

# The role of HIF1 $\alpha$ in renal cell carcinoma tumorigenesis

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## Abstract

The transcription factor HIF1 $\alpha$  is implicated in the development of clear cell renal cell carcinoma (ccRCC). Although HIF1 $\alpha$  was initially believed to be essential for ccRCC development, recent studies hypothesize an oncogenic role for HIF2 $\alpha$  in ccRCC, but a tumor suppressor role for HIF1 $\alpha$  [1], leading to uncertainty as to the precise roles of the different HIF transcription factors in this disease. Using evidence available from studies with human ccRCC cell lines, mouse xenografts, murine models of ccRCC, and human ccRCC specimens, we evaluate the roles of HIF1 $\alpha$  and HIF2 $\alpha$  in the pathogenesis of ccRCC. We present a convergence of clinical and mechanistic data supporting an important role for HIF1 $\alpha$  in promoting tumorigenesis in a clinically important and large subset of ccRCC. This indicates that current

understanding of the exact roles of HIF1 $\alpha$  and HIF2 $\alpha$  is incomplete and that further research is required to determine the diverse roles of HIF1 $\alpha$  and HIF2 $\alpha$  in ccRCC.

## Key messages

- The TRACK mouse ccRCC model with constitutively active HIF1 $\alpha$  but not HIF2 $\alpha$  expressed in proximal tubules develops RCC.
- HIF1 $\alpha$  protein is expressed in the majority of human ccRCC specimens.
- Elevated HIF1 $\alpha$  in ccRCC correlates with a worse prognosis.
- Many publications do not support a tumor suppressor role for HIF1 $\alpha$  in ccRCC.
- HIF1 $\alpha$ , but not HIF2 $\alpha$ , is expressed in some types of cancer stem cells.

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## Clear cell renal cell carcinoma and von Hippel-Lindau disease

Approximately 75–85 % of all kidney cancers are clear cell renal cell carcinomas (ccRCC). Known for many years to be hypervascular tumors, it was not until the discovery and characterization of the Von Hippel-Lindau (VHL) gene that researchers gained a better understanding of the pathogenesis of ccRCC. Families with VHL disease have a high incidence of ccRCC that occurs as a consequence of VHL inactivation. However, most patients with sporadic ccRCC acquire a single deleted or inactivated VHL allele in normal kidney proximal tubule cells and over time, loss of heterozygosity occurs and

both VHL alleles become inactivated. This is believed to lead to the development of sporadic renal ccRCCs [2].

The VHL protein is a member of the E3 ubiquitin ligase complex that reduces the levels of the transcription factors HIF1 $\alpha$  and HIF2 $\alpha$  during normoxia [3–5]. Both HIF1 $\alpha$  and HIF2 $\alpha$  normally function in part to transcriptionally activate target genes in response to hypoxic conditions. In the normal rat kidney, hypoxia results in high levels of HIF1 $\alpha$  protein in proximal and distal tubules and in collecting ducts, while high HIF2 $\alpha$  protein levels are found in peritubular endothelial cells and in renal fibroblasts [5]. However, in the absence of active VHL protein, as is the case in the majority of ccRCCs, the VHL-associated proteolysis of HIF1 $\alpha$  and HIF2 $\alpha$  that occurs in the *absence* of hypoxia is lost, leading to constitutive activity of HIF1 $\alpha$  and HIF2 $\alpha$  independent of the oxygen level ([6, 7]; for review [8]). Whereas the VHL protein normally functions as an E3 ubiquitin ligase that targets HIF1 $\alpha$ , studies show that VHL may have other functions, such as in metabolism and inflammation, as judged by various studies in model organisms in addition to mice [9].

