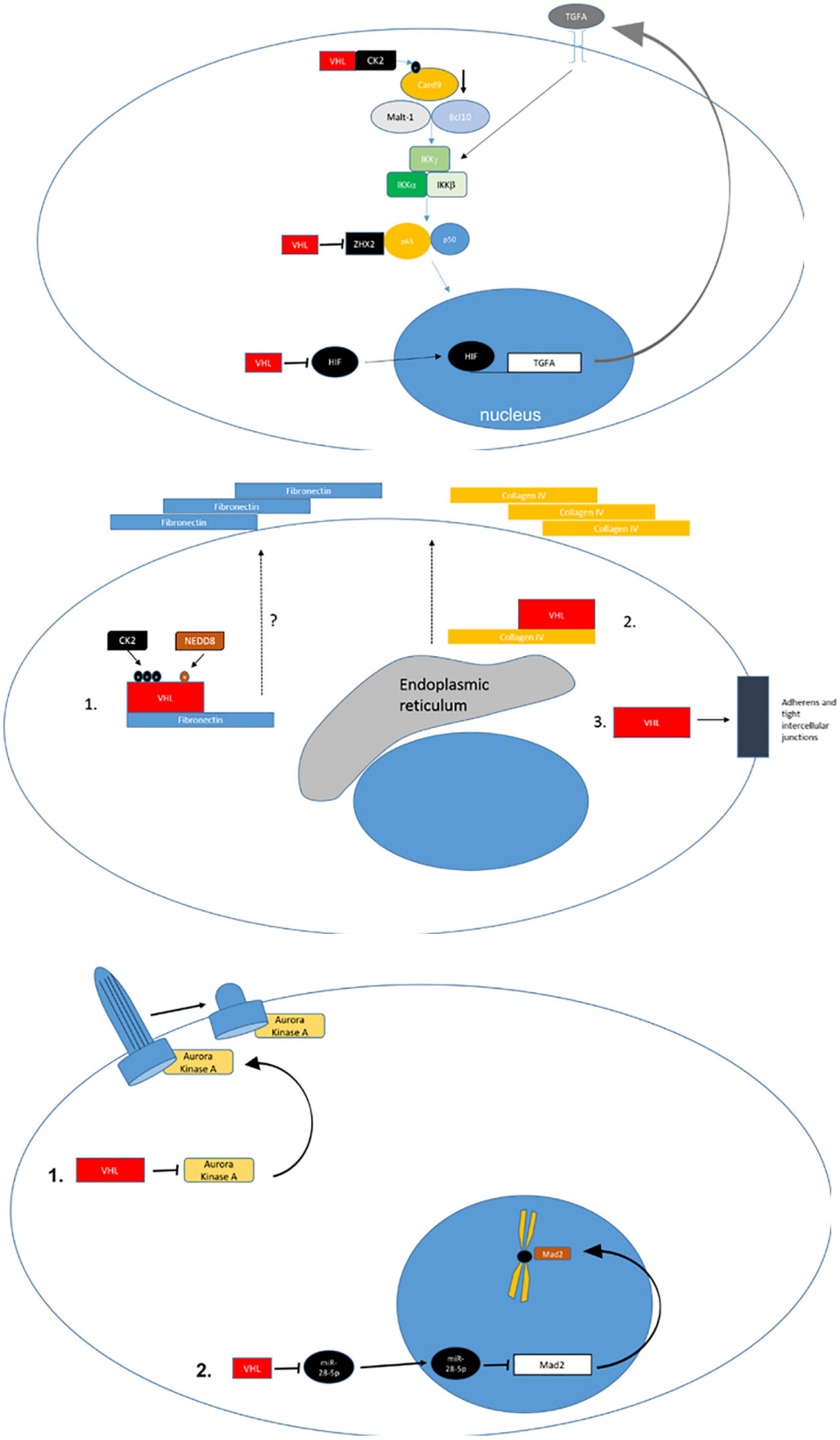
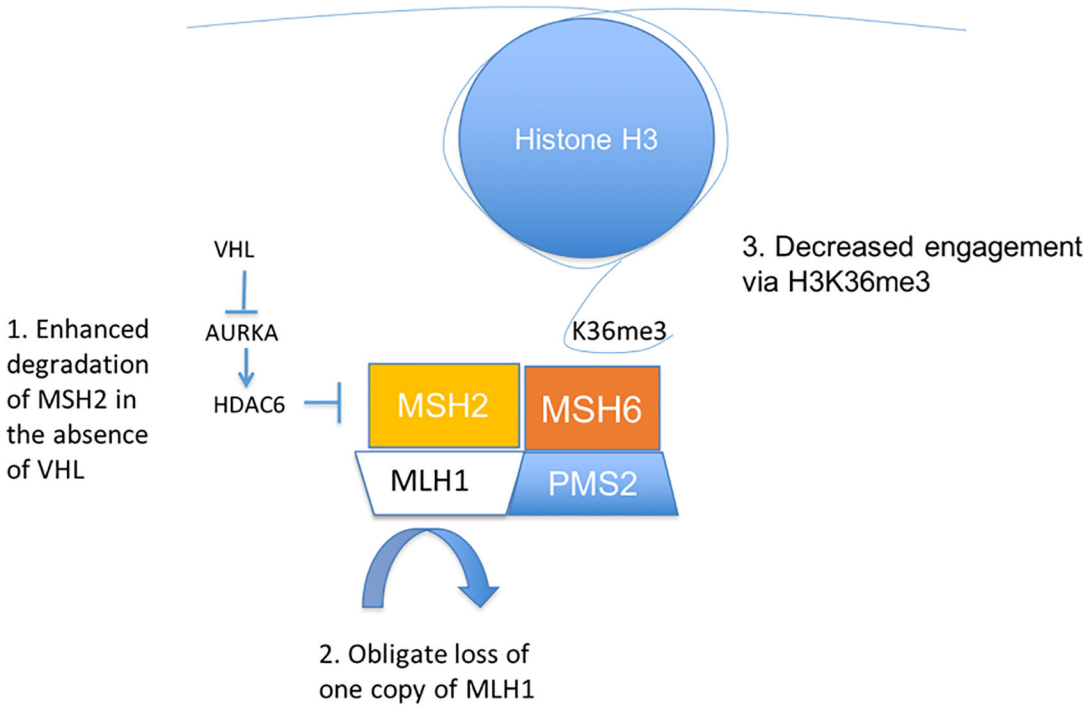


