HIF-1α and HIF-2α differently regulate tumour development and inflammation of clear cell renal cell carcinoma in mice

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Mutational inactivation of VHL is the earliest genetic event in the majority of clear cell renal cell carcinomas (ccRCC), leading to accumulation of the HIF-1α and HIF-2α transcription factors. While correlative studies of human ccRCC and functional studies using human ccRCC cell lines have implicated HIF-1α as an inhibitor and HIF-2α as a promoter of aggressive tumour behaviours, their roles in tumour onset have not been functionally addressed. Herein we show using an autochthonous ccRCC model that Hif1a is essential for tumour formation whereas Hif2a deletion has only minor effects on tumour initiation and growth. Both HIF-1α and HIF-2α are required for the clear cell phenotype. Transcriptomic and proteomic analyses reveal that HIF-1α regulates glycolysis while HIF-2α regulates genes associated with lipoprotein metabolism, ribosome biogenesis and E2F and MYC transcriptional activities. HIF-2α-deficient tumours are characterised by increased antigen presentation, interferon signalling and CD8+ T cell infiltration and activation. Single copy loss of HIF1A or high levels of HIF2A mRNA expression correlate with altered immune microenvironments in human ccRCC. These studies reveal an oncogenic role of HIF-1α in ccRCC initiation and suggest that alterations in the balance of HIF-1α and HIF-2α activities can affect different aspects of ccRCC biology and disease aggressiveness.
More than 400,000 new cases of kidney cancer arose worldwide in 2018. Clear cell renal cell carcinoma (ccRCC) represents 70–80% of all cancers of the kidneys. Biallelic inactivation of the von Hippel–Lindau (VHL) tumour suppressor gene is a truncal genetic event that arises in the majority of cases of ccRCC, demonstrating that loss of one or more of the various tumour suppressor functions of the pVHL protein isoforms is central to the earliest steps in the initiation of tumour formation. Subsequent mutations or chromosomal copy number alterations in epigenetic regulatory genes (including PRDM1, BAP1, SETD2, and KDM5C), cell-cycle regulatory genes (including TP53, CDKN2A, and MYC) or PI3K pathway genes (including PIK3CA, PTEN, MTOR, and TSC1) arise recurrently in ccRCC and are believed to cooperate with VHL inactivation to promote the development and evolution of ccRCC tumours.

Numerous mouse models have supported this notion of genetic cooperation by showing that renal epithelial cell-specific inactivation of different combinations of Vhl together with Pten, Tsc1, Phrm1, Bap1, Trp53, Trp53/Rb1, Cdkn2a, or with Myc overexpression causes the formation of cystic and solid precursor lesions or ccRCC tumours. The best characterised tumour suppressor function of pVHL relates to its role in targeting the alpha subunits of the hypoxia-inducible transcription factors (HIF-1α and HIF-2α) for oxygen-dependent, ubiquitin-mediated proteolytic degradation. Genetic inactivation of VHL causes the constitutive stabilisation of HIF-1α and HIF-2α, which induce gene expression programmes that play a central role in the pathogenesis of ccRCC by altering cellular metabolism, inducing angiogenesis, promoting epithelial-to-mesenchymal transition, invasion, and metastatic spread. Numerous lines of evidence argue that HIF-2α plays a major proto-oncogenic role in established human ccRCCs, whereas HIF-1α appears to function rather to inhibit aggressive tumour behaviour. Loss of the region of chromosome 14 harbouring HIF1A correlates with poor survival and is commonly found in ccRCC metastases. ccRCC tumours that express only HIF-2α have higher proliferation rates than those expressing HIF-1α and HIF-2α, ccRCC tumour cell lines frequently display intragenic deletions of HIF1A but express wild-type (WT) HIF-2α. HIF-2α is necessary for the formation of ccRCC xenografts while knockdown of HIF-1α enhances xenograft tumour formation in cell lines that express both HIF-1α and HIF-2α.

This prompted the development of HIF-2α-specific inhibitors which show excellent on-target efficacy in ccRCC xenograft models, efficacy in a subset of patient-derived xenograft models and clinical responses in some patients in phase I clinical trials. These pharmacological studies in patient-derived xenograft models however also indicate that HIF-2α specific inhibition is not sufficient to inhibit the growth of all ccRCCs, suggesting that other oncogenic drivers may be important in some or all tumours. It should be noted that all of the functional and genetic data described above largely relates to either studies of established, later stage ccRCC human tumours or to the somewhat artificial setting of xenograft tumour formation by cultured ccRCC cell lines or patient-derived xenograft models. These studies have necessarily been unable to adequately assess the involvement of HIF-1α and HIF-2α throughout the entire process of tumour evolution beginning with VHL mutant cells in the context of a normal renal tubular epithelium.

To address the roles of HIF-1α and HIF-2α in the development of ccRCC we take advantage of an accurate mouse model of ccRCC based on tamoxifen-inducible renal epithelial cell-specific deletion (Ksp-CreERT2) of Vhl, Trp53, and Rb1. This mouse model at least partly reflects the complex patterns of chromosomal copy number gains and losses of cell-cycle regulatory genes in human ccRCC and reproduces many aspects of the evolution of human ccRCC by first developing cystic and solid precursor lesions that progress to tumours over the course of 5–12 months following gene deletion in adult mice. Tumours arising in this model exhibit histological, immunohistochemical, transcriptional, and mutational similarities to human ccRCC. We introduce floxed alleles of Hif1α and Hif2α (also known as Epas1) into this genetic background and show that HIF-1α is essential for tumour formation whereas deletion of HIF-2α has only moderate effects on tumour onset and growth rate but leads to increased intra-tumoural immune activation. This study defines differing roles of HIF-1α and HIF-2α in ccRCC formation and progression and suggests a model in which alterations in their relative activities affect different aspects of tumour biology and immunology.

## Results

### ccRCC formation is strongly dependent on Hif1α

We fed 6-week-old mice tamoxifen-containing food for 2 weeks to induce gene deletion in cohorts of Ksp-CreERT2, Vhlfl/fl, Trp53fl/fl, Rb1fl/fl (hereafter termed VhlΔΔ/Trp53ΔΔ/Rb1ΔΔ in the text and VpR in figures), Ksp-CreERT2; Vhlfl/fl, Trp53fl/fl, Rb1fl/fl, Hif1αfl/fl (hereafter termed VhlΔΔ/Trp53ΔΔ/Rb1ΔΔ/Hif1αΔΔ in the text and VpRH1 in figures) and Ksp-CreERT2; Vhlfl/fl, Trp53fl/fl, Rb1fl/fl, Hif2αfl/fl (hereafter termed VhlΔΔ/Trp53ΔΔ/Rb1ΔΔ/Hif2αΔΔ in the text and VpRH2 in figures) mice. Tumour onset, volume and numbers were monitored over time using contrast-assisted μCT imaging and mice were sacrificed at individual time points based on the presence of rapid tumour growth. These data were added to, or compared to, our previously published analyses of separate VhlΔΔ/Trp53ΔΔ/Rb1ΔΔ and Trp53ΔΔ/Rb1ΔΔ termed pR cohorts, respectively. All animals from both cohorts were housed in the same animal facility. We first determined that tumour growth curves (Supplementary Fig. 1a) showed an excellent goodness of fit (Supplementary Fig. 1b) to the exponential linear regression $e^t$ where $a$ describes the coefficient of exponential growth, a mathematical description of the tumour growth rate, and $t$ represents time in days after gene deletion. These analyses showed that Vhl deletion accelerates tumour onset (Fig. 1a), increases tumour number (Fig. 1b) and increases tumour growth rate (Fig. 1c) in the Trp53ΔΔ/Rb1ΔΔ background. Hif1α co-deletion completely abolished these tumour-promoting effects of Vhl deletion (Fig. 1a) and these mice developed very few tumours (Fig. 1b), which grew slowly when they did develop (Fig. 1c). In contrast, Hif2α deletion caused more moderate, yet statistically significant effects, partly delaying tumour onset (Fig. 1a), partly reducing the number of tumours per mouse (Fig. 1b) and average tumour growth rates (Fig. 1c). Metastases were not observed in any of the genotypes. These data indicate that HIF-1α is very important for the efficient evolution and growth of Vhl mutant ccRCCs, while HIF-2α is only partly required and many tumours still develop in the Vhl/Trp53/Rb1/Hif2α quadruple mutant background.

Since Hif1α deletion provided such a strong phenotypic rescue we next investigated whether the Hif1α and Hif2α genes are indeed deleted in the relevant tumours to exclude that tumours might be escapers in which Cre activity failed to correctly recombine the floxed Hif1α or Hif2α alleles. PCRs specific for the recombinated Hif1α and Hif2α alleles revealed that tumour DNA exhibited Cre-induced recombinated alleles of these genes in tumours from the relevant mice (Supplementary Fig. 2a).