The loss of VHL tumor suppressor function and the resulting loss of regulated HIF degradation in ccRCC cells results in the increased expression of several proteins transcriptionally activated by HIF $\alpha$  that are involved in angiogenesis, such as vascular endothelial growth factor (VEGF) and platelet-derived growth factor B chain (PDGF-B). The increased expression of VEGF in ccRCCs explains the vascularity of these tumors, and directly led to the development of a variety of therapies that specifically target the VEGF pathway. Currently, sunitinib, pazopanib, sorafenib, and axitinib, all small molecule inhibitors of receptor tyrosine kinases, including the VEGF receptor, are in use for the treatment of advanced ccRCC [10]. The humanized monoclonal antibody (bevacizumab) that recognizes and inactivates VEGF, a HIF target gene, is also widely used to treat advanced ccRCC [11, 12].

Two other small molecular weight drugs approved to treat ccRCC, temsirolimus and everolimus, act by inhibiting the mammalian target of rapamycin (mTOR) [13]. mTOR consists of two enzymatically active complexes, mTOR complex 1 (mTORC1) and mTORC2 [14]. Activation of mTOR complexes leads to the stimulation of ribosomal translation of various messenger RNAs (mRNAs), including the translation of HIF1 $\alpha$  message, whereas inhibition of mTOR results in decreased HIF1 $\alpha$  translation [15]. Thus, the successful treatment of ccRCC today involves direct and indirect targeting of the HIF pathway, though it is becoming clear that significant intratumoral heterogeneity exists within primary and metastatic ccRCCs in the same patient, and this heterogeneity makes successful eradication of ccRCC more difficult [16].

## The roles of HIF1 $\alpha$ and HIF2 $\alpha$ in human clear cell renal cell carcinoma

Over the past 10 years, numerous researchers have studied the roles of the VHL target genes HIF1 $\alpha$  and HIF2 $\alpha$  in renal carcinogenesis (for review [17]). Many of these studies directly implicate the overexpression of HIF1 $\alpha$  as a critical factor in ccRCC tumorigenesis. In contrast, others have reported that HIF2 $\alpha$  is more tumorigenic than HIF1 $\alpha$  in ccRCC [1, 18], as well as implicating HIF1 $\alpha$  as a tumor suppressor in ccRCC [1]. We recently developed transgenic mouse models that specifically express either a mutated, constitutively active HIF1 $\alpha$  or HIF2 $\alpha$  in mouse proximal tubule cells, the normal progenitor cells of ccRCC (see below). In these models, we observed the development of ccRCC in mice expressing constitutively active HIF1 $\alpha$  but not in mice expressing constitutively active HIF2 $\alpha$  [19, 20]. These results have led us to critically re-examine the evidence for the specific roles of HIF1 $\alpha$  and HIF2 $\alpha$  in human renal clear cell carcinogenesis.

## Cell and animal model data

There are numerous, somewhat contradictory reports concerning the results of HIF1 $\alpha$  and HIF2 $\alpha$  overexpression and/or shRNA knockdown in tumor cell lines and xenograft models of human tumor cell proliferation. Xu et al. [21] demonstrated that the silencing of HIF1 $\alpha$  in the human RCC lines Caki-1 and OS-RC-2 *inhibited* growth in cell culture and inhibited tumorigenicity in tumor xenograft experiments in athymic mice. In another xenograft model, the apoptosis repressor with a caspase recruitment domain ARC gene was shown to be activated directly by HIF1 $\alpha$  at the transcriptional level in human renal cell carcinoma cell lines. Loss of expression of ARC led to a great reduction in RCC proliferation in SCID mice *in vivo* [22], indicating that this HIF1 $\alpha$  target gene regulates the growth of human RCC cells. The data from these two publications implicate HIF1 $\alpha$  in *driving* RCC cell proliferation. In contrast, the knockdown of HIF2 $\alpha$  *prevented* the growth of renal tumors in numerous xenograft models, whereas HIF1 $\alpha$  knockdown did not prevent the growth of tumors in xenografts [23–28]. Conversely, overexpression of HIF2 $\alpha$  also caused increased growth rates of tumors in xenografts, but not in cell culture experiments [29].

It is important to note, however, that in all of these experiments, only the *proliferation* of kidney tumor cells was examined, and not the actual process of tumor development. The striking differences observed in these cell culture and xenograft assays with respect to HIF1 $\alpha$  and HIF2 $\alpha$  actions on cancer cell proliferation, i.e., the contradictory results obtained by various laboratories, are not easily explained and may be related to the particular human tumor cell lines used in the assays [18, 21–28].