Figure 3: Non-HIF VHL Targets.

A. VHL regulates NFkB through HIF dependent and independent mechanisms. VHL loss will increase TGFA transcription which induces cell autonomous increase in NFkB activation via growth factor receptor mediated IKK inhibition. CK2 mediated CARD9 inhibition is enhanced by VHL, and ZHX2 is inhibited by VHL. **B.** 1. Extracellular fibronectin homeostasis is dependent on interaction with CK2-phosphorylated and neddylatedVHL and loss of VHL results in defective or deficient extracellular matrix. 2. VHL is involved in collagen IV homeostasis. 3. VHL regulates intercellular and adherens junctions. **C**. VHL regulates primary cilium and mitosis. 1. VHL maintains primary cilium homeostasis by blocking AURKA mediated reduction of primary ciliary stability. 2. VHL supports mitotic function by enhancing Mad2 function by downregulating Mad2 inhibitory miR-28–5p.



1. Zhang and Zhang *Mol Cell* 2014
2. TCGA *Nature* 2013
3. Li and Li *Cell* 2014

Figure 4: Factors influencing mismatch repair in ccRCC.

Mismatch repair mechanisms are affected by multiple axes in ccRCC. 1. Loss of VHL itself alters HDAC6 regulation via AURKA, effectively suppressing MSH2 via protein degradation. 2. MLH1 may exhibit haploinsufficiency due to 3p deletion. 3. Loss of MSH6 targeting to H3K36me3 can result in uncoupling of transcription coupled repair mechanisms for efficient targeting of DNA repair mechanisms to essential segments of the genome.

