Combined mutation in Vhl, Trp53 and Rb1 causes clear cell renal cell carcinoma in mice

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Clear cell renal cell carcinomas (ccRCCs) frequently exhibit inactivation of the von Hippel–Lindau tumor-suppressor gene, VHL, and often harbor multiple copy-number alterations in genes that regulate cell cycle progression. We show here that modeling these genetic alterations by combined deletion of Vhl, Trp53 and Rb1 specifically in renal epithelial cells in mice caused ccRCC. These tumors arose from proximal tubule epithelial cells and shared molecular markers and mRNA expression profiles with human ccRCC. Exome sequencing revealed that mouse and human ccRCCs exhibit recurrent mutations in genes associated with the primary cilium, uncovering a mutational convergence on this organelle and implicating a subset of ccRCCs as genetic ciliopathies. Different mouse tumors responded differently to standard therapies for advanced human ccRCC, mimicking the range of clinical behaviors in the human disease. Inhibition of hypoxia-inducible factor (HIF)-α transcription factors with acriflavine as third-line therapy had therapeutic effects in some tumors, providing preclinical evidence for further investigation of HIF-α inhibition as a ccRCC treatment. This autochthonous mouse ccRCC model represents a tool to investigate the biology of ccRCC and to identify new treatment strategies.

Kidney cancers represent approximately 2–3% of all human cancers, and ccRCC accounts for roughly 70% of all renal malignancies. Metastatic spread of ccRCC occurs in about half of all affected individuals, and, despite ongoing improvements in clinical management due to the availability of several therapeutic options in the form of targeted therapies and immune-checkpoint-blocking agents, 5-year survival rates for individuals with metastatic disease are still around 8–12% (refs. 2,3). Between 82–92% of ccRCC tumors harbor biallelic inactivation of VHL4,5,6, and VHL mutations occur at the earliest stage of tumor formation7. Many individuals with von Hippel–Lindau disease (VHL) inherit a mutant VHL allele that predisposes them to develop ccRCC. However, second-hit loss-of-function mutations in VHL in the renal epithelial cells of individuals with VHL are insufficient to produce ccRCC in humans8 and numerous mice with renal epithelial cell–specific Vhl knockout also failed to develop ccRCC (reviewed in ref. 9), arguing that ccRCC formation requires mutations in addition to those in VHL. The presence of recurrent mutations in PBRM1, BAP1, SETD2, KDM5C, PIK3CA, PTEN, MTOR and TP53, as well as copy-number losses of CDKN2A and RB1 and gains of MDM4 and MYC, in human ccRCCs6,10 provides evidence that these genetic alterations may act cooperatively with VHL loss to induce ccRCC formation. In accordance with this hypothesis, kidney epithelial cell–specific co-deletion of Vhl with Pten in mice reduced the frequency of ciliated epithelial cells and caused the formation of simple and atypical cystic lesions11, which mimicked the proposed precursor lesions observed in a subset of ccRCC. Deletion of Vhl together with Kif3a, to genetically ablate primary cilia, caused a similar phenotype12. Deletion of Vhl and Trp53 gave rise to simple and atypical cystic lesions, as well as small tumors containing cells that displayed cytoplasmic clearing and elevated mechanistic target of rapamycin complex 1 (mTORC1) activity13, recapitulating some of the cellular and molecular changes that are characteristic of human ccRCC. Similar phenotypes were observed in mice with combined Vhl deletion and heterozygous loss of Bap1 (ref. 14). Although these mouse models reproduce precursor lesions of ccRCC and small tumors with some of the features of ccRCC, they do not fully reproduce all of the characteristics of human ccRCC. A very recent study15 showed that combined deletion of Vhl and Pbrm1 causes renal cysts with subsequent development of ccRCCs after approximately 10 months, providing an autochthonous model that reflects the genetic subset of human ccRCC with mutations in both VHL and PBRM1. In this study, we identify another genetic combination that gives rise to ccRCC, furthering understanding of the spectrum of molecular causes of this disease and providing an autochthonous mouse model to test new therapeutic strategies.

RESULTS

Human ccRCCs exhibit recurrent copy-number gains and losses of genes regulating p53 and the G1/S cell cycle checkpoint

To gain further insight into the genetic changes that arise in ccRCC, we utilized cBioPortal16,17 to reanalyze genomic data from 448 sporadic ccRCC.
ccRCCs from The Cancer Genome Atlas (TCGA) data set. We focused on alterations in the network of genes that regulate p53 and the G1/S cell cycle transition, including TP53, MDM2, MDM4, cyclin-dependent kinase (CDK)-inhibitor-family genes, RB-family genes, cyclins and CDKs. While loss- or gain-of-function single-nucleotide mutations in these genes rarely occurred in ccRCC,