## Murine models of kidney cancer with constitutive HIF1 $\alpha$ expression

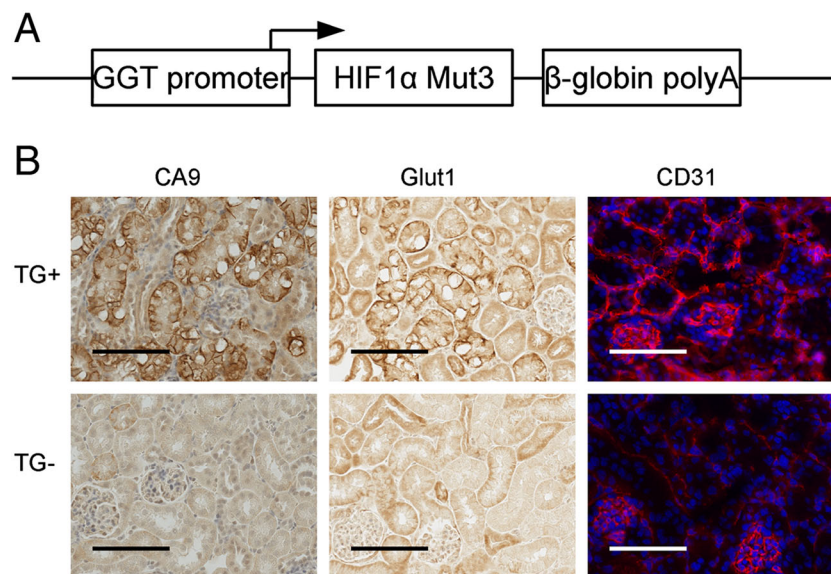
Currently, few mouse models of RCC possess the major features of human RCC (for review [30–32]). Attempts to recapitulate human kidney carcinogenesis by inactivation of the human tumor suppressor gene VHL have not been successful [33–38]. Clear cells, renal cysts, and tumors characteristic of human RCC are not generally seen in these models, but why these VHL animal models lack the ability to mimic the human disease is not understood. Other RCC models have long latency times [39, 40]. Models of adenomatous polyposis coli (APC) deficiency in mice lead to RCC, but this occurs over an extended period and is associated with activation of the  $\beta$ -catenin pathway, an event that is not common in human RCC [41]. Currently, murine xenograft models using human RCC-derived cell lines or human ccRCC explants are used to test new drugs for efficacy (e.g., [42–46]).

We recently developed the transgenic cancer of the kidney (TRACK) murine model of kidney cancer in which constitutively active HIF1 $\alpha$  is expressed specifically in renal proximal tubules, and this expression drives tumorigenesis that recapitulates many pathological and molecular features of early human ccRCC [20] (Fig. 1). For instance, expression of CA9, Glut1, and CD31 (as a marker of angiogenesis) is highly elevated in TRACK kidneys, and these proteins are similarly highly expressed in human ccRCC [47, 48]. In contrast, we and another research group have shown that constitutive HIF2 $\alpha$  expression specifically in the proximal tubules does not lead to neoplastic transformation in ccRCC [19, 49].

Furthermore, we showed by genome-wide RNA-seq that the transcripts expressed in the mice that constitutively express HIF2 $\alpha$  in their kidneys do not resemble the transcripts highly expressed in human ccRCC samples assessed in Oncomine, a cancer array database [19, 50]. Crossing the TRACK mice with the mice that express constitutively active HIF2 $\alpha$  in their kidneys did not make the tumorigenic phenotype more severe; the double transgenic TRACK-HIF2 $\alpha$ -mut3 mice resembled mice expressing only the TRACK-HIF1 $\alpha$ -mut3 gene [19]. Therefore, taken together, the transgenic animal models of kidney cancer are most consistent with HIF1 $\alpha$  having a major role in driving ccRCC tumorigenesis.