To compare the extent of Cre-mediated deletion of Vhl, Trp53, Rb1, Hif1α, and Hif2α we conducted quantitative real-time PCR using primers to specifically amplify floxed exons of each gene.
as well as non-flxed exons of the Vhl, Trp53, and Hif2a genes (which served as normalisation controls) from genomic DNA from cortex samples from non-Cre mice (WT cortex), as well as tumours from Vhl Δ/Δ, Trp53 Δ/Δ, Rb1 Δ/Δ, and Vhl Δ/ΔTrp53 Δ/ΔRb1 Δ/ΔHif1a Δ/Δ and Vhl Δ/ΔTrp53 Δ/ΔRb1 Δ/ΔHif2a Δ/Δ mice. These analyses showed that the allelic ratios of flxed exons normalised to the average of the three non-flxed exons were reduced for Vhl, Trp53, and Rb1 compared to WT cortex and for Hif1a and Hif2a when compared to WT cortex or to mouse genotypes that did not harbour the flxed allele (Supplementary Fig. 2b). Residual flxed exons (~10–15% allelic burden) in the genomic DNA of tumours likely reflect DNA...
derived from non-Cre-expressing cells of the tumour stroma. These analyses demonstrated that all genes are deleted by Cre and importantly that Hif1α and Hif2α are deleted to similar extents to Vhl, Trp53, and Rbl1 in the relevant tumour samples. We additionally analysed RNA-sequencing data (see experiments described below) which showed that VhlΔΔ Trp53ΔΔRbl1ΔΔ tumours displayed lower mRNA levels of Hif1α and Hif2α than WT cortex but that there was no compensatory upregulation of Hif2α in VhlΔΔTrp53ΔΔRbl1ΔΔHif1αΔΔ tumours, nor of Hif1α in VhlΔΔTrp53ΔΔRbl1ΔΔHif2αΔΔ tumours (Supplementary Fig. 3a, b). This data also revealed the specific reduction in the relative numbers of sequencing reads in the floxed exons compared to adjacent non-floxed exons. This was true for all floxed genes in the mouse genotypes that contain the floxed alleles but not in those that do not (Supplementary Fig. 2c), consistent with specific Cre-mediated recombination occurring equivalently for all genes in all genotypes. In the case of Vhl, the deletion of the first exon including the first intronic mRNA splice site leads to sequencing read-through into the intron. Since the intronic sequencing reads do not start at the same position in different tumour samples, it is difficult to assess the effect of this read-through on potential translation of the resulting mRNA transcript, however western blotting of primary cells derived from Vhlflox/flox mice demonstrated that Cre-mediated recombination results in complete loss of the pVHL protein isoforms15 and Supplementary Fig. 5b. The slightly varying degrees of residual sequencing reads in the floxed exons of the different genes likely reflects gene expression in various types of tumour stromal cells, which likely differentially express the different genes.

Tumours in VhlΔΔTrp53ΔΔΔRbl1ΔΔHif1αΔΔΔlox mice lacked the clear nuclear HIF-1α signal that was present in tumours from VhlΔΔTrp53ΔΔRbl1ΔΔ and VhlΔΔTrp53ΔΔRbl1ΔΔHif2αΔΔΔlox mice when staining with an anti-HIF-1α antibody (Fig. 1d). RNA-sequencing analyses identified Car9 as a HIF-1α specific target gene (Supplementary Fig. 3c) and the protein product of this gene, CA9, showed membrane staining in tumours from VhlΔΔTrp53ΔΔΔRbl1ΔΔΔ and VhlΔΔTrp53ΔΔΔRbl1ΔΔΔHif2αΔΔΔlox mice but not in tumours from VhlΔΔΔTrp53ΔΔRbl1ΔΔHif1αΔΔΔlox mice (Fig. 1d). Nuclear HIF-2α staining was present in tumours from VhlΔΔΔTrp53ΔΔΔRbl1ΔΔΔ and VhlΔΔΔTrp53ΔΔΔRbl1ΔΔΔHif1αΔΔΔΔlox mice but absent in all tumours from VhlΔΔΔTrp53ΔΔΔRbl1ΔΔΔHif2αΔΔΔlox mice. Collectively these analyses demonstrate that Cre activity occurs equivalently for all floxed genes and that Hif1α and Hif2α are deleted in the tumours arising in the relevant mouse backgrounds.

Characterisation of Hif1α- and Hif2α-deficient mouse ccRCC. Histomorphological analyses and comparisons were performed for the different genetic backgrounds on a total of 26 (VhlΔΔΔTrp53ΔΔΔRbl1ΔΔΔHif1αΔΔΔlox), 16 (VhlΔΔΔTrp53ΔΔΔRbl1ΔΔΔHif1αΔΔΔlox), and 21 (VhlΔΔΔTrp53ΔΔΔRbl1ΔΔΔHif2αΔΔΔlox) H&E stained tumours. In line with our previous report16, all VhlΔΔΔTrp53ΔΔΔRbl1ΔΔΔΔlox tumours were classified as mid-to-high grade (46% grade 2; 54% grade 3) tumours. The malignant lesions in the VhlΔΔΔTrp53ΔΔΔRbl1ΔΔΔHif1αΔΔΔlox (19% grade 2; 75% grade 3; 6% grade 4) or VhlΔΔΔTrp53ΔΔΔRbl1ΔΔΔHif2αΔΔΔlox background (14% grade 2; 81% grade 3; 5% grade 4) displayed on average higher degrees. Since tumour grade up to grade 3 is classified mostly based on nucleous size, these data hint that loss of HIF-1α or HIF-2α may modify processes such as transcription of ribosomal DNA genes that affect the nucleolus28. Potentially relevant mechanisms that have been previously linked to HIF-α activities and that might contribute to nucleolar alterations include metabolite generation of ATP and deoxynucleotides to fuel transcription, epigenetic regulatory mechanisms, and DNA repair29. In order to analyse similarities to the classical human ccRCC clear cell phenotype, we established a scoring system (Fig. 1e) based on a three-tiered classification of tumours with completely clear cytoplasm (score 1), partly clear or weakly stained cytoplasm (score 2), or stronger cytoplasmic eosin staining (score 3). Fifty-seven percent of the VhlΔΔΔTrp53ΔΔΔRbl1ΔΔΔlox tumours were classified with a clear cell score of one or two, whereas the vast majority of tumours in the other genetic backgrounds showed a score of three (86% of VhlΔΔΔTrp53ΔΔΔRbl1ΔΔΔHif1αΔΔΔlox and 83% of VhlΔΔΔTrp53ΔΔΔRbl1ΔΔΔHif2αΔΔΔlox) (Fig. 1f). This observation is consistent with our previous findings that HIF-1α is necessary for the clear cell phenotype of normal renal epithelial cells following Vhl deletion29 but also implicates HIF-2α in the clear cell phenotype in this tumour model. Intra-tumoural histomorphological heterogeneity was observed mainly in VhlΔΔΔTrp53ΔΔΔRbl1ΔΔΔlox (23%) and VhlΔΔΔTrp53ΔΔΔRbl1ΔΔΔHif2αΔΔΔlox tumours (28%) (Supplementary Fig. 4a) and is a well-known characteristic of human ccRCC30. Other typical histopathological features of ccRCC like necrosis (Supplementary Fig. 4b) or intra-tumoural haemorrhage (Supplementary Fig. 4c) were equally distributed throughout the different genotypes and were mainly detected in larger tumours. The vast majority of tumours showed a solid and spherical growth pattern with pushing rather than infiltrating borders. Hemangioinvasion with direct tumour infiltration of blood vessels or extra-parenchymal invasion of the peri-tumoral renal tissue was not observed in any of the cases. A subset of VhlΔΔΔTrp53ΔΔΔRbl1ΔΔΔlox (15%), VhlΔΔΔTrp53ΔΔΔRbl1ΔΔΔHif1αΔΔΔlox (25%), and VhlΔΔΔTrp53ΔΔΔRbl1ΔΔΔHif2αΔΔΔlox tumours (19%) exhibited cystic features (Supplementary Fig. 4d). While all ccRCC in the VhlΔΔΔTrp53ΔΔΔRbl1ΔΔΔlox background showed strong phospho-4E-BP1-staining, indicative of PI3K/mTOR-pathway activation, only 50% of the VhlΔΔΔTrp53ΔΔΔRbl1ΔΔΔHif1αΔΔΔlox and 88% of the VhlΔΔΔTrp53ΔΔΔRbl1ΔΔΔHif2αΔΔΔlox tumours were positive (Fig. 1d). Irrespective of the genetic background, all malignant lesions stained positively for the proximal tubule marker CD10 (Fig. 1d).
Cancer assays do not reflect HIF-1α’s in vivo oncogenic role. Since our genetic experiments demonstrated that HIF-1α is necessary for the efficient initiation of ccRCC formation we wondered firstly if HIF-1α is generally required for cellular proliferation following loss of Vhl and secondly whether established VhlΔ/ΔTrp53Δ/ΔRb1Δ/Δ tumours remain dependent on HIF-1α or whether they might lose this dependency during tumour evolution. To mimic the earliest events in ccRCC formation in a genetically tractable cellular system that allows long-term proliferation assays, we derived mouse embryonic fibroblasts (MEFs) from wild type, VhlΔ/Δ, VhlΔ/ΔHif1aΔ/Δ, VhlΔ/ΔHif2aΔ/Δ, and VhlΔ/ΔHif1aΔ/ΔHif2aΔ/Δ embryos, infected them with Adeno-GFP as control or Adeno-Cre-GFP and confirmed the deletion of the floxed genes by real-time PCR and western blotting (Supplementary Fig. 5a, b). Long-term proliferation assays confirmed previous findings that loss of Vhl in MEFs induces an early loss of proliferative capacity, however, in contrast to the initial claim that this senescence phenotype is independent of HIF-a activity31, our results clearly demonstrate the dependency on Hif1a but not on Hif2a (Fig. 2a). This proliferative rescue due to Hif1a deletion contrasts with the suppression of ccRCC initiation by Hif1a deletion in vivo. To remove the potential confounding factor of senescence we took advantage of the fact that deletion of Trp53 overcomes the phenotype of loss of proliferative capacity associated with loss of Vhl23,32. To investigate the effect of loss of HIF-1α function in immortalised cells we infected VhlΔ/ΔTrp53β/β MEFS with lentiviruses expressing either non-silencing control shRNA or expressing two different shRNAs against Hif1a and infected the cells with Adeno-GFP or Adeno-Cre-GFP. Western blotting confirmed the reduced abundance of pVHL, p53, and HIF-1α (Supplementary Fig. 5c). Knockdown of HIF-1α further increased the proliferation rate of immortalised Vhl/Trp53 null MEFs (Fig. 2b), furthering illustrating that HIF-1α generally acts to inhibit proliferation in the context of Vhl deletion.

We next used a cell line derived from a mouse VhlΔ/ΔTrp53Δ/ΔRb1Δ/Δ ccRCC (termed 2020 cells) (Supplementary Fig. 5d) and introduced human pVHL30 to rescue Vhl function (Supplementary Fig. 5e) as well as knocked down Hif1a with two independent shRNAs (Supplementary Fig. 5f). We confirmed the efficient functional re-introduction of pVHL30 and knockdown of Hif1a mRNA in reducing HIF-1α protein (Supplementary Fig. 5g) and showed that the knockdowns reduced the abundance of the PDK1 and LDH-A proteins, that are encoded by the HIF-1α transcription target genes Pdk1 and LDha, equivalently to pVHL30 re-introduction (Supplementary Fig. 5h). Proliferation assays revealed that neither pVHL30 re-introduction (Fig. 2c), nor Hif1a (Fig. 2d) knockdown affected cellular proliferation of 2020 cells growing in renal epithelial medium on cell culture plastic but that either of these manipulations were sufficient to increase the growth of 2020 cells as spheroids in non-adherent cell culture conditions (Fig. 2e–g), a common readout of cellular transformation. Since re-introduction of pVHL into human ccRCC cell lines does not affect proliferation rates in culture, but does inhibit tumour formation in the xenograft setting33, we conducted allograft studies in SCID-Beige mice. pVHL re-introduction into 2020 cells significantly delayed tumour growth (Fig. 2h) but HIF-1α knockdown did not (Fig. 2i).