Figure 1 Renal epithelial cell–specific co-deletion of Vhl, Trp53 and Rb1 permits the evolution of ccRCCs. (a,b) Examples of mild renal tubular disorganization in an Rb1Δ/Δ mouse aged 12 months (a) and a VhlΔ/ΔRb1Δ/Δ mouse aged 12 months (b). (c–e) Examples of a simple cyst (c), dysplasia associated with cysts (d) and solid dysplasia (e) in a Trp53Δ/ΔRb1Δ/Δ mouse aged 12 months. (f) Kidneys from a VhlΔ/ΔTrp53Δ/ΔRb1Δ/Δ mouse aged 10 months; arrowheads point to tumors. (g–i) Examples of histological sections from VhlΔ/ΔTrp53Δ/ΔRb1Δ/Δ mice aged 10 months, with tumors outlined with dashed lines. (j) Example of longitudinal µCT imaging of tumor development in a VhlΔ/ΔTrp53Δ/ΔRb1Δ/Δ mouse; the time points are days following tamoxifen feeding. (k) Tumor onset in cohorts of VhlΔ/ΔTrp53Δ/ΔRb1Δ/Δ and Trp53Δ/ΔRb1Δ/Δ mice. The two-sided P value was calculated by log-rank Mantel–Cox test. (l–o) Histological appearances of tumors 1–4, marked in g–i, respectively; the tumors show pure clear cytoplasm (l), clear cytoplasm with weak eosinophilic staining (m), strong cytoplasmic eosinophilic staining (n) and a mixture of clear and eosinophilic cytoplasm (o). (p) Example of a papillary-like ccRCC. (q,r) Sinusoidal vascular networks in a tumor with a clear cell appearance (q) and a tumor with an eosinophilic appearance (r); endothelial cells are highlighted by arrowheads. (s,t) CD31 (s) and vWF (t) immunohistochemical staining in ccRCCs from VhlΔ/ΔTrp53Δ/ΔRb1Δ/Δ mice. (u) Example of a tumor margin of a mouse ccRCC. Scale bars: 50 µm in a–e and l–u, and 5 mm in f–j.
copy-number losses of negative regulators or gains of positive regulators of the network were common. Sixty-eight percent of human ccRCCs harbored a chromosomal copy-number alteration in at least one of these genes, and most (78%) of these tumors harbored multiple, simultaneous alterations (Supplementary Fig. 1a). Individuals with tumors that displayed alteration in at least one gene in the p53–G1/S network signature had a worse outcome than those without these genetic alterations (Supplementary Fig. 1b). Alterations in other frequently mutated ccRCC-associated genes, including VHL, PBRM1, BAP1 and SETD2, were not enriched in tumors with a p53–G1/S network signature (Supplementary Fig. 1a), providing evidence that alterations of the genes in this network arise independently of other putative mutational selection pressures during ccRCC evolution.

These data give rise to the hypothesis that during the evolution of the majority of ccRCCs there is selection for multiple copy-number alterations that are predicted to alter the integrity of the G1/S cell cycle checkpoint, promoting tumor initiation and progression.

**Vhl, Trp53 and Rb1 deletion allows the evolution of ccRCC in mice**

To functionally test this idea in mice, we deleted *Vhl* together with two tumor-suppressor genes that encode proteins that function as the key controllers of cell cycle entry in the p53–G1/S network, namely *Trp53* (encoding p53) and *Rb1* (encoding retinoblastoma protein (pRb)). We generated mice that allow inducible renal epithelial cell–specific (Ksp1.3-CreERT2)18 homzygous deletion ofloxP-flanked alleles of *Rb1, Vhl/Rb1, Trp53/Rb1* and *Vhl/Trp53/Rb1* to complement our previous analyses of the effects of *Vhl* and *Vhl/Trp53* deletion11,13. Epithelial cell–specific gene deletion throughout the nephron, including in proximal tubule epithelial cells18, the likely cell of origin of ccRCC19, was induced in pups by injecting nursing dams with tamoxifen or by feeding mice aged 6 weeks with tamoxifen–containing food. These two treatments yielded identical results, and data from them have been pooled for the purposes of the following descriptions. Hereafter, ∆/∆ refers to deleted alleles. Littermate mice lacking the Ksp1.3-CreERT2 transgene served as wild-type controls, denoted by +/+.