## Protein expression data in human RCCs

When tumor specimens are examined for HIF1 $\alpha$  and HIF2 $\alpha$  protein expression, generally nuclear expression is expected since both HIF1 $\alpha$  and HIF2 $\alpha$  are transcription factors that should act in the nucleus. Numerous studies have examined the expression of HIF1 $\alpha$  and HIF2 $\alpha$  by immunostaining in ccRCCs (Table 1) [28, 51–62]. Although the definition of high HIF1 $\alpha$  expression varied among studies, on average, HIF1 $\alpha$  protein was detected in 70 % (range 17 to 97 %) of ccRCC specimens. In a number of studies, HIF1 $\alpha$  expression was associated with an inferior survival [55, 56, 61, 63]. Schultz et al. [61] showed that HIF1 $\alpha$  levels were significantly higher in primary and metastatic ccRCCs compared with benign tissues ( $p < 0.0001$ ), and that tumor size and HIF1 $\alpha$  expression were independent predictors of both reduced disease free



**Fig. 1** **a** Simplified construction map of the linearized  $\gamma$ -HIF1 $\alpha$ -M3 plasmid used to create the TRACK mouse. The expression of the triple mutant (P402A, P564A, N803A) HIF1 $\alpha$  ORF is driven by the proximal tubule specific type 1 gamma-glutamyl transpeptidase (GGT) promoter. **b** Immunostaining of CA-9, Glut-1, and CD31 in TRACK kidney proximal

tubule cells. Increased expression levels of CA-9, Glut-1, and CD31 proteins are shown as stronger immunohistochemistry (CA-9, Glut-1) or immunofluorescence (CD31, red) signals in the TRACK TG<sup>+</sup> mouse compared to the TG<sup>-</sup> mouse. Scale bars represent 100  $\mu$ m

**Table 1** Evaluation of HIF1 $\alpha$  and HIF2 $\alpha$  protein expression in renal cell carcinoma samples

Reference	Number of patients	% HIF1 $\alpha$ positive	% HIF2 $\alpha$ positive	% HIF negative	Remarks
Biswas et al. [51]	106	97	99		Positivity was determined from intensity of staining and percentage of cells stained positive (histoscore). For HIF1 $\alpha$ , the histoscore cutoff >2 was used, and for HIF2 $\alpha$ , the histoscore cutoff >0 was used.
Chintala et al. [52]	88	41	79	13	HIF1 $\alpha$ positive samples have staining in more than 66 % of nuclei. HIF2 $\alpha$ positive samples have an average of 15 % of nuclei stained positive.
Gordan et al. [28]	57	61	88	12	Positive samples have more than 40 % of nuclei stained positive.
Nyhan et al. [53]	17	88	100	0	Positive samples have expression of HIF1/2 $\alpha$ as determined by Western blot analysis.
Sato et al. [54]	106	84	73	7	Positive samples have low, moderate, or high expression of HIF1/2 $\alpha$ .
Reference	Number of patients	% HIF1 $\alpha$ positive			Remarks
Di Cristofano et al. [55]	136	95			Positive samples have cytoplasmic staining in more than 50 % of cells.
Dorević et al. [56]	94	Not specified			Immunoreactivity was evaluated as a percentage of nuclear or cytoplasmic positivity. The median value for positive nuclei is 47.1 and for positive cytoplasm is 12.9.
Klatte et al. [57]	308	17			Positive samples have specific nuclear staining in more than 35 % of cells.
Lidgren et al. [58]	66	67			Positive samples have high HIF1 $\alpha$ expression as determined by Western blot analysis.
Lidgren et al. [59]	176	58			Positive samples have medium or strong HIF1 $\alpha$ staining.
Medina Villaamil et al. [60]	80	73			HIF1 $\alpha$ positive samples have strong staining (in an average of 80 % of cells) or moderate positivity.
Schultz et al. [61]	176	Not specified			Mean H-score (0–300) was calculated by taking into account the intensity of staining and the percentage of positive cells. For primary tumors, H-score mean is 76.2 and for metastatic ccRCC, H-score mean is 99.8. Benign tissue has an H-score mean of 0.6.
Reference	Number of patients	% HIF2 $\alpha$ positive			Remarks
Kroeger et al. [62]	300	97			Positive samples have >32 % cells with HIF2 $\alpha$ staining in the nuclei.