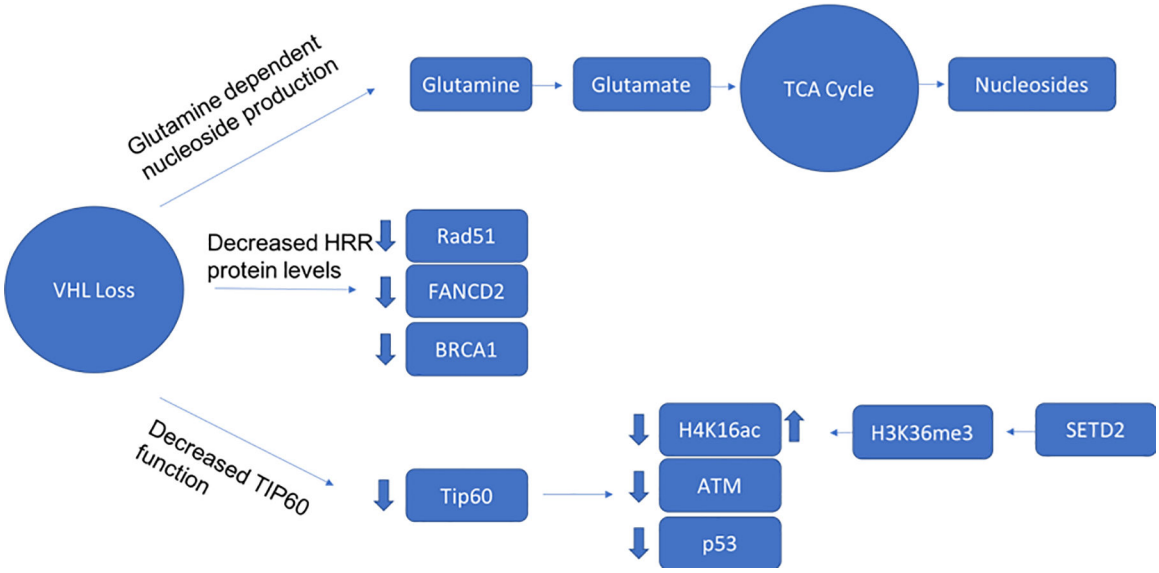


Figure 5: Factors Influencing HRR in ccRCC.

1. Loss of VHL creates glutamine dependence for nucleoside production and increased risk for nucleoside shortfall, which can create vulnerabilities during mitosis dependent DNA repair. 2. VHL loss impacts key HRR genes through a HIF dependent mechanism. 3. VHL interacts with KAT5/Tip60, which acetylates and regulates ATM, p53, and H4K16.