p53 was not detectable by immunohistochemical staining, loss of immunoreactivity for pRb confirmed *Rb1* deletion in all genotypes, and the nuclear accumulation of HIF-1α in the kidneys of *Vhl/∆Rb1/∆* and *Vhl/∆Trp53/∆Rb1/∆* mice confirmed *Vhl* deletion (Supplementary Fig. 2a). The kidneys of *Rb1/∆* (n = 35) and *Vhl/∆Rb1/∆* (n = 29) mice displayed occasional sites of subtle disorganization of renal tubular epithelia 50–57 weeks after gene deletion (Fig. 1a,b). The kidneys of *Trp53/∆Rb1/∆* (n = 12) mice displayed small cysts (1–5 cysts per kidney section) (Fig. 1c), as well as occasional sites of cystic or tubular dysplasia (Fig. 1d,e), within 46–54 weeks of gene deletion. In contrast, within 30–47 weeks of gene deletion, 10 of 25 *Vhl/∆Trp53/∆Rb1/∆* mice developed a total of 64 tumors (Fig. 1f–i). Mice that did not develop tumors showed an equivalent extent of tubular immunoreactivity to carbonic anhydrase 9 (CA9) (Supplementary Fig. 2b), a marker of *Vhl* deletion and HIF-1α activation, as histologically normal tubules in kidneys with tumors, showing that the absence of tumors in these mice was not caused by failure of Cre activation. The presence of putative small precursor lesions in these mice (Supplementary Fig. 2b) implies that tumors may potentially have developed at later time points. To follow up these observations in new cohorts of mice, we first established that the emergence of tumors in *Vhl/∆Trp53/∆Rb1/∆* mice could be monitored using contrast-assisted micro computed tomography (µCT) imaging (Fig. 1j) and followed tumor onset over time in larger cohorts of *Trp53/∆Rb1/∆* and *Vhl/∆Trp53/∆Rb1/∆* mice (Fig. 1k). Thirty-one of 38 (82%) *Vhl/∆Trp53/∆Rb1/∆* mice that were fed tamoxifen at 6 weeks of age developed a total of 159 tumors within 25–61 weeks of tamoxifen treatment. In contrast, 6 of 25 (24%) *Trp53/∆Rb1/∆* mice developed a total of 13 tumors within 50–70 weeks of tamoxifen treatment. We conclude that *Vhl* deletion accelerates the onset and increases the incidence of tumor formation, as well as increases the number of tumors per mouse. Interestingly, male *Vhl/∆Trp53/∆Rb1/∆* mice developed tumors at earlier time points and developed more tumors in comparison to female *Vhl/∆Trp53/∆Rb1/∆* mice (Supplementary Fig. 3a,b), which is consistent with the fact that human ccRCC is almost twice as likely to occur in males than in females20.

On the basis of the World Health Organization (WHO) 2016 criteria, all tumors in *Vhl/∆Trp53/∆Rb1/∆* mice were classified as grade 3 or grade 4 ccRCCs growing in acinar, solid or pseudopapillary growth patterns (Fig. 1j–p). Some tumors exhibited a cystic component (Fig. 1h, tumor 2). Eosin staining revealed that 60% of tumors contained cells with either completely optically clear cytoplasm (Fig. 1l) or weakly stained cytoplasm (Fig. 1m). Twenty-eight percent of tumors exhibited more cytoplasmatic eosin staining (Fig. 1n) and resembled the eosinophilic variant of ccRCC. Five tumors had regions with obvious transitions between clear cell and eosinophilic morphology, providing evidence that these cytoplasmatic appearances represent a continuous phenotypic spectrum (Fig. 1o). Indeed, similar eosinophilic cells can be found in high-grade human ccRCC or in hypoxic regions of human ccRCC tumors. Regions of necrosis (data not shown) were observed in many of the mouse tumors, and two tumors exhibited intratumoral hemorrhage (Fig. 1h, tumor 3). Necrosis and hemorrhage are also common features of human ccRCC. In addition, two clear cell tumors showed papillary-like features similar to tumors that

![Figure 2](https://example.com/figure2.png)

**Figure 2** ccRCCs in *Vhl/∆Trp53/∆Rb1/∆* mice exhibit HIF-α and mTORC1 pathway activation. Examples of immunohistochemical staining using antibodies against the indicated proteins in normal kidney tissue from a wild-type (WT) mouse, histologically normal tissue from a tumor-bearing *Vhl/∆Trp53/∆Rb1/∆* mouse and ccRCCs from human ccRCC tumors (data not shown) were observed in many of the mouse tumors, and two tumors exhibited intratumoral hemorrhage (Fig. 1h, tumor 3). Necrosis and hemorrhage are also common features of human ccRCC. In addition, two clear cell tumors showed papillary-like features similar to tumors that...
rarely arise in individuals with VHL (Fig. 1p) but did not show features of the newly described clear cell papillary renal cell carcinoma entity. These tumors were also distinguished from true papillary renal cell carcinomas by the absence of a fibrovascular core and cytokeratin 7 (CK7) staining (data not shown). Irrespective of cytoplasmic morphology, all tumors displayed a highly developed vascular network of CD31- and von Willebrand factor (vWF)-positive thin-walled blood vessels enveloping clusters of carcinoma cells in a pseudoalveolar fashion (Fig. 1q–t). These sinusoidal vascular structures are a characteristic diagnostic feature of human ccRCC. All tumors were...
confined to the kidney and exhibited pushing, rather than infiltrative, margins (Fig. 1u). There was no evidence of invasion into blood vessels, perirenal fat or the renal pelvis. No metastases were observed in the lungs, liver, spleen, bones or brain of tumor-bearing mice (n = 19). In contrast to the ccRCC tumors in VhlΔ/ΔTrp53Δ/ΔRb1Δ/Δ kidneys, the tumors that arose in Trp53Δ/ΔRb1Δ/Δ kidneys exhibited a range of non-ccRCC phenotypes and were variously characterized by sarcomatoid and rhabdoid tumor cell morphologies, eosinophilic cytoplasm, comedonecrosis, atypical giant tumor cells and aberrant mitosis (Supplementary Fig. 4).