% positive indicates the percentage of tumor samples in which HIF1 $\alpha$  or HIF2 $\alpha$  protein expression was detected. % negative indicates the percentage of tumor samples in which both HIF1 $\alpha$  and HIF2 $\alpha$  protein were not detected. All studies used immunohistochemistry to evaluate protein expression unless otherwise specified in the remarks. For studies in which the % positive was not specified, details regarding their findings are noted in the remarks. Information regarding how the different studies determined positivity and negativity is also included in the remarks. The table is divided into three sections: the top section cites publications in which both HIF1 $\alpha$  and HIF2 $\alpha$  were assayed; the middle section includes publications in which only HIF1 $\alpha$  was assessed; the bottom section includes one publication in which only HIF2 $\alpha$  was assayed

survival and tumor progression in primary ccRCC. In another recent report, the highest level of HIF1 $\alpha$  expression in ccRCCs was associated with the worst prognoses [60]. Studies have also examined both HIF1 $\alpha$  and HIF2 $\alpha$  in the same tissue samples. Gordan et al. [28] reported HIF immunostaining patterns that separated ccRCCs into three distinct classes:

no HIF protein detected (“VHL WT,” 12 %), both HIF1 $\alpha$  and HIF2 $\alpha$  detected (61 %), and HIF2 $\alpha$  only detected (27 %). Their data suggested that high HIF expression was inversely correlated with VHL expression. In another recent study, HIF1 $\alpha$ <sup>HIGH</sup>/HIF2 $\alpha$ <sup>LOW</sup> renal tumors had a worse overall survival compared with HIF1 $\alpha$ <sup>LOW</sup>/HIF2 $\alpha$ <sup>LOW</sup> tumors [51].



Thus, numerous analyses of human ccRCC specimens show that HIF1 $\alpha$  is highly expressed in the majority of primary renal tumors, either alone or with HIF2 $\alpha$ . Sato et al. [54] recently reported expression of HIF1 $\alpha$  protein by immunostaining in 84 % of 106 primary surgical ccRCC specimens.

In a tissue microarray (TMA) study of 308 ccRCC patient specimens, high HIF2 $\alpha$  nuclear (N) (cutoff >32 %) expression correlated with smaller tumor sizes ( $p=0.002$ ) and lower Fuhrman grades ( $p=0.044$ ), whereas tumors with high cytoplasmic (C) HIF2 $\alpha$  staining had a higher frequency of positive lymph nodes ( $p=0.004$ ), distant metastases ( $p=0.021$ ), and higher Fuhrman grades ( $p<0.0001$ ). The localization of HIF2 $\alpha$  in the cytoplasm rather than the nucleus associated with increased ccRCC severity, indicating a complex and poorly understood role for HIF2 $\alpha$  in the cytoplasm in ccRCC [62].

Of note, HIF1 $\alpha$  expression correlates significantly with the “clear” histological subtype of renal cell carcinoma ( $p<0.01$ ) [60], possibly because HIF1 $\alpha$  (and not HIF2 $\alpha$ ) increases the expression of lipin 1, a phosphatidate phosphatase that catalyzes the last step in triglyceride biosynthesis [64]. Triglycerides form lipid droplets, the major neutral lipid stores in cells, and these lipid droplets cause the “clear” phenotype in human ccRCC. HIF1 $\alpha$  activation also increases glutamine-dependent lipid synthesis in tumors by reducing the activity of the enzyme  $\alpha$ -ketoglutarate dehydrogenase via SIAH-targeted