Collectively, these studies show that HIF-1α in fact antagonises cellular proliferation of normal mouse cells lacking Vhl and is dispensable for proliferation and allograft tumour formation of a mouse ccRCC cell line, highlighting the specificity of the requirement for HIF-1α for tumour onset in the autochthonous setting. This argues that the oncogenic role of HIF-1α is evident only in the context of the physiological environment of the renal epithelium.

Impact of HIF-1α and HIF-2α on mouse ccRCC transcriptome. Our previous analyses demonstrated that the mouse ccRCC model exhibits an excellent overlap with human ccRCC at the global transcriptional level19. To gain further insight into in vivo relevant functions of HIF-1α and HIF-2α we compared the molecular features of tumours that developed in the presence of both HIF-1α and HIF-2α to those that were genetically restricted to develop in the absence of either HIF-1α or HIF-2α. We conducted RNA sequencing of six WT cortex samples, six VhlΔ/ΔTrp53Δ/ΔRb1Δ/Δ tumour samples, eight VhlΔ/ΔTrp53Δ/ΔHif1aΔ/Δ tumour samples, and ten VhlΔ/ΔTrp53Δ/ΔRb1Δ/ΔHif2aΔ/Δ tumour samples, and combined these data with our previously obtained RNA-sequencing data from three WT cortex samples and six VhlΔ/ΔTrp53Δ/ΔRb1Δ/Δ tumour samples. After read trimming and mapping (Supplementary Fig. 6a, b) the mRNA abundance of 19,723 genes was determined in each sample. All normalised gene expression values are provided in Supplementary Data 1. Transcriptomic profile principal component analysis and unsupervised hierarchical clustering by sample Euclidean distance matrix (Supplementary Fig. 6c) suggested minimal batch effect amongst different sequencing runs. Principal component analysis (Fig. 3a) also revealed clear separation of WT cortex from all tumour samples on the PC1 axis and this accounted for 36% of the overall variability. VhlΔ/ΔTrp53Δ/ΔRb1Δ/Δ tumours and VhlΔ/ΔTrp53Δ/ΔHif2aΔ/Δ tumours tended to segregate from one another on the PC2 axis, which represented 9% of total variability, suggesting that they are the most distinct in terms of gene expression patterns, whereas VhlΔ/ΔTrp53Δ/ΔRb1Δ/ΔHif1aΔ/Δ tumours were more widely distributed along the entire axis. These analyses are consistent with the deletion of Vhl, Trp53, and Rb1 in all three tumour genotypes inducing large transcriptional changes, with more limited and specific contributions of HIF-1α and HIF-2α to the regulation of specific sets of genes.

We focused analyses on genes that were differentially expressed between VhlΔ/ΔTrp53Δ/ΔRb1Δ/Δ tumours and the VhlΔ/ΔTrp53Δ/ΔRb1Δ/ΔHif1aΔ/Δ and VhlΔ/ΔTrp53Δ/ΔRb1Δ/ΔHif2aΔ/Δ tumour genotypes and identified 396 differentially expressed genes that are dependent on HIF-1α (Supplementary Fig. 7a), 804 differentially expressed genes that are dependent on HIF-2α (Supplementary Fig. 7b) and 131 differentially expressed genes that are dependent on both HIF-1α and HIF-2α (Supplementary Fig. 7c). To begin to identify biological processes that these sets of genes are likely to reflect or regulate, we conducted generally applicable gene-set enrichment (GAGE) analyses using the pathway databases from ConsensusPathDB, the Biological Processes from Gene Ontology and MSigDB terms for Chemical and Genetic Perturbations and Transcription Factor Targets. The full list of statistically significantly altered (P adj <0.05) gene sets of these GAGE analyses are provided in Supplementary Data 2. VhlΔ/ΔTrp53Δ/ΔRb1Δ/ΔHif1aΔ/Δ tumours in comparison to VhlΔ/ΔTrp53Δ/ΔRb1Δ/Δ and VhlΔ/ΔTrp53Δ/ΔRb1Δ/ΔHif2aΔ/Δ tumours show low expression of glycolytic genes (Fig. 3b), as well as signatures associated with hypoxia and known HIF-1α targets. This is consistent with a large body of previous work in human ccRCC cells14 and in mouse models29 that shows that HIF-1α is the primary transcription factor that promotes Warburg-like metabolism of high rates of glycolysis and low oxidative phosphorylation. Additional HIF-1α-dependent signatures include reduced expression of genes involved in cell adhesion (Fig. 3c) and focal adhesion and receptor signalling (Fig. 3d). Genes that were expressed at low levels in VhlΔ/ΔTrp53Δ/ΔRb1Δ/Δ Hif2aΔ/Δ tumours compared to the other two tumour genotypes included those involved in different DNA repair processes (e.g. Fanc—DNA interstrand cross link repair, Rad52—homologous
recombination repair, Ogg1—oxidative stress induced base excision repair, Ercc2—transcription coupled nucleotide excision repair) (Fig. 3e), cholesterol uptake and lipoprotein metabolism (Fig. 3f), which may relate to the observed dependency of the clear cell phenotype on HIF-2α, and ribosome biogenesis (Fig. 3g), potentially consistent with the slower rate of growth of VhlΔ/Δ Trp53Δ/ΔRb1Δ/ΔHif2aΔ/Δ tumours. Other HIF-2α-dependent GAGE terms that might be relevant to the evolution and proliferation of VhlΔ/Δ Trp53Δ/ΔRb1Δ/ΔHif2aΔ/Δ tumours included genes that are targets of the MYC and E2F transcription factors.
**Fig. 2 HIF-1α is dispensable for cellular proliferation and for allograft tumour formation.** a 3T3 proliferation assays of MEFs derived from mice of the indicated genotypes infected with adenoviruses expressing GFP or Cre. Mean ± std. dev. are derived from three independent cultures. b 3T3 proliferation assays of MEFs derived from Vhlfl/flTrp53fl/fl mice infected with non-silencing shRNA (shRNA-ns) or shRNA against Hif1a (shRNA-Hif1a #1 and shRNA-Hif1a #2), followed by infection with adenoviruses expressing GFP or Cre. Mean ± std. dev. are derived from three independent cultures. c, d Proliferation assays of mouse ccRCC cell line 2020 expressing empty vector control or human pVHL30c or non-silencing shRNA (shRNA-ns) or shRNA against Hif1a (shRNA-Hif1a #1 and shRNA-Hif1a #2). Mean ± std. dev. are derived from two independent experiments each with replicates of six cultures. e–g Representative images (scale bars depict 200 μm) e and size distributions f, g of spheres formed by the cells described in c, d when grown in non-adherent cell culture plates. Mean ± std. dev. of the total number of colonies pooled from three independent experiments are shown, P values were calculated by two-sided Student’s t test. h, i Survival of mice following subcutaneous allograft tumour assays of the cells described in c into SCID-Beige mice. P values were calculated by two-sided log-rank Mantel-Cox test.

**Fig. 3 HIF-1α and HIF-2α deletion affect different transcriptional programmes and inflammatory responses.** a Principal component analysis of RNA sequencing of WT Cortex and VpR, VpRH1, and VpRH2 tumours. Gene expression heatmaps for selected differentially regulated genes from the indicated GSEA terms glycolysis (b), cell adhesion molecules (c), focal adhesion and receptor signalling (d), DNA repair (e), lipoprotein metabolism (f), ribosome biogenesis (g), T-cell activation (h), and response to IFN-β (i). Rows represent row-normalised z-scores of mRNA abundance, each column represents an individual sample from WT cortex or VpR, VpRH1, and VpRH2 tumours. Source data is provided in Supplementary Data 1 and 2.
factors, consistent with previous studies showing that HIF-2α promotes the activity of the MYC transcription factor. GAGE analyses also identified a significant downregulation of the gene set HIF-2α Transcription Network, including Eph, Egln1, Egln3, Lgfbp1, and Pfkβ3. To further investigate potential overlap with recently defined HIF-2α-dependent genes in human ccRCC, we used a set of 277 genes that were identified as being inhibited specifically in tumour cells in ccRCC tumourgrafts in mice treated with the HIF-2α inhibitor PTZ299. Analyses of the expression levels of the mouse orthologues of this set of HIF-2α target genes revealed that many of these genes are highly upregulated in VhlΔΔTprp3ΔΔRb1ΔΔ tumours compared to WT cortex, but that the loss of either HIF-1α or HIF-2α did not broadly affect the upregulation of these genes (Supplementary Fig. 7d). Nonetheless, 11 genes, marked in red in Supplementary Fig. 7d and shown in Supplementary Fig. 7e, were expressed at significantly lower levels (fold change < -1.7, P < 0.05) in VhlΔΔTprp3ΔΔRb1ΔΔHif2aΔΔ tumours than in VhlΔΔTprp3ΔΔRb1ΔΔΔ tumours. The relatively small overlap between mouse and human HIF-2α-dependent genes may be due to inherent differences between mice and humans, to the very different experimental settings of acute pharmacological inhibition versus tumour evolution in the genetic absence of Hif2a, or to specific features of this particular model of ccRCC. In this latter context, it is noteworthy that many human HIF-2α-dependent ccRCC genes are related to the cell cycle and to DNA damage responses. These signatures are highly represented in the comparison VhlΔΔTprp3ΔΔRb1ΔΔΔ versus WT cortex (see GAGE signatures in Supplementary Data 2). We speculate that it is likely that these genes are not dependent on HIF-2α in the mouse model due to the fact that the deletion of Rb1 and Tprp3 already strongly affects these classes of genes.