In addition to ccRCCs, the kidneys from VhlΔ/ΔTrp53Δ/ΔRb1Δ/Δ mice exhibited a spectrum of lesions (Supplementary Fig. 5) that recapitulated the putative precursor lesions that are found in the kidneys of individuals with VHL, namely cysts with a single epithelial layer and cysts with proliferation of atypical cells growing in multilayered structures in the cystic lumen, as well as small solid lesions that appeared to have no cystic component. ccRCCs in this model therefore appear to arise via both cystic and solid precursor lesions.

**Mouse ccRCCs are molecularly similar to human ccRCCs**

Immunohistochemical staining revealed numerous similarities between VhlΔ/ΔTrp53Δ/ΔRb1Δ/Δ mouse ccRCCs and human ccRCCs (Fig. 2). All mouse ccRCCs displayed nuclear accumulation of HIF-2α (n = 41) and 75% displayed nuclear accumulation of HIF-1α (n = 47), which is consistent with the fact that, although most human ccRCCs express only HIF-1α and HIF-2α, approximately 30% express only HIF-2α and not HIF-1α (ref. 21). All tumors stained positively for the HIF-α target CA9 (n = 46). It is noteworthy that histologically normal tubules in VhlΔ/ΔTrp53Δ/ΔRb1Δ/Δ kidneys frequently exhibited HIF-1α, HIF-2α and CA9 immunoreactivity (Fig. 2), demonstrating that these mutant cells had not become tumors within 9–11 months of gene deletion. All tumors had strong staining for 4E–BP1 phosphorylated at Thr37/Thr46 (n = 44), which is indicative of strong mTORC1 activation, a common feature of human ccRCC. In contrast, antibodies against ERK1 and ERK2 (ERK1/2) phosphorylated at Thr202/Tyr204, a marker of activation of the RAS–mitogen-activated protein kinase (MAPK) pathway, labeled only rare cells in most tumors (n = 44).

All tumors showed strong immunoreactivity for paired-box gene 8 (PAX8) (n = 47) (Fig. 2) and pan-cytokeratin (n = 47) (Supplementary Fig. 6), which are clinical diagnostic markers for ccRCC. Thirty-nine of 43 tumors stained positively for CD10, 2 of 11 were positive for the proximal tubule marker CD10, 2 of 11 were positive for THP, implicating the proximal tubule as the likely cell of origin (Supplementary Fig. 4).

We next used RNA sequencing to compare global mRNA abundance between six mouse ccRCCs and three nontransgenic kidney cortices from littermate VhlΔ/ΔTrp53Δ/ΔRb1Δ/Δ mice. Multidimensional scaling analysis demonstrated a clear segregation of global gene expression profiles between normal kidney and tumors (Supplementary Fig. 7a,b), with 1,852 genes significantly upregulated more than twofold and 2,010 genes significantly downregulated more than twofold (Fig. 3a and Supplementary Table 1). Of the human orthologs corresponding to these differentially expressed genes in mouse ccRCC, 37% were also present in a list of differentially expressed genes identified by comparing 72 samples from normal human kidneys and 533 human...
ccRCC samples (Fig. 3b). Moreover, correlation analysis of the average expression values for all unique orthologous gene pairs between human ccRCC and mouse ccRCC revealed a strong correlation in global transcriptional profiles, particularly for those genes that were highly expressed (Fig. 3c). These data demonstrate that there are strong transcriptional similarities between the mouse ccRCC model and human ccRCCs. Predominant transcriptional signatures in mouse ccRCCs included upregulation of a set of HIF-1α and HIF-2α target genes that we previously identified as being upregulated following deletion of Vhl in primary renal epithelial cells22 (Fig. 3d) and upregulation of numerous genes that regulate cell cycle progression, DNA replication and mitosis (Fig. 3e), as well as upregulation of a set of genes that regulate immune responses and inflammation (Fig. 3f). Global comparisons of mRNA expression in mouse ccRCCs with the gene expression profiles of microdissected normal mouse nephron segments23 revealed strong expression correlations of all tumors with S1 and S3 proximal tubule segments, but not with other nephron segments (Fig. 3g). These data provide strong evidence that the proximal tubule is the origin of ccRCCs in this model, which is consistent with similar gene expression correlation analyses of human ccRCC19 and with our immunohistochemical stainings.