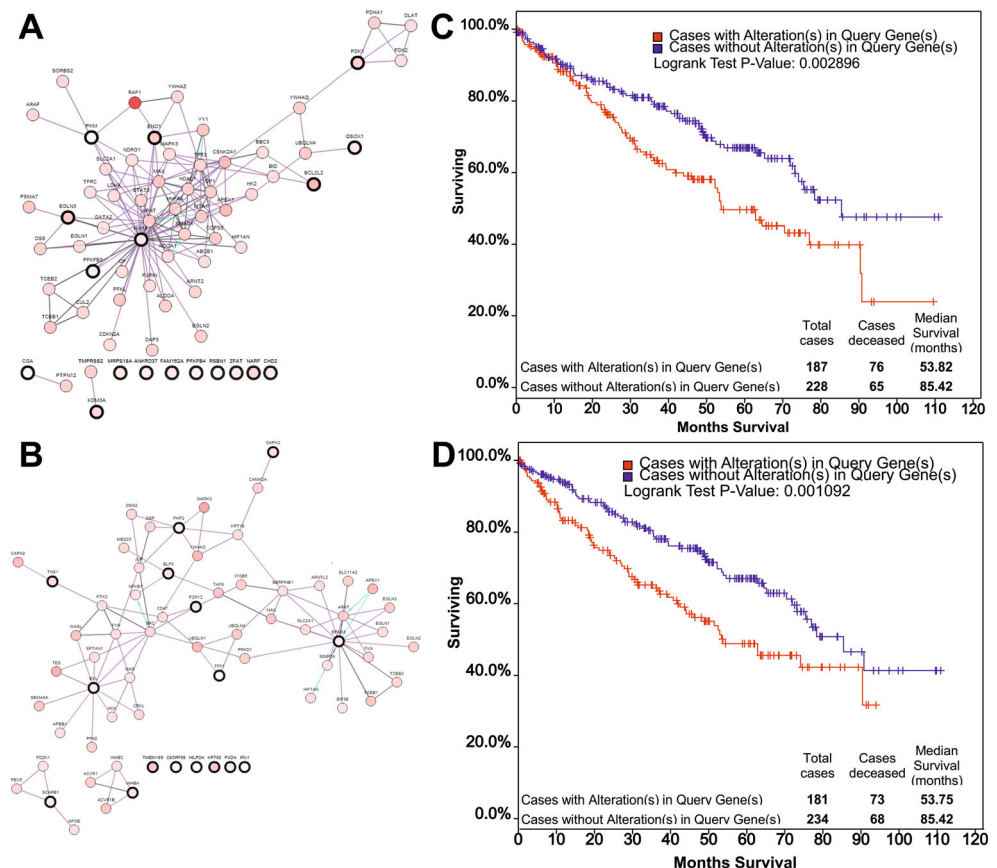
ubiquitination; no effects of HIF2 $\alpha$  on this enzyme were presented in this study [65].

### Gene expression and HIF mutation/deletion in human RCCs

Genome-wide chromatin immunoprecipitation, coupled with next-generation sequencing (ChIPseq), has been used to identify HIF1 $\alpha$ - and HIF2 $\alpha$ -regulated gene networks [66]. Of the high stringency genomic regions identified for HIF1 $\alpha$  (359 sites) and HIF2 $\alpha$  (301 sites) in MCF-7 cells, only 157 are common to both HIF1 $\alpha$  and HIF2 $\alpha$  [66].

Analysis of the published renal cancer data from The Cancer Genome Atlas (TCGA) research network [67] shows that *HIF1 $\alpha$*  and *HIF2 $\alpha$*  mRNA expression is altered in 3 and 5 % of patients, respectively, and that these genes are mutated in <1 % of patients. However, an analysis of the expression in the same renal cancer cohort of putative HIF1 $\alpha$ - and HIF2 $\alpha$ -regulated gene targets [66] demonstrates that HIF1 $\alpha$  (Fig. 2a) and HIF2 $\alpha$  (Fig. 2b) regulate distinct networks which are both associated with poorer overall survival (Fig. 2c, d). It is important to note that in the TCGA patients [67], only mRNA levels and no protein expression data are available. Furthermore, in these TCGA data, the expression of *HIF1 $\alpha$*  and