Interestingly, genes that were upregulated in HIF-2α-deficient tumours include those enriched in diverse GAGE terms for interferon signalling, T-cell activation, innate immunity, adaptive immunity, antigen processing and presentation, and NF-kB as well as IRF transcription factor targets, suggestive of an altered immune environment in these tumours. Supplementary Fig. 8a shows a selection of these enriched immune signatures and highlights that the signatures are upregulated in VhlΔΔTprp3ΔΔΔ Rb1ΔΔΔ tumours compared to WT cortex, that there are very few or no statistically significant differences in these signatures between VhlΔΔTprp3ΔΔΔRb1ΔΔΔHif1aΔΔΔ and VhlΔΔTprp3ΔΔΔRb1ΔΔΔ tumours (i.e. that HIF-1α deficiency does not strongly alter the inflammatory tumour environment in VhlΔΔTprp3ΔΔΔ Rb1ΔΔΔ tumours) and that all of these signatures are further highly statistically significantly upregulated in VhlΔΔTprp3ΔΔΔRb1ΔΔΔHif2aΔΔΔ tumours in comparison to VhlΔΔTprp3ΔΔΔ Rb1ΔΔΔ tumours. Gene expression heatmaps of differentially expressed genes associated with GAGE terms for T-cell activation (Fig. 3h), response to IFN-β (Fig. 3i), and IFN-γ production (Supplementary Fig. 8b) are shown as examples of these inflammatory signatures. We conclude that these analyses suggest that there is a complex inflammatory response in VhlΔΔTprp3ΔΔΔ Rb1ΔΔΔ tumours that is further modified by HIF-2α deficiency. These phenotypes were further investigated in experiments described in the following sections.

Impact of HIF-1α and HIF-2α on mouse ccRCC proteome.

In order to further explore whether the biological alterations predicted by transcriptomic analyses are also reflected at the protein expression level, as well as to attempt to capture differences in the proteomes of the tumours that might not be reflected in their transcriptomes, we used exploratory quantitative proteomic analyses of six samples of WT cortex and six tumours each from VhlΔΔTprp3ΔΔΔRb1ΔΔΔ, VhlΔΔTprp3ΔΔΔRb1ΔΔΔHif1aΔΔΔ, and VhlΔΔTprp3ΔΔΔRb1ΔΔΔHif2aΔΔΔ mice as an independent discovery tool. These analyses allowed the quantification of 4257 proteins that were present in at least four of six samples of each genotype (Supplementary Data 3). As is commonly observed in comparisons of proteome and transcriptome data, the overall correlations of protein abundance and mRNA abundance were low (Supplementary Fig. 9a). However, there were strong correlations between fold changes in mRNA abundance and fold changes in protein abundances when analysing only those proteins that showed differential expression between genotypes (Supplementary Fig. 9b–d). Through comparison with previously conducted analyses of the proteome of eight human ccRCC tumours, we identified a strong correlation in the relative abundance of proteins in mouse VhlΔΔTprp3ΔΔΔRb1ΔΔΔ ccRCC and in human ccRCC (Fig. 4a). Of the differentially expressed proteins identified in the comparison between mouse VhlΔΔTprp3ΔΔΔRb1ΔΔΔ ccRCC and WT cortex, 82% were also identified as differentially expressed proteins in comparisons of human ccRCC to normal kidney (Fig. 4b), further emphasising that the VhlΔΔTprp3ΔΔΔRb1ΔΔΔ model accurately reflects the molecular features of human ccRCC. Using a less stringent cut-off for statistical significance (P < 0.01), we identified 884 proteins that are upregulated in VhlΔΔTprp3ΔΔΔRb1ΔΔΔ ccRCCs compared to WT cortex (Fig. 4c).

To characterise biological pathways that are altered in tumour compared to normal tissue, we conducted two complementary analyses; ROAST (rotation gene set testing) analysis was used to assess gene set enrichment based on the expression levels of all measured proteins and gene set enrichment analysis using the online platform of MSigDB (https://www.gsea-msigdb.org/gsea/msigdb/index.jsp) was performed using only the lists of statistically differentially upregulated proteins. These analyses revealed many overlaps with one another as well as with GAGE gene set terms that emerged from the analyses of the transcriptome, including glycolysis, hypoxia, DNA repair, mTORC1 signalling, EZF, and MYC targets and IFNγ response (Supplementary Data 3 and Supplementary Fig. 10a).

To compare the effect of the absence of HIF-1α or HIF-2α on the proteome, we first conducted principal components analysis (Supplementary Fig. 10b), which revealed that all tumour samples clustered separately from the WT cortex samples, but that the tumour samples of all of the different genotypes largely overlapped with one another, suggesting a relatively high degree of similarity in the overall protein expression patterns of tumours from the different genetic backgrounds. ROAST analyses as well as gene set enrichment analyses of the lists of proteins that are differentially expressed between VhlΔΔTprp3ΔΔΔRb1ΔΔΔHif1aΔΔΔ and VhlΔΔTprp3ΔΔΔΔ Rb1ΔΔΔ tumours (Fig. 4d) and between VhlΔΔTprp3ΔΔΔRb1ΔΔΔHif2aΔΔΔ and VhlΔΔTprp3ΔΔΔΔ Rb1ΔΔΔ tumours (Fig. 4e and Supplementary Data 3) both also highlighted numerous similarities to the transcriptomic analyses. HIF-1α deficiency reduced expression of glycolytic enzymes and increased expression of proteins associated with oxidative phosphorylation and respiratory electron transport (Fig. 4f), while HIF-2α deficiency reduced the expression of MYC targets and resulted in increased expression of genes associated with immune responses, interferon signalling, cytokine signalling, and antigen presentation (Fig. 4g). In conclusion, the analyses of the proteomes strongly align with the analyses of the transcriptomes, providing independent validation for the predicted biological differences between the tumour genotypes.
Fig. 4 Proteomic analyses of the effects of HIF-1α and HIF-2α deletion in mouse ccRCCs. a Correlation of protein abundance in mouse and human ccRCC samples. Each dot represents a unique pair of orthologous proteins between the two species. Spearman’s correlation coefficient is depicted. b Venn diagram showing the overlap of differentially expressed proteins derived from comparison of mouse ccRCC with WT cortex and human ccRCC with normal kidney. Volcano plots showing differentially expressed proteins (green dots) between VpR and WT cortex (c), VpRH1 and VpR (d), and VpRH2 and VpR (e). Protein expression heatmaps showing differentially expressed proteins between VpRH1 and VpR (f) and VpRH2 and VpR (g) as well as a summary of GSEA terms associated with the down- and upregulated proteins. Rows represent row-normalised z-scores of protein abundance, each column represents an individual sample from WT cortex or VpR, VpRH1, and VpRH2 tumours. Source data is provided in Supplementary Data 3.
levels of expression of many MHC class I (Fig. 5a) and MHC class II (Fig. 5b) genes, as well as other genes involved in antigen processing and presentation (Fig. 5c) in VhlΔ/ΔTrp53Δ/ΔRb1Δ/Δ Hif2αΔ/Δ tumours than in the tumours of the other genotypes. Immunohistochemical staining with an anti-MHC class II antibody revealed that all tumour genotypes displayed cases in which the tumour cells were either negative, partly positive, or almost entirely positive (Fig. 5d). However, tumour cells in VhlΔ/Δ Trp53Δ/ΔRb1Δ/ΔHif2αΔ/Δ tumours were more frequently positive than the other genotypes (Fig. 5e). Since MHC class II expression is upregulated by interferon-γ signalling, these results are consistent with the fact that interferon signalling terms were upregulated in VhlΔ/ΔTrp53Δ/ΔRb1Δ/ΔHif2αΔ/Δ tumours in our transcriptomic and proteomic analyses. Tumour cells in at least half of all human ccRCCs are immunohistochemically positive for MHC class II expression42,43 and ccRCC cells have been shown to present class II ligands44. Building upon these observations, we next sought to determine in human ccRCC whether HIF2A (also known as EPAS1) expression correlates with expression of MHC class I and class II genes, as well as other genes involved in antigen processing and presentation. Analyses of TCGA mRNA expression data revealed that HIF2A, but not HIF1A, is more highly abundant in ccRCC in comparison to normal kidney and that HIF2A shows a wide distribution of expression levels amongst tumours (Fig. 5f, g). This upregulation and wide expression level distribution is not observed in chromophobe RCC or papillary RCC (Fig. 5f, g). The wide expression distribution provided a good basis to investigate potential correlations between HIF2A mRNA abundance and the abundance of mRNAs involved in antigen presentation. Examples of correlations of MHC class I (Fig. 5h, i), MHC class II (Fig. 5j, k) and antigen processing and presentation (Fig. 5l, m) genes are shown. The Spearman correlation analyses of the full lists of these classes of genes are provided in Supplementary Data 4. Consistent with our mouse tumour data, Spearman correlation analyses revealed statistically significant negative correlations between HIF2A expression and the expression of 3 of 6 MHC class I genes, 12 of 15 MHC class II genes, and 26 of 51 non-MHC genes from the GO antigen processing and presentation gene set. While highly statistically significant, the relatively small magnitudes of some of these correlations suggest that HIF2A expression levels may be one of several factors that influence the overall expression of antigen presenting genes in ccRCC. Collectively the mice and human data suggest that HIF-2α suppresses antigen presentation.