Mouse ccRCCs exhibit genetic mutational profiles similar to those of human ccRCCs

The observation that ccRCCs arise in vivo after a relatively long latency implies that additional mutational events likely accumulate to allow tumor formation. To identify such cooperating genetic alterations, we conducted exome sequencing of DNA isolated from seven mouse ccRCCs and normal liver tissue from these six mice. An average of 4.52 ± 0.56 x 10⁷ total reads were obtained per sample, giving mean target coverages of 56–5-fold. Specific losses of sequencing coverage of the loxP-flanked regions of Vhl, Trp53 and Rb1 confirmed that all three genes were mutated in the tumors (Supplementary Fig. 7c).

Copy-number variants in tumors were determined by comparison with the matched normal liver samples. All tumors exhibited a normal autosomal karyotype without evidence of whole-chromosome aneuploidy. Large regions of gain and loss were rare, but a total of 55 regions of copy-number variation were identified. We focused analyses on named genes that were present in the minimal overlapping chromosomal regions of gain or loss between different tumors or that were amplified (present in four or more copies) in individual tumors (Fig. 4a and Supplementary Table 2). Notably, two tumors harbored amplifications (estimated copy numbers of 59 and 66) of the Myc oncogene (Fig. 4a and Supplementary Fig. 7d), and these tumors exhibited very high levels of Myc mRNA (Fig. 3e). Copy-number gains or amplifications of MYC occur in 8–15% of human ccRCCs and are associated with poor survival10,24,25 (Fig. 4c). We identified regions of human–mouse synten that investigated whether similar copy-number variations arise in human ccRCCs. Interestingly, when human tumors exhibited copy-number gains of genes that were gained in the mouse ccRCCs or losses of genes that were lost in the mouse ccRCCs, these tumors almost invariably (with the exception of the DYNT1L1 (DYNLT1) syntenic region) also displayed alteration in at least one gene in the p53–G1/S network signature (Fig. 4b), providing evidence that the copy-number alterations in the mouse model are nonrandom and that they may participate in the evolution and progression of a subset of ccRCCs. In accordance with this notion, copy-number gain of SYCP1 in human ccRCC predicts poor survival (Fig. 4c).

Single-nucleotide variants (SNVs) and insertions and deletions (indels) were identified in mouse ccRCCs versus matched liver samples. The most frequent SNVs were C>A/G>T transversions, C>T/G>A transversions and A>G/T>C transversions (Fig. 5a). These three classes of mutations are also the most frequently occurring in human ccRCC16, demonstrating that the mouse model reproduces the same classes of mutations that arise in human ccRCC. Mouse ccRCCs
enables the analysis of genetic changes in tumors (T1, T2, T3) in two different mice before and during therapy; tumors were visualized with contrast-assisted CT imaging. Information regarding treatment type and dosage (in mg per kg bodyweight per day) is provided under the images. Scale bars, 5 mm. (b) Quantification of the volumes of T1, T2 and T3 from a. (c) Summary of the therapeutic effects of sunitinib, everolimus and acrilavine (each row represents an independent tumor) in four mice.

Fig. 6 ccRCCs in Vhl<sup>Δ/Δ</sup>Trp53<sup>Δ/Δ</sup>Rb1<sup>Δ/Δ</sup> mice exhibit varying patterns of therapeutic sensitivity and resistance. (a) Examples of development of three tumors (T1, T2, T3) in two different mice before and during therapy; tumors were visualized with contrast-assisted μCT imaging. Information regarding treatment type and dosage (in mg per kg bodyweight per day) is provided under the images. Scale bars, 5 mm. (b) Quantification of the volumes of T1, T2 and T3 from a. (c) Summary of the therapeutic effects of sunitinib, everolimus and acrilavine (each row represents an independent tumor) in four mice.