**Fig. 2** The cBio database was used to compare expression and clinical correlation in renal cancer of putative HIF1 $\alpha$  and HIF-2 $\alpha$ /EPAS1 target genes [66]. HIF1 $\alpha$  (a) (RSBN1, CHD2, PFKFB3, QSCN6, PKM2, CGA, BCL2L2, EGLN3, MRPS18A, PFKFB4, PDK1, ANKRD37, C3orf28, ZFAT1, ENO1, NARF, JMJD1A/KAT3A), and HIF2 $\alpha$  (b) (TFF1, HIG2, TNS1, P2RY2, KRT80, EVL, C8orf58, PXDN, INHBA, IRX1, Kua, ELF3, SCARB1, PKP2, DAPK2) target genes form distinct networks. Kaplan-Meier survival estimates for HIF1 $\alpha$  (c) and HIF-2 $\alpha$ /EPAS1 (d) target gene expression are associated with poorer overall survival



*HIF2 $\alpha$*  transcripts is compared to the *average* gene expression in kidney *tumor* tissue rather than to the levels of *HIF1 $\alpha$*  and *HIF2 $\alpha$*  in the normal kidney; this suggests that *HIF1 $\alpha$*  and *HIF2 $\alpha$*  levels are even higher in tumor tissue relative to the levels in normal kidney tissue. Of importance, mRNA expression data do not reflect the levels of HIF protein, which is regulated primarily posttranscriptionally by protein degradation.

The identities of protein partners shown to interact with *HIF1 $\alpha$*  and *HIF2 $\alpha$*  also differ, supporting distinct roles for the *HIF1 $\alpha$*  and *HIF2 $\alpha$*  protein complexes in normal physiology and in carcinogenesis. For example, the protein interactions summarized in NCBI (<http://www.ncbi.nlm.nih.gov/gene/>; Fig. 3) indicate that *HIF1 $\alpha$*  can interact with multiple, distinct epigenetic regulatory proteins, including the coactivators KAT2B/pCAF, NCoA1/SRC-1, and NCoA2/SRC-2/TIF2. In addition, multiple histone deacetylases (HDACs), which are typically associated with transcriptional repression, interact with *HIF1 $\alpha$* . This suggests that *HIF1 $\alpha$*  can have both positive and repressive effects on transcriptional regulation. Interestingly, while there is evidence that *HIF2 $\alpha$*  can interact with the mediator transcriptional complex and the CBP histone acetyltransferase [68], there is little overlap in known protein interactions of *HIF1 $\alpha$*  and *HIF2 $\alpha$*  in the diverse cell lines studied (Fig. 3).

### Is the *HIF1 $\alpha$* gene deleted in a high proportion of human ccRCCs?

The report by Shen et al. [1] implicating “*HIF1 $\alpha$*  as a 14q kidney cancer suppressor gene” has been widely quoted in the literature, but these and related data [69] have not been carefully analyzed and compared to other published integrated molecular analyses of ccRCC. The deletions reported in Shen et al. [1] and Beroukhim et al. [69] in ccRCC specimens are at 14q31.1. It was also recently reported that “loss of chromosome 14q, associated with loss of *HIF1 $\alpha$* , occurs frequently in ccRCC,” with single nucleotide polymorphism (SNP) arrays

indicating loss of 14q24.3 (encompassing *NRXN3* and ~462 other genes) in 45 % of patients [67]. However, *HIF1 $\alpha$*  maps at 14q23.2 (chr14:62162117–62214976), whereas *NRXN3* is located at chr14:79745682–80334633 (HG19/GRCh37), some 17 million nucleotides distant. Moreover, RNAseq analysis of ccRCC specimens [67] identified a transcriptional hub related to *HIF1 $\alpha$* /ARNT that is believed to contribute to the metabolic changes in ccRCC. Furthermore, RNAseq data suggest that *HIF1 $\alpha$*  expression persists at a level within the range of diploid *HIF1 $\alpha$*  tumors in most ccRCC specimens affected by *HIF1