**HIF-1α and HIF-2α alter the ccRCC immune microenvironment.**

Since diverse and complex inflammatory signatures were observed in the transcriptomes and proteomes of ccRCC tumours, we next applied three different bioinformatic methods to our RNA-sequencing data to attempt to further deconvolve the relative abundance of different types of immune cells or specific gene signatures associated with inflammation in the different tumour genotypes. We applied two methods that were previously used to deconvolve the immune microenvironment of human ccRCC12,43. These methods are based on single-sample gene set enrichment analyses (ssGSEA) using the mouse orthologues of an expanded number of immune-specific gene signatures to those initially described by Bindea et al.44, which we term Bindea et al. (described in ref. 43), and a set of gene signatures that were identified by analyses of human ccRCC tumourgrafts, termed eTME (described in ref. 42). The genes in these signatures and their overlap are listed in Supplementary Data 5. The third method is CIBERSORT with the mouse specific ImmuCC gene panel, which uses a matrix-weighted score, based on both high and low expressed genes in each immune subset, to assess the relative abundance of each immune cell population45. For all three methods we generated z-scores representing the degree of enrichment of each signature and statistically compared the immune infiltration scores of all tumours of a given genotype in a series of pairwise comparisons to WT cortex and to the other genotypes (Fig. 6a). In general, comparisons of all three tumour genotypes to WT cortex revealed enrichment of gene sets associated with myeloid cell inflammation, including dendritic cells, monocytes, macrophages, and neutrophils, but not mast cells or eosinophils. Terms associated with different types of T cells and B cells revealed inconsistent results, varying depending on the deconvolution method used. VhlΔ/ΔTrp53Δ/ΔRb1Δ/ΔHif2αΔ/Δ tumours showed greater enrichment of a number of different immune cell signatures when compared to VhlΔ/ΔTrp53Δ/ΔRb1Δ/Δ tumours and to VhlΔ/ΔTrp53Δ/ΔRb1Δ/ΔHif1αΔ/Δ tumours, including several types of T cells, monocytes, and macrophages, and notably for interferon-γ signalling (REACTOME.IFNG), consistent with the previous GAGE analyses. VhlΔ/ΔTrp53Δ/ΔRb1Δ/ΔHif1αΔ/Δ tumours showed statistically lower average z-scores, or trends to lower scores, for myeloid cell signatures than VhlΔ/ΔTrp53Δ/ΔRb1Δ/Δ and VhlΔ/ΔTrp53Δ/ΔRb1Δ/ΔHif2αΔ/Δ tumours.

To further characterise the immune microenvironments of the three different tumour genotypes, we next conducted immunohistochemical stainings for a series of markers of different types of immune cells to permit analyses of a larger set of tumours of each genotype (n = 14–26 tumours). We stained sections of whole tumour-bearing kidneys with antibodies against CD3 to label T cells, CD4 to label helper T cells, CD8 to label effector T cells, CD69 as an early activation marker of T cells and NK cells, pericentrin to label activated cytotoxic T cells and NK cells, PD-1 to label antigen-exposed activated or exhausted T cells, B220 to label B cells, CD68 to label monocytes and macrophages, F4/80 to label differentiated macrophages, and Ly-6G to label granulocytes and neutrophils. These markers revealed considerable inter-tumoural heterogeneity in terms of the density of infiltrating cells, even within the same kidney (Supplementary Fig. 11a–j). We quantified the densities of positively stained cells either by manual counting, via automated detection and quantification algorithms, or we calculated the average relative staining intensity for F4/80 where it was not possible to identify individual cells in the network of macrophages, within the tumours as well as in unaffected regions of kidney tissue (normal) within the same mouse (Supplementary Fig. 11k, l). Consistent with HIF-2α deficient tumours showing the highest GAGE mRNA signatures of T-cell inflammation, VhlΔ/ΔTrp53Δ/ΔRb1Δ/Δ Hif2αΔ/Δ tumours displayed increased densities of CD3 (Fig. 6b), CD4 (Fig. 6c), and CD8 (Fig. 6d) positive T cells compared to normal tissue, whereas only CD8 positive T-cell densities were significantly increased in VhlΔ/ΔTrp53Δ/ΔRb1Δ/Δ and VhlΔ/Δ Trp53Δ/ΔRb1Δ/ΔHif1αΔ/Δ tumours compared to the respective normal tissues. Notably, both VhlΔ/ΔTrp53Δ/ΔRb1Δ/ΔHif1αΔ/Δ and VhlΔ/ΔTrp53Δ/ΔRb1Δ/ΔHif2αΔ/Δ tumours exhibited higher densities of CD8 positive T cells than VhlΔ/ΔTrp53Δ/ΔRb1Δ/Δ tumours, in line with the Bindea et al. ssGSEA CD8 T-cell signature results. VhlΔ/ΔTrp53Δ/ΔRb1Δ/ΔHif1αΔ/Δ but not VhlΔ/ΔTrp53Δ/ΔRb1Δ/ΔHif2αΔ/Δ tumours showed increased densities of CD4 positive cells compared to VhlΔ/ΔTrp53Δ/ΔRb1Δ/Δ tumours. This result is not reflected by any of the bioinformatic immune deconvolution methods. Analyses of the T-cell activation markers CD69 (Fig. 6e) and perforin (Fig. 6f) revealed that all tumours showed increased T-cell activation compared to normal tissue, and that VhlΔ/ΔTrp53Δ/ΔRb1Δ/ΔHif2αΔ/Δ tumours showed higher levels of T-cell activation than VhlΔ/ΔTrp53Δ/ΔRb1Δ/Δ or VhlΔ/ΔTrp53Δ/ΔRb1Δ/ΔHif1αΔ/Δ tumours, consistent with our conclusions from the GAGE analyses. There was no statistically significant enrichment of PD-1 positive cells, a marker of
exhausted T cells, in any of the tumour genotypes (Fig. 6g). B220 staining revealed increased B cell density in all tumour genotypes compared to normal tissue, and higher densities in VhlΔ/ΔTrp53Δ/Rb1Δ/ΔHif1aΔ/Δ tumours than in VhlΔ/ΔTrp53Δ/Rb1Δ/Δ or VhlΔ/ΔTrp53Δ/Rb1Δ/ΔHif2aΔ/Δ tumours (Fig. 6h). Interestingly, these observations do not reflect the results of the bioinformatic immune cell deconvolutions for B cells. In contrast to the relatively low numbers of tumour-infiltrating lymphocytes, myeloid lineage cells were much more abundant within tumours. CD68 positive monocytes/macrophages (Fig. 6i), F4/80 positive macrophages (Fig. 6j), and Ly-6G-labelled granulocytes/neutrophils (Fig. 6k) were highly abundant.
in tumours compared to normal tissue but there were no differences in abundance of these cells between tumour genotypes. Given that HIF-1α and HIF-2α deficiencies increase CD8 positive T-cell infiltration, we first sought to gain insight into potential mechanisms that might explain these observations by examining our RNA-seq data. We previously demonstrated that VhlΔ/ΔTrp53Δ/ΔRb1Δ/Δ tumours show upregulation of numerous cytokines in comparison to WT cortex,16 however analyses of the new dataset did not reveal any cytokines that were specifically altered by the absence of HIF-1α or HIF-2α (Supplementary Fig. 12a) that might be expected to influence T-cell infiltration or activation. To functionally investigate whether signalling molecules or metabolic factors that are released by mouse or human ccRCC cells might directly influence the proliferation of CD8+ T cells, we activated mouse splenic CD8+ T cells and incubated them for 3 days in conditioned medium from mouse 2020 ccRCC cells (including VHL30 rescue and Hif1a knockdown cells), human renal proximal tubule epithelial cells (RPTEC), human A498 ccRCC cells or human and papillary (KIRC, n = 290) renal cell carcinomas and associated normal renal tissues (Normal_KIRC n = 25, Normal_KIRC n = 72, Normal_KIRC n = 32). Box–whisker plots depict median, bounded by Q1 (25% lower quartile) and Q3 (75% upper quartile) and whiskers depict 1.5 times the Q3 range. Spearman’s correlation analyses between HIF2A mRNA abundance and mRNA abundance of two MHC class I (h, i), class II (j, k), and antigen processing/presentation (l, m) genes in ccRCC (TCGA KIRC dataset). Source data is provided in Supplementary Data 4.

Fig. 5 HIF-2α influences the expression of MHC class I and II genes. Gene expression heatmaps for MHC class I (a), class II (b), and other antigen processing and presenting (c) genes. Rows represent row-normalised z-scores of mRNA abundance, each column represents an individual sample from WT cortex or VpR, VpRHI, and VpRHI2 tumours. Source data is provided in Supplementary Data 1 and 2. d Examples of different scores for MHC class II immunohistochemical staining. All panels are the same magnification, scale bar = 100 μm. e Distribution of MHC class II staining scores in the indicated (n) number of VpR, VpRHI, and VpRHI2 tumours. P values are derived from the two-sided Mann–Whitney U test without adjustments for multiple comparisons. f, g Relative HIF1A and HIF2A mRNA abundance in TCGA datasets of human chromophobe (KICH, n = 66), clear cell (KIRC, n = 533), and papillary (KIRP, n = 290) renal cell carcinomas and associated normal renal tissues (Normal_KICH n = 25, Normal_KIRC n = 72, Normal_KIRP n = 32). Box–whisker plots depict median, bounded by Q1 (25% lower quartile) and Q3 (75% upper quartile) and whiskers depict 1.5 times the Q3 range. Spearman’s correlation analyses between HIF2A mRNA abundance and mRNA abundance of two MHC class I (h, i), class II (j, k), and antigen processing/presentation (l, m) genes in ccRCC (TCGA KIRC dataset). Source data is provided in Supplementary Data 4.

which was detected in 9% of all ccRCC tumours (Supplementary Fig. 13e, f). However, BAP1 mutation status alone did not significantly affect survival (Supplementary Fig. 13g) in this cohort and removal of all BAP1 mutant tumours from the cohort did not alter the correlation of HIF1A loss with poor prognosis (Supplementary Fig. 13h, i). The conclusion that BAP1 mutation status is not a relevant co-variant that affects survival outcome in the HIF1A loss cohort was also demonstrated by COX univariate (HR 1.839, 95% CI 1.332–2.539, P = 0.00021) and multivariate proportional hazards analyses (HR 1.776, 95% CI 1.274–2.474, P = 0.00069). These findings suggest that loss of one allele of HIF1A, which is predicted to lead to diminished HIF-1α abundance, may be selected for during the evolution of some ccRCC tumours and that this correlates with aggressive disease.