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Therapeutic studies using the mouse ccRCC model
Having established many molecular similarities between mouse and human ccRCC, as well as demonstrated that different mouse ccRCCs are genetically distinct from one another, we next asked whether this model is useful for preclinical therapeutic studies. Patients with metastatic ccRCC typically receive antiangiogenic therapy in the form of receptor tyrosine kinase inhibitors such as sunitinib. Up to 20% of tumors are refractory to these therapies, and the majority of patients typically develop resistance within 1 year. Second-line therapy often involves mTOR inhibition (for example, treatment with everolimus), which confers a moderate increase in progression-free survival in comparison to treatment with placebo. We first mimicked this therapeutic regime by monitoring tumor initiation and progression in Vhl<sup>Δ/Δ</sup>Trp53<sup>Δ/Δ</sup>Rb1<sup>Δ/Δ</sup> mice using contrast-assisted μCT imaging, and we initiated therapy once the largest tumor in each mouse reached a volume of approximately 20–70 mm<sup>3</sup> (Fig. 6a,b). Of 19 independent tumors in four mice under sunitinib treatment, 6 tumors grew at a rapid rate, 3 tumors regressed and 10 tumors were stable with no or slow growth (Fig. 6c). Of these latter tumors, 6 developed resistance within 2–3 weeks and grew rapidly. Mice undergoing second-line therapy with everolimus showed stable disease or regression in 18 of 23 tumors (several new tumors developed during sunitinib therapy), 4 of which developed resistance (Fig. 6c). Thus, individual ccRCCs in Vhl<sup>Δ/Δ</sup>Trp53<sup>Δ/Δ</sup>Rb1<sup>Δ/Δ</sup> mice have different sensitivities to clinical agents used to treat human ccRCC, further validating the accuracy of one another and of PTEN mutations. We have previously shown that mutated PTEN cooperates with mutated Vhl to reduce the frequency of ciliated renal epithelial cells and to induce renal cysts. Similar to human ccRCCs, all mouse ccRCCs exhibited a reduced frequency of ciliated tumor cells (Fig. 5d). These data uncover a mutational convergence on genes associated with the primary cilium in mouse and human ccRCCs.

exhibited 161 ± 17 nonsynonymous mutations per tumor, and these were almost entirely attributable to SNVs rather than indels (Fig. 5b). Human ccRCCs exhibited 50.6 ± 20.3 nonsynonymous mutations per tumor (n = 382, cBioPortal), suggesting that Vhl<sup>Δ/Δ</sup>Trp53<sup>Δ/Δ</sup>Rb1<sup>Δ/Δ</sup> mouse ccRCCs do not harbor fewer mutations than the human counterparts, which is a limitation of some genetically engineered mouse tumor models. To narrow the search for potential truncal or clonal mutations that cooperate in tumor evolution, we focused further analyses on the set of truncating and frameshift mutations that were present at a variant allele frequency (VAF) greater than or equal to 5% plus nonsynonymous coding mutations that were present at a VAF greater than or equal to 5%. The set enrichment analysis demonstrated striking enrichment for mutations in genes associated with Gene Ontology (GO) terms for cilium biology, we found that 40% of human ccRCCs harbored one or more mutations in primary-cilium-related genes (Supplementary Fig. 8). Notably, these mutations were largely mutually exclusive of each other, suggesting that human ccRCCs may exhibit a limited genetic complexity in the primary cilium.
of the mouse model and suggesting that this model will be useful to interrogate mechanisms or identify biomarkers that are associated with therapeutic sensitivity or resistance.

Acriflavine, a drug that was given orally as a urinary antiseptic in the 1920s, was recently identified as an inhibitor of the dimerization of HIF-1α and HIF-2α with HIF-1β, blocking transcriptional activation. Acriflavine treatment was shown to reduce the growth of various cancer cell lines in xenograft assays and to reduce colorectal cancer growth in an autochthonous mouse model. Given the important pathogenic role of dysregulated HIF-α activity in human ccRCC, we tested acriflavine in the mice described above as a third-line therapy after sunitinib and everolimus. Daily intraperitoneal injection slowed the growth of 2 of 23 tumors, and 1 large tumor that was resistant to everolimus showed initial regression but developed resistance after 3 weeks of acriflavine therapy (Fig. 6b,c). Three smaller tumors regressed during acriflavine therapy, while other similarly sized tumors in the same mice increased in size (Fig. 6c and Supplementary Fig. 9). Although these proof-of-principle studies show that this drug is effective in only a subset of tumors, these results provide further preclinical evidence to support the ongoing development and clinical testing of various HIF-α inhibitors with better specificities and pharmacological properties as therapies for ccRCC. This mouse model represents an experimental platform that should assist in the identification of biomarkers that could be used to predict which human ccRCC tumors are likely to respond to inhibition of HIF-α.