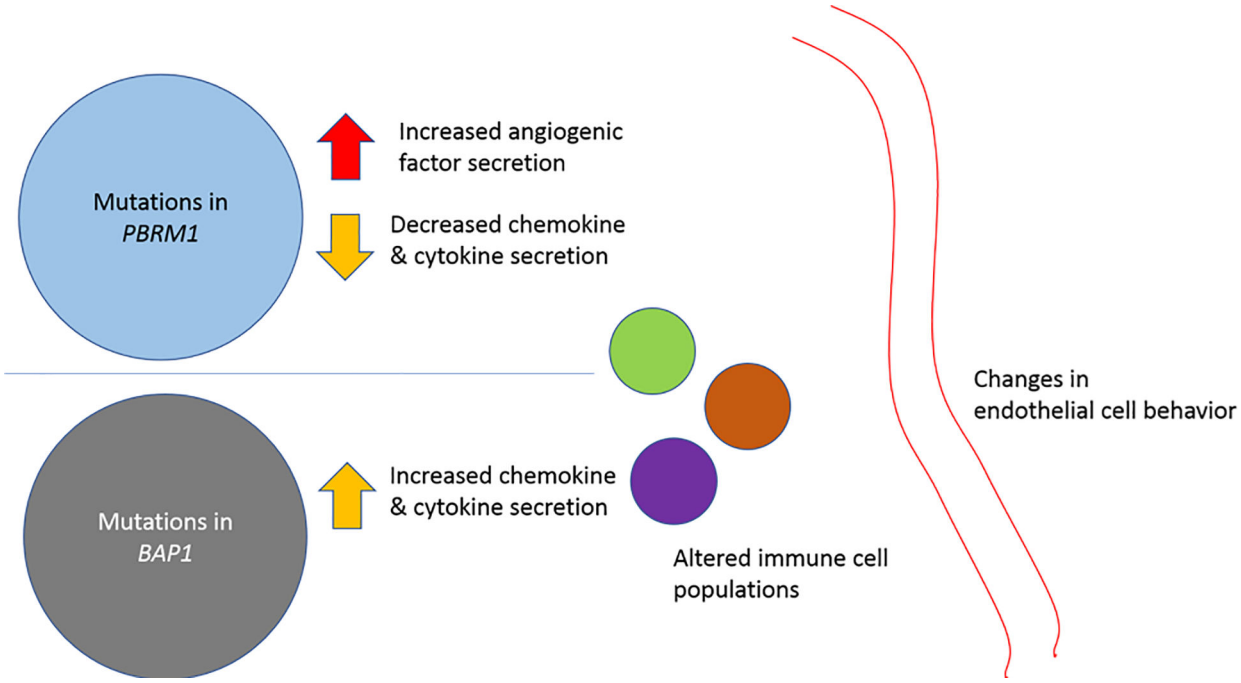


Figure 6: Alterations in the immune microenvironment.

Tumor cell genomic features may influence cell surface receptor expression as well as synthesis and secretion of immunomodulatory chemokines, cytokines and proangiogenic factors. *PBRM1* mutations have been shown to increase angiogenesis, and decrease chemokine and cytokine production, whereas tumors bearing *BAP1* mutations are associated with an inflamed microenvironment.

Table 1:

Tumor immune microenvironmental studies in RCC

Paper/First Author	Tissue	Study Goal	Analytes and analytical tools	Key Findings
Chevrier	-Primary ccRCC	Define ccRCC macrophage and T-cell subtypes	Single cell mass cytometry; single cell RNAseq	-Described 17 tumor-associated macrophage- and 22 T-cell phenotypes -Identified prognostically significant macrophage subcategories
Wang	-Primary ccRCC -Tumorgrafts	Characterize RCC TME	Bulk and single cell RNAseq, IHC, WES	-Identified novel immune/stromal transcripts -Defined an inflamed pan-RCC subtype
Hakimi	-Primary ccRCC	Create clinically significant ccRCC subtypes using RNA	RNA microarray, WES	-Defined four biologically distinct clusters, with specific angiogenic and immune features - Linked subtypes to clinical outcome and treatment response
McDermott	-Primary ccRCC	Assess response to immunotherapy, and identify genomic and microenvironmental response determinants	Bulk RNAseq, WES	-Combined immunotherapy plus anti-VEGF therapy showed trend towards improved outcome vs TKI -Response to IO therapy higher in patients whose tumor transcriptomes showed T-cell infiltrates -Presence of myeloid population attenuated IO therapy response; addition of anti-VEGF therapy rescued IO effect
Miao	-Primary ccRCC	Link genomic status to immune response	WES	-Showed PBRM1 deficiency was associated with improved response to IO therapy
Clark	-Primary ccRCC	Use proteomic and phosphoproteomic data to subclassify ccRCC	Bulk RNAseq, WES, reversed phase liquid chromatography, immobilized metal affinity chromatography	-Identified genomically unstable ccRCC subtype -Mapped metabolic changes in Krebs cycle and OXPHOS pathways -Defined CD8+ inflamed, CD8- inflamed, metabolic immune desert and VEGF immune desert subtypes
Liu	-Primary ccRCC -Isogenic Renca cell lines	Assess impact of PBRM1 loss in immune response	WES, RNAseq, multispectral IHC	-Revealed *Pbrm1* KO tumors less immunogenic in a Renca murine model, and in human tissue -PBRM1 deficiency reduced *Ifgr2* transcription

Table 2:

Opportunities for intervention in ccRCC.

Tumor alteration	Therapeutic Opportunities	Knowledge Gap
VHL/3p loss	Prevention Early detection	Initiating factors for 3p loss Sensitive assays for early clonal evolution
Mutations in chromatin remodeling genes	Synthetic lethal strategies	Mechanism(s) of oncogenesis
Upregulation of oncogenic pathways	Targeted agents	Key drivers of pathway upregulation/mechanisms of resistance
Modulation of immune microenvironment	Tailored intervention(s) to modulate immune response	Mechanism of microenvironmental alteration(s), genotype/phenotype association