To investigate whether HIF1A loss correlates with altered inflammation, we first demonstrated that HIF1A loss tumours exhibit on average between 1.9- and 2.1-fold higher levels of mRNA of CD3D, CD3E, CD8A, and CD88 and 1.4-fold higher levels of CD4 than unaltered tumours (Fig. 7d, f, h, j, l), suggesting that this group of tumours has higher CD8+, and to a lesser extent CD4+, T-cell infiltration. This observation is consistent with the mouse analyses in which Hif1a-deficient tumours on average display approximately double the number of CD8+ and CD4+ T cells. In contrast, HIF2A gain tumours show no differences in the expression of any of these T-cell marker genes compared to unaltered tumours (Fig. 7e, g, i, k, m). To gain a more in-depth overview of the effects of HIF gene copy number or expression level alterations on the immune microenvironment, we performed immune deconvolution analyses of RNA-seq data, again using three independent methods of immune cell deconvolution; ssGSEA using the Bindera et al. and eTMGE gene signatures and using the CIBERSORT method. We compared HIF1A loss and HIF2A gain tumours to diploid human ccRCCs (Fig. 7n) and also took advantage of the wide distribution of mRNA expression levels of HIF2A to compare tumours in the top (Q4) and bottom (Q1) quartiles of HIF2A mRNA abundance. While HIF2A gain tumours exhibited very few alterations in immune scores, HIF1A loss tumours showed statistically significant increases or decreases in 57 of 83 immune signatures of a variety of lymphoid and myeloid lineage cells. Notable amongst these are consistently upregulated scores for T helper cells and for B cells, mirroring our immunohistochemical findings of the comparison of VhlΔ/ΔTrp53Δ/ΔRb1Δ/Δ and VhlΔ/Δ Trp53Δ/ΔRb1Δ/ΔHif1aΔ/Δ and VhlΔ/ΔHif2aΔ/Δ tumours. It should however be noted that the magnitude of the z-scores are generally low, suggesting that this group of ccRCCs on average exhibits numerous subtle differences in immune inflammation compared to tumours with normal HIF1A copy number. In contrast, high HIF2A mRNA expressing tumours displayed downregulation of scores for interferon-γ and for APM2 (measuring MHC class II antigen presentation), consistent with the upregulation of these features in mouse ccRCC tumours lacking HIF-2α. Somewhat paradoxically, high HIF2A expressing tumours also displayed general
upregulation of CD8 T-cell scores and some NK cell scores and downregulation of all three scores for regulatory T cells and for the immune checkpoint proteins PD-1 and CTLA4. These scores might be predicted to reflect elevated CD8 T-cell activity. However, these tumours also display elevated scores for monocytes, neutrophils, and mast cells, which in some settings contribute to suppression of anti-tumour CD8 T-cell responses. Immunosuppressive mast cells were shown to correlate with HIF2A mRNA abundance in human ccRCC. Thus, the extent of T cell mediated anti-tumour immunity is likely to be determined by the balance of the abundance and activities of several different immune cell types in a manner that is partly influenced by HIF2A.
expression. Finally, it is also noteworthy that HIF1A copy loss and HIF2A mRNA high tumours showed opposite effects on signatures for pericytes, endothelial cells and angiogenesis, implying that both HIF-1α and HIF-2α may act as positive factors that promote blood vessel formation in ccRCC tumours.

Collectively, the mouse and human analyses demonstrate that the genetic copy number or expression level status of the HIF genes in ccRCC tumour cells correlate with the composition and activation state of the innate and adaptive immune systems in the tumour microenvironment.

Discussion

Herein we show by introducing floxed alleles of Hif1a or Hif2a into an autochthonous mouse model of ccRCC that HIF-1α is necessary for tumour formation whereas HIF-2α deficiency has only a moderate effect on tumour initiation and growth. While it cannot be excluded that there might be differences between mice and humans, and the findings may be contextual to the Vhl/Trp53/Rb1 mutant background, this result seemingly contrasts with several independent lines of evidence from the study of human ccRCC tumours and ccRCC cell lines, which have demonstrated that HIF-2α possesses strong oncogenic activity and HIF-1α acts in the manner of a tumour suppressor to suppress aggressive tumour behaviour. How can these apparent discrepancies be reconciled? There are several possible explanations which suggest that these observations may not in fact be discrepancies. In addition, our current study also suggests that there may be caveats to the interpretation of previous studies, arguing against an oversimplified, binary oncogene/tumour suppressor model of the contributions of these proteins to ccRCC development. We argue that HIF-1α and HIF-2α are likely to play different roles at different stages of tumour formation and progression and that these roles might potentially be modulated by the spectrum of mutations present in each individual ccRCC. We further argue that it is possible that the balance of the relative strengths of the activities of the different HIF-α proteins might be dynamically modulated via different mechanisms throughout the lifetime of a ccRCC tumour to tailor their combined transcriptional outputs to provide an evolutionary advantage at the given stage of the tumour.

In addition to genetic mechanisms that can irreversibly affect the balance of HIF-1α and HIF-2α activities, such as loss of one copy of chromosome 14q, encoding HIF1A, or intragenic deletions of HIF1A22, several mechanisms exist that potentially provide tumour cells with a more dynamic mode of fine-tuning the relative strengths of HIF-1α and HIF-2α expression and activities. These include mutual epigenetic suppression50, mutual suppression of protein levels53, HIF-2α-mediated suppression of HIF-1α translation51 and HAF-mediated ubiquitination and degradation of HIF-1α to promote the switch from HIF-1α towards HIF-2α activity52. Analyses of expression patterns in human ccRCC support the notion that HIF-1α plays an oncogenic role at early and late stages of ccRCC development and progression. In VHL patients, the earliest VHL-null multi-cellular renal tubule lesions tend to strongly express HIF-1α and weakly express HIF-2α, but later lesions such as cysts and tumours express HIF-1α as well as higher levels of HIF-2α23,24,25. While HIF-2α protein is present in the vast majority of all sporadic ccRCC tumours, HIF-1α protein expression is not detected in about 30% of cases and this correlates with increased tumour cell proliferation21. Nonetheless, HIF-1α is detected in about 70% of ccRCC tumours and several studies have correlated higher HIF-1α expression levels with poor patient survival (reviewed in ref. 54). Consistent with our current findings of an obligate oncogenic role of HIF-1α, transgenic overexpression of constitutively stabilised HIF-1α55, but not HIF-2α56 in mouse renal tubules causes the formation of small lesions that have some features of precursor lesions of ccRCC. Interestingly, combined overexpression of HIF-1α and HIF-2α did not cause a more severe phenotype than HIF-1α overexpression alone56, demonstrating that even the combined actions of both HIF-1α or HIF-2α are insufficient to induce tumour formation. In agreement with this conclusion, numerous previous studies showed that deletion of Vhl in mouse renal epithelial cells in vivo57–62, resulting in abrogation of the many different tumour suppressor functions of pVHL2,7,63, was insufficient to cause tumour initiation either when both HIF-1α and HIF-2α were stabilised, or when the balance of HIF-1α and HIF-2α activities were genetically altered by co-deletion of Hif1a or Hif2a29. Nonetheless, deletion of either Hif1a or Hif2a was sufficient to inhibit the formation of cysts and tumours induced by Vhl/Trp53 double mutation29, demonstrating that both HIF-1α and HIF-2α have pro-tumourigenic activities. In this context, it is noteworthy that we now show that Hif2a deletion fails to strongly inhibit tumour formation in the Vhl/Trp53/Rb1 deletion model. One explanation for these different findings may relate to our RNA-sequencing observations which highlighted that HIF-2α increases expression of MYC and EZF target genes, consistent with previous findings that HIF-2α stimulates MYC activity21. This predicted cell cycle promoting activity of HIF-2α is likely to be necessary for tumour formation in the Vhl/Trp53 background, but may become at least partly redundant due to the additional mutation of Rb1, which promotes the cell cycle by removing the negative regulation of EZF transcription factors.

The patterns of copy number alterations that arise in the TCGA ccRCC dataset are consistent with the idea that the balance of HIF-1α and HIF-2α activities may be differently selected for, or tolerated, depending on the status of the network of G1-S cell cycle controlling genes. We have previously described a copy number signature in which multiple genes involved in the p53 pathway and G1-S checkpoint are altered in about two thirds of ccRCC tumours16. While HIF1A copy number losses or mutations (45%) occur in ccRCC more frequently than gains (2.5%), HIF2A losses are very rare (4%), and gains more common (15%) (Supplementary Fig. 14a). Interestingly, of the rare tumours with HIF2A losses or mutations, only 2 of 16 did not exhibit a copy number alteration in one or more genes that control the G1-S checkpoint (Supplementary Fig. 14b). Thus, rare genetic events that might be predicted to result in lowered HIF-2α activity almost always arise in the background of genetic alterations that
are predicted to abrogate or weaken normal cell-cycle control. We speculate that this may allow the tumours to grow in the absence of a lowered cell cycle promoting activity of HIF-2α. In contrast, the distribution of copy number losses of HIF1A does not correlate with the presence or absence of alterations in the G1-S network: 52 tumours harbouring HIF1A copy loss showed no alteration in G1-S genes, while 146 tumours with HIF1A copy loss did display alterations (Supplementary Fig. 1c).

Since our current and previous mouse genetic studies collectively show that the requirement for HIF-1α and HIF-2α is dependent on the underlying genotype of the tumour (Vhl/Trp53/Rb1 mutations), it will be important to test the generality of our findings in other genetic mouse models of ccRCC, particularly as epigenetic modifications resulting from mutations in Pbrm1 have been shown to alter HIF-1α transcriptional outputs. It is likely that other ccRCC-
relevant epigenetic tumour suppressors such as BAP1, SETD2, and KDM5C also influence HIF-α transcriptional outputs. Thus, the combinations of mutations present in ccRCC cells might represent another mechanism for altering the balance of the relative activities of HIF-1α and HIF-2α, potentially affecting the genetic dependency on the two HIF-α proteins.