**DISCUSSION**

This study describes an autochthonous mouse model of ccRCC that accurately recapitulates the cellular and molecular features of human ccRCC. Although the exact combination of biallelic inactivation of VHL, TP53 and RB1 is not common in human ccRCC tumors, VhlΔ/Δ Trp53Δ/Δ Rb1Δ/Δ mice represent a genetic background that models the predicted effects of the many combinatorial copy-number alterations in regulators of the interconnected networks governing the p53 pathway and the G1/S cell cycle control machinery that arise in human ccRCC. The VhlΔ/Δ Trp53Δ/Δ Rb1Δ/Δ genetic background does not automatically confer tumor-forming capacity on renal epithelial cells in vivo but rather permits the evolution of genetically distinct ccRCC tumors. The dependency of these tumors on Vhl mutation is clearly shown by the accelerated and increased incidence of tumor formation in VhlΔ/Δ Trp53Δ/Δ Rb1Δ/Δ mice as compared to Trp53Δ/Δ Rb1Δ/Δ mice, as well as the fact that only VhlΔ/Δ Trp53Δ/Δ Rb1Δ/Δ mice develop ccRCCs. These functional genetic data are consistent with the fact that VHL is bi-allelically inactivated as an initiating event in the vast majority of ccRCCs. We identified mutational convergence on genes that regulate the structure or function of the primary cilium in mouse ccRCCs and in 40% of human ccRCCs. Owing to the relative rarity of these mutations when genes are considered individually, they had previously been missed by statistical analyses to identify recurrent mutations, but, when these mutations are considered in the larger biological context, we propose that at least a subset of ccRCCs can be viewed as genetic ciliopathies. Although further functional studies will be necessary to establish whether the mutations that we identified in ccRCC play a pathogenic role in the disease, given that a common phenotypic outcome of genetic alterations in diverse genes that are important for cilia biology is the induction of renal epithelial cell proliferation and cyst formation, it appears likely that mutations in primary-cilium-related genes might either permit or enhance the proliferation of VHL-mutant cells. In this context, our previous studies have shown cooperation between loss of Vhl and loss of primary cilia in causing uncontrolled renal epithelial cell proliferation and development of simple and atypical cystic precursor lesions in mice. It is plausible that the combination of mutation in VHL, mutation in primary-cilium-related genes and additional genetic alterations—such as those in the p53 and G1/S cell cycle network or in other frequently mutated tumor-suppressor genes, like PBRM1, BAP1 or SETD2—act cooperatively to cause the evolution of ccRCC. It is noteworthy that none of the seven mouse ccRCCs examined in this study showed mutations in these tumor-suppressor genes, providing evidence that this model may reflect the approximately 50% of human ccRCCs that do not harbor mutations in other known or suspected tumor-suppressor genes associated with kidney cancer.

The VhlΔ/Δ Trp53Δ/Δ Rb1Δ/Δ ccRCC mouse model will provide an opportunity to assess genetic dependencies and identify mutational modifiers that enhance tumor initiation or promote invasion and metastasis. Our copy-number analyses identified several candidate genes, including the Myc oncogene and Loxl2, that have been implicated in various aspects of the pathogenesis of ccRCC. It is also noteworthy that all VhlΔ/Δ Trp53Δ/Δ Rb1Δ/Δ mouse ccRCCs showed high levels of mTORC1 activation, as evidenced by staining for phosphorylated 4E-BP1. PI3K–mTORC1 pathway activation was also observed in cysts or tumors in mice with mutations in Vhl Ptten, Vhl/Trp53 (ref. 13), Vhl/Trp53/Kif3a, Vhl/Bap1 (ref. 14) and Vhl/Pbrm1 (ref. 15), suggesting that PI3K–mTORC1 pathway activation might generally promote ccRCC evolution in the context of diverse cooperating mutations.

The fact that different ccRCCs in VhlΔ/Δ Trp53Δ/Δ Rb1Δ/Δ mice are genetically distinct from one another, coupled with our observations that different ccRCCs within and between mice respond differently to the therapeutic regimes used to treat human metastatic ccRCC, suggests that this experimental system will be useful for the identification of molecular determinants of tumor sensitivity and resistance. Our observations of the therapeutic effects of the HIF-1α and HIF-2α inhibitor acriflavine on at least some tumors support recent findings that a new HIF-2α-specific inhibitor shows good therapeutic activity in various preclinical models toward approximately half of human ccRCC-derived cell lines or tumour grafts. Finally, the availability of this and other autochthonous models of ccRCC will allow investigations of the role of the tumor microenvironment in tumor progression and therapeutic responses, particularly in the context of the ongoing optimization of immune-checkpoint-blocking therapies, which have already yielded good clinical responses in subfractions of patients with ccRCC.

**METHODS**

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

**Note:** Any Supplementary Information and Source Data files are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

I.J.F. and S.H. designed the study; S.H., D.S., A.C. and L.B. conducted experiments; N.C.T., M.P., I.J.F. and S.H. conducted bioinformatic analyses; P.J.W. and H.M. conducted pathological analyses; I.J.F. wrote the manuscript with input from all authors.
COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mice. *Rb1*fl/fl (FVB, 129P2 background), *Vhl*fl/fl (C.129S background) and *Trp53*∆/∆ (FVB, 129P2 background) mice were crossed with *Ksp1.3-CreERT2* (B6.Cg background) mice to generate *Ksp1.3-CreERT2*, *Vhl*fl/fl, *Trp53*∆/∆, *Rb1*fl/fl, *Ksp1.3-CreERT2*, *Vhl*fl/fl, *Rb1*fl/fl, *Ksp1.3-CreERT2*, *Vhl*fl/fl, *Rb1*fl/fl and *Ksp1.3-CreERT2* Fl/Fl, *Rb1*fl/fl animals. Littermate mice that lacked the Cre transgene served as wild-type controls. Gene deletion in mice aged 6 weeks was achieved by feeding them food containing tamoxifen (400 ppm) for 2 weeks. For gene deletion in pups, nursing dams were injected intraperitoneally with tamoxifen (0.1 mg per gram body weight per day) from postnatal days 2–4. Mouse crosses and phenotyping were conducted under the breeding license of the Laboratory Animal Services Center, University of Zurich, and tumor monitoring studies were conducted under license ZH116/16 of the Canton of Zurich. Investigators were not blinded to the genotype of the mice. No statistical method was used to predetermined sample size. The experiments were not randomized.

Immunohistochemistry and immunofluorescence. Immunohistochemistry and immunofluorescence were conducted using previously described methods41 using antibodies against the following antigens: AQP1 (1:500, Abcam, ab51080), AQP2 (1:4,000, gift from J. Lofng58), CA9 (1:2,000, Invitrogen, PA-16195), CD10 (1:2,000, Thermo Fisher Scientific, PA-5-47075), CD31 (1:2,000, Abcam, ab28364), pan-cytokeratin (1:1,000, DAKO, M3515), HIF-1α (1:20,000, Novus Biotechnologies, NB-100-105), HIF-2α (1:2,500, PM8, gift from P. Pollard44), NAP1A2 (1:250, gift from J. Biber30), NCC (1:500, Millipore, AB3553), PAX8 (1:800, Protein Tech Group, 10336-1-AP), phospho-ERK1/2 (1:1,000, Ths202/204; Cell Signaling Technologies, 9101), phospho-4E-BP1 (1:800, Thr37/Thr46; Cell Signaling Technologies, 2855), pRB (1:10,000, BD Biosciences, 14794), pRB (1:10,000, BD Biosciences, 14794), p38 (1:1,000, Cell Signaling Technologies, 9212), p38 (1:1,000, Cell Signaling Technologies, 9212), and pERK1/2 (1:1,000, Cell Signaling Technologies, 4695). Validations of the primary antibodies are provided on the manufacturers’ websites or in the referenced citations.

Therapeutic studies and μCT imaging. Imaging of animals was performed as previously described12. Mice were imaged monthly, beginning 5 months after tamoxifen feeding and every week or every 2 weeks during therapeutic studies. Tumor volumes were determined from the product of an ellipsoid (\(V = \frac{1}{3}\pi x y z\)) and using the formula for the volume of a cylinder (\(V = \pi r^2 h\)). For μCT imaging, mice were scanned using the NanoFocus digital x-ray microtomography (μCT) system (GEWA Technology, Thum, Germany) at 120 kV and 200 μA. Images were reconstructed using the software CTView 4.1 (NanoFocus, Germany). Tumor volumes were determined from the raw fastq files were mapped against the mouse reference (GRCm38/mm10) using BWA-MEM (http://github.com/lh3/bwa). Subsequently, the Picard tool MarkDuplicates and the GATK IndelRealigner were used to improve the mapping quality. The Picard tools AlignmentSummaryMetrics and HSMetrics were used to perform quality control on the raw sequencing data and the alignments. Somatic SNVs were called for each tumor–normal combination using the following two tools: mutect and strelka. Small somatic indels were called using strelka. Merging and filtering of variant calls was performed using GATK31, and only variants with variant allele frequencies that were greater than 5% were included in the analyses. SnpEff32, SnpSift33 and BEDTools34 were used for annotations. EXACVATOR35 was used to call copy-number variations with a 95% probability cutoff in tumors against the matched normal liver samples. Lists of copy-number variants were manually filtered to remove calls in which multiple tumors showed the exact start and end points of the gain or loss, as these are highly likely to represent artifacts of the calling algorithm. Result files were annotated with mouse and human genes using BEDTools34. We used the non-mouse regef track (xenoRefGene) from the UCSC Genome Browser56 (http://genome.ucsc.edu/) to map human genes to the mouse genome. Gene set enrichment analysis was conducted using the online GSEA software (http://software.broadinstitute.org/gsea/index.jsp).

Survival analyses. Human ccRCC Kaplan–Meier survival analyses were conducted using the online tools of the cBioPortal for Cancer Genomics (http://www.cbioportal.org/).

Statistics. All sample size (n) values used for statistical analyses are provided in the relevant figures and supplementary figures. Differences in tumor onset between different mouse genotypes and sexes were assessed using the log-rank Mantel–Cox test, and differences in tumor numbers between mice were assessed using Student’s one-tailed paired t-test. Pearson correlation analyses were used to compare global mouse and human ccRCC mRNA expression as well as to compare mouse ccRCC mRNA expression with nephron-segment-specific mRNA expression profiles.

Data availability. Exome sequencing or RNA sequencing data sets (beyond the summaries that are deposited as supplementary information) are available from the corresponding author upon reasonable request.

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