Our present study also highlights a potential general caveat to the interpretation of the results of studies of human ccRCC cell lines, or patient-derived tumour lines, in cell culture and in xenograft assays. In contrast to the clear requirement for Hif1α in tumour formation in the autochthonous setting, we find that HIF-1α rather exhibits putative tumour suppressor activities in cell culture-based assays, including inducing the early loss of proliferative capacity of MEFs following Vhl deletion, inhibiting the proliferation of immortalised Vhl/Trp53 null MEFs and inhibiting anchorage-independent growth of a Vhl/Trp53/Rb1 mutant mouse ccRCC cell line. Furthermore, HIF-1α knockdown did not affect the growth of allograft tumours generated with this ccRCC cell line. This latter experiment argues against the idea that removal of HIF-1α activity in an established mouse ccRCC tumour, to mimic the loss of HIF-1α function that arises as a later event in tumour progression in a subset of human ccRCCs, has a potent effect on tumour aggressiveness. Collectively, our studies show that in the same genetic ccRCC tumour model, the cell culture and allograft assays do not reflect the in vivo requirement in the autochthonous setting. Previous studies which have interpreted the oncogenic and tumour suppressive roles of HIF-α factors in ccRCC based on studies of cultured ccRCC cells and xenograft models may therefore not necessarily be reflective of the true in vivo functions of HIF-1α and HIF-2α at different stages of tumour development in the physiological context of the kidney.

Our transcriptomic and proteomic analyses suggest that one potential oncogenic contribution of HIF-1α to tumour initiation is the induction of expression of genes that induce Warburg metabolism of high rates of conversion of glucose to lactate and low rates of pyruvate entry into mitochondrial respiration. These findings of HIF-1α dependency are in accordance with numerous studies in mice and humans. This mode of Warburg tumour metabolism has recently been proven to occur in vivo in human ccRCC tumours and is in stark contrast to the normal mode of metabolism of RPTECs, the cell of origin of ccRCC, which generate ATP predominantly through mitochondrial oxidation of substrates other than glucose, allowing them to fuel solute transport, including the transport of glucose, from the tubular fluid back to the blood stream. It remains to be functionally elucidated if and how this shift to glucose metabolism contributes to ccRCC formation by proximal tubule cells and whether this represents a true driver of tumour formation or acts as an enabler of tumour formation once additional transforming genetic mutations arise. Previously published mouse genetic data suggest that the latter is likely to be the case as induction of Warburg metabolism in mice through Vhl deletion alone did not cause tumour formation. We identified that both HIF-1α and HIF-2α are necessary for the clear cell phenotype of Vhl/Trp53/Rb1-deficient ccRCCs, implying that they might differently contribute to the accumulation of cytoplasmic glycogen and lipids. HIF-1α-dependent alterations in glucose and glycogen utilisation and reduction of mitochondrial abundance and activity might lead to accumulation of glycogen and failure to efficiently metabolise fatty acids. We also identified a HIF-2α-dependent metabolic signature involving genes involved in cholesterol and lipoprotein uptake and metabolism, which may account for the requirement of HIF-2α for the clear cell phenotype.

A major advantage of the study of autochthonous models of ccRCC is that the tumours develop in the presence of a functioning immune system. In general, the activities of cytotoxic innate and adaptive immune cells can act to inhibit or delay tumour formation and progression, but an inflamed tumour microenvironment can also promote tumour formation through a variety of mechanisms. The immune microenvironment of human ccRCC appears to be unusual in some respects as higher levels of T-cell infiltration have been shown to correlate with disease recurrence and worse survival. In almost all other tumour types the degree of T-cell inflammation is a good prognostic factor. ccRCC tumours are also frequently infiltrated with immature myeloid lineage cells that at least partly reflect different states of differentiation along the path from myeloid progenitor to differentiated dendritic cells, macrophages, and neutrophils. At least some of these myeloid lineage cells have been proposed to promote tumour formation by suppressing T-cell activation. Our bioinformatic immune deconvolution and immunohistochemical analyses of the immune microenvironment of mouse ccRCC tumours is broadly consistent with this latter observation, namely that the immune microenvironment is dominated by myeloid lineage cells with a modest degree of T-cell inflammation and activation. An unexpected finding was that tumours with Hif2a deletion showed stronger mRNA signatures associated with tumour immune cell infiltration, antigen presentation, and interferon activity, as well as higher densities of CD8 T cells and cells expressing the T-cell activation markers CD69 and perforin, compared to the other two tumour genotypes. Furthermore, MHC class I and II genes, as well as other genes involved in antigen processing and presentation, were upregulated and tumour cells in VhlΔ/ΔTrp53Δ/ΔRb1Δ/ΔHif2aΔ/Δ tumours were more frequently immunohistochemically positive for MHC class II expression, suggestive of increased presentation of intracellular and extracellular epitopes in this tumour genotype. Collectively, these observations reflect a generally higher degree of immune activity directed against Hif2a-deficient tumour cells and it is plausible that this immune control may at least partly contribute to the reduced numbers of tumours and delayed tumour onset seen in this genotype. Interestingly, we demonstrated not only in VhlΔ/ΔTrp53Δ/ΔRb1Δ/ΔHif2aΔ/Δ tumours but also in VhlΔ/Δ...
Trp53Δ/ΔRb1Δ/ΔHif1αΔ/Δ tumours that the density of intra-tumoural CD8+ T cells were approximately double the density in VhlΔ/ΔTrp53Δ/ΔRb1Δ/Δ tumours. The densities of CD8+ T cells observed in the mouse ccRCC tumours (~40–80 cells/mm²) are within the range seen in most cases of human ccRCC (~20–160 cells/mm²), providing support for the relevance of our mouse model. Consistent with our findings of HIF-2α acting as a suppressor of T-cell inflammation in mouse ccRCC, it was shown that HIF-2α expression levels in human ccRCCs anti-correlate with T-cell abundance and markers of T-cell activation97. We speculate that this apparent suppression of anti-tumour immune responses by HIF-2α might reflect a mechanism of positive selection that maintains HIF-2α expression in ccRCCs. Moreover, we show that loss of one copy of HIF1A correlates with a worse survival outcome, higher mRNA signatures of T-cell abundance and a broadly altered immune microenvironment in human ccRCCs. This observation is consistent with the fact that higher levels of T-cell infiltration have been shown to correlate with disease recurrence and worse survival in ccRCC93,74.

Thus, genetic and likely transcriptional and translational mechanisms that alter the balance of HIF-1α and HIF-2α abundance and activities appear to affect T-cell inflammation. The mechanisms that underlie the increased T-cell infiltration and/or activity in the absence of HIF-1α or HIF-2α will require further study. Analyses of RNA sequencing revealed that the mRNA levels of many cytokine-encoding genes are upregulated in tumours compared to normal cortex, but none of these genes were differentially regulated by HIF-1α or HIF-2α. Our functional tests to investigate if T-cell proliferation might be suppressed by ccRCC cells in general, for example through the secretion of cytokines or factors that show high levels of mRNA expression of cytokines or that inhibit the activation of T cells. Indeed, immune deconvolution analyses showed that different methods and different signatures can lead to different results for the same immune cell type, as well as the fact that many other complex factors such as the presence and activation states of other immune microenvironmental cells (such as macrophages, MDCs, dendritic cells, and regulatory T cells), antigen presentation and the presence or absence of co-activating or inhibitory ligands by ccRCC cells, as well as the composition of the extracellular matrix might all play a role in the trafficking and activation of T cells. Indeed, immune deconvolution analyses revealed that the group of tumours that exhibit loss of one copy of HIF1A or that show high levels of mRNA expression of HIF2A show medium in different signatures of different types of cells in the immune microenvironment. Since our own studies comparing multiple bioinformatic immune cell deconvolution methods showed that different methods and different signatures can lead to different results for the same immune cell type, as well as the fact that we observed both consistencies and inconsistencies between bioinformatic predictions and direct immune cell enumeration using immunohistochemistry, it will be important to treat these bioinformatic observations as hypothesis-generating starting points that will need to be orthogonally tested by staining for specific immune cell markers in larger cohorts of human ccRCC tumours. Given the clinical importance of immune checkpoint-based therapies for ccRCC and the fact that not all patients respond to these regimes, we believe that further investigation of the relationship between HIF-1α and HIF-2α status and the immune microenvironment is of potential therapeutic relevance. A further corollary of our findings is that inhibition of HIF-1α and HIF-2α transcription factor activities in ccRCC cells could be investigated therapeutically to inhibit tumour cell proliferation and simultaneously to attempt to increase T-cell infiltration and activation. The potential direct effects of pharmacological inhibition of HIF-α factors on different immune cells would also have to be considered in this strategy. Finally, while specific inhibitors of HIF-2α are available and are currently being tested in clinical trials97,98, our findings demonstrating the importance of HIF-1α for ccRCC formation argue that the development of specific inhibitors of HIF-1α or of new specific dual HIF-1α/HIF-2α inhibitors would also be desirable and may have therapeutic benefit in ccRCC. Proof-of-principle that these approaches are likely to be tolerable and effective comes from previously reported therapeutic effects of Acriflavine, which inhibits the binding of both HIF-1α and HIF-2α to haemiporphyrin in the VhlΔ/ΔTrp53Δ/ΔRb1Δ/Δ ccRCC model99, as well as in xenograft and autochthonous mouse models of different types of tumours100,101.
Allograft assay. Single-cell suspensions were prepared with Accutase (Gibco) and 5 x 10^6 cells were resuspended in 75 µl RPMI following transfer into a precooled 30°C insulin syringe mixed with 75 µl Matrigel (Corning). Syringes with cell suspension were kept on ice to avoid hardening of the Matrigel. SCID-Beige mice (Charles River Laboratories) were anaesthetised by inhalation of 3% isoflurane using oxygen as carrier gas. Mice were shaved and cells were injected subcutaneously in the flank. Tumour volumes were measured weekly with a calliper. Experiments were conducted under license G-17/165 of the Regierungspräsidium Freiburg.

Conditioned medium T-cell proliferation assay. In total, the T cells from one mouse (cCre) were seeded in triplicates in a six-well plate with 2 ml RPMI + 10% FCS and kept incubated in a humidified 5% (v/v) CO2 and 20% O2 incubator at 37 °C for 2 days. Two days later, splenocytes from each group of mice were harvested, washed in PBS and pooled. The splenocytes from each group were stained with the Live/Dead Fixable Aqua Dead Cell Stain Kit (Thermo Fisher) and counted. The splenocytes from each group were added to the triplicates of conditioned medium T cells for 3 days and incubated at 37 °C. After the incubation time the T cells were resuspended and centrifuged in a 2 ml reaction tube for 5 min at 1600 rpm and 4 °C. The dead cells within the pellet were stained with the Live/Dead Fixable Aqua Dead Cell Stain Kit (Thermo Fisher), washed twice with MACS buffer and the pellet was resuspended in 100 µl MACS buffer. Via flow cytometry (BD LSRII) dead/living cells were measured with a 405 nm Extinction Laser (LaserCyto), T cells were measured with a 640 nm Extinction Laser (APC), and the Proliferation Dye was measured with a 405 nm Extinction Laser (APC). Data were gated and analysed using FlowJo software.

Immunohistochemistry. Immunohistochemical stainings were performed as previously described10. Primary antibodies against the following proteins or epitopes were used at the following dilutions and antigen retrieval conditions: B220 (1:3000, BD Biosciences, 553084, Tris/EDTA 20 min, 100 °C), CA9 (1:2000, Invitrogen, PA1-18640, citrate, 10 min, 110 °C), CD3 (1:250, Zytomed, RB0224, citrate, 30 min, 95 °C), CD4 (1:1000, eBioscience, 14-9796, citrate, 30 min, 100 °C), CD8α (1:200, Invitrogen, 14-0880-82, citrate buffer, 15 min, 114 °C), CD10 (1:2000, Thermo Fisher Scientific, PS-A57075, citrate buffer, 10 min, 110 °C), CD8a (1:600, abcam ab125212, citrate buffer, 30 min, 95 °C), CD69 (1:1000, Bioss, bs-2499R, Tris/EDTA 20 min, 100 °C), CA9 (1:2000, Invitrogen, PA5-47075, citrate buffer, 10 min, 110 °C), CD68 (1:100, abcam ab199, Aivia Systems Biology ARP32253, Biorbyt orb96817, Sigma MAB3472, GeneTex GTX0114. For analyses of immune cell markers sections were scanned using a Nanozoomer Scansystem (Hamamatsu Photonics). Automatic quantifications of B220, CD3, CD4, CD8a, and CD68 positive cells were carried out from duplicate stains (average values were determined) as previously described85 using the Vis software suite (Visiopharm, Hoersholm, Denmark). Each tumour was outlined manually. Immune cell densities were calculated as cells per mm² based on surface area and immune cell quantification. The quantifications of cells stained with F4/80 was performed using a positive threshold based on chosen image and positron emission tomography (PET) images. The average number of reads was 45451780 ± 13975818 [20629675,70032834]. Real-time PCR was performed using the LightCycler 480 SYBR Green Master mix (Roche) using a LightCycler 480 (Roche) and annealing temperature of 55 °C. The following sets of primer pairs (sequences provided as 5'-3') were used to amplify the following floxed and non-floxed exons: Vhl Exon 1 (floxed) (fwd ATAAATCCGGCAGAGGACG, rev CTGGTGACAGGGCAGAC) Vhl Exon 2 (floxed) (fwd ACGCTTGAGAAGGCTTG, rev CTGCTGTCGTCCGAC) Vhl Exon 3 (floxed) (fwd TGGCGTCTTACACCTCTCCTC, rev TTGGGACTGCAAGGGACAGAGC) Vhl Exon 1 (flox) (fwd AGAAGGAGGCAGTCTACTCTCC, rev AA AAGCAGAGAGAAGGACCC) Rb1 Exon 19 (flox) (fwd AATACAGGAGCAAGAGAGCCG, rev GAGGACCAACTTACCTGCTCTGG) Vhl Exon 2 (flox) (fwd CTGAAGGCCCCGTCAGTG, rev AGCCAGGTTGACAGTAG) Vhl Exon 3 (flox) (fwd CTGGAGGAGAAGGACTCCG, rev AGCCAGGTTGACAGTAG) Western blotting. Antibodies against the following proteins were used for western blotting: HIF-1α (1:500, Sigma-Aldrich, A2228), HIF-1α (1:500, Novus Biologicals, NB-100-479), LAMIN-A/C (1:500, Santa Cruz, sc-376248), LDH-A (1:500, Santa Cruz Biotechnology, sc-27230), PD1 (1:100, Assay Designs, KAP-PK112-0), VHL (1:1000, Cell Signalling Technologies, #68547), VINCULIN (1:5000, Abcam, ab130007).

RNA-sequencing. RNA was isolated from powdered frozen samples of WT kidney cortex controls from Cre negative mice in the Vhlfl/flRb1fl/fl background and from tumours of the different genetic backgrounds using the NucleoSpin RNA kit (Machery Nagel). Paired-end RNA-sequencing was performed on an Illumina HiSeq4000 device. For each tumour sample, 3 replicates were performed. Overall, 60 ng genomic DNA per reaction was subjected to real-time PCR using the LightCycler 480 SYBR Green Master mix (Roche) using a LightCycler 480 (Roche) and annealing temperature of 55 °C. The following sets of primer pairs (sequences provided as 5'-3') were used to amplify the floxed and non-floxed exons: Vhl Exon 1 (flox) (fwd ATAAATCCGGCAGAGGACG, rev CTGGTGACAGGGCAGAC) Vhl Exon 2 (flox) (fwd ACGCTTGAGAAGGCTTG, rev CTGCTGTCGTCCGAC) Vhl Exon 3 (flox) (fwd TGGCGTCTTACACCTCTCCTC, rev TTGGGACTGCAAGGGACAGAC) Vhl Exon 1 (flox) (fwd AGAAGGAGGCAGTCTACTCTCC, rev AA AAGCAGAGAGAAGGACCC) Rb1 Exon 19 (flox) (fwd AATACAGGAGCAAGAGAGCCG, rev GAGGACCAACTTACCTGCTCTGG) Vhl Exon 2 (flox) (fwd CTGAAGGCCCCGTCAGTG, rev AGCCAGGTTGACAGTAG) Vhl Exon 3 (flox) (fwd CTGGAGGAGAAGGACTCCG, rev AGCCAGGTTGACAGTAG) Western blotting. Antibodies against the following proteins were used for western blotting: HIF-1α (1:500, Sigma-Aldrich, A2228), HIF-1α (1:500, Novus Biologicals, NB-100-479), LAMIN-A/C (1:500, Santa Cruz, sc-376248), LDH-A (1:500, Santa Cruz Biotechnology, sc-27230), PD1 (1:100, Assay Designs, KAP-PK112-0), VHL (1:1000, Cell Signalling Technologies, #68547), VINCULIN (1:5000, Abcam, ab130007).
Gene-set enrichment analysis. Enrichment of signalling pathways was performed as implemented in the R/Bioconductor package GAGE\(^92\) with signalling pathways from Gene Ontology\(^93,94\), ConsensusPathDB\(^95\) and MsigDB\(^96\). The human gene identifiers from the MsigDB pathways were mapped on mouse homologues with the R/Bioconductor package GeneAnswers (R package version 2.28.0). Pathways were considered significant with an adjusted p value < 0.05 (Benjamini–Hochberg).

ssGSEA immune deconvolution analysis. RNA-seq raw read sequences were aligned against mouse genome assembly mm10 by STAR 2-pass alignment\(^89\). QC metrics, for example general sequencing statistics, gene feature, and body coverage, were then calculated based on the alignment result through RSeQC. RNA-seq gene level count values were computed by using the R package GenomicAlignments\(^8\) over aligned reads with UCSC KnownGene\(^98\) in mm10 as the base gene model. The Union counting mode was used and only mapped paired reads after alignment quality filtering were considered. Gene level FPKM (fragments per kilobase million) and raw read count values were computed by using the R package DESeq\(^27\). Single-sample GSEA\(^96\) was utilised for immune deconvolution analyses based on FPKM expression values to estimate the abundance of immune cell types\(^96\). MHC class I antigen presenting machinery expression, T-cell infiltration score, immune infiltration score\(^98\), and immune cytolytic score\(^99\) as well as the eTME signatures\(^42\) which was developed from leveraging RCC patient-derived xenograft RNA-sequencing data. In addition to the gene signature-based deconvolution approach, CIBERSORT\(^48\) which is a regression-based method using Support Vector Machine was also employed using either the human gene panel or the mouse reviewed sequences (downloaded from Uniprot on Aug 26th, 2018) for immune cell type deconvolution. More unique peptides for protein quantitation. MaxQuant output was further aligned against mouse genome assembly mm10 by STAR 2-pass alignment\(^89\).

Mass spectrometry-based proteome profiling. Tissue sections of WT kidney cortex controls from Cre negative mice in the Vhl\(^+/–\)Prx3\(^+\)Psp3\(^+\)Ppx3\(^+\) background and from tumours of the different genetic backgrounds were homogenised by sonication (Qiagen, USA) using an UltraTurrax (IKA, Germany) at 100 mM HEPEs, pH 8.0 with subsequent trypsinization of 100 μg proteome based on the filter aided sample preparation protocol\(^101\). Overall, 24 samples (six mice from four genotypes) were split into eight fractions across three pools and differentially labelled by amine-reactive tandem-mass tags (TMT11plex, Thermo/Pierce, Rockford, IL, USA) including a pooled normalisation sample. A summary of the labelling scheme can be found online as part of the ProteomeXchange submission (see below for details).

Data availability

Raw proteomics data are available via PRIDE/ProteomeXchange with identifier PXD016630. Raw RNA-sequencing data have been uploaded to GEO with identifier GSE150983. The source data underlying analyses in Figs. 3b–1, 4c–g, 5a–c, 6a, 7a and Supplementary Figs. 53, S7a–e, S8a, b, S9a–d, S10a, and S12a are provided as Supplementary Data 1–6. Full scan blots for Supplementary Figs. 2 and 5 are provided as Source Data file. All remaining relevant data are available in the article, Supplementary Information, or from the corresponding author upon reasonable request. Source data are provided with this paper.

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Author contributions

R.H., S.H., S.S., D.S., M.A., A.P., F.U., P.S., D.H., C.C., and I.F., designed and conducted experiments, P.M., C.C., M.C.-C., R.H., O.S., F.K., and I.F., analysed RNA seq and proteomic data, C.S. contributed pathological analyses, A.J., B.L., N.G., T.C., J.D., R.Z., M.H., O.S., A.H., M.B., and I.F. provided technical assistance and scientific advice, I.F. designed the study and wrote the paper in collaboration with all authors.

Competing interests

The authors declare no other conflicts of interest.

Additional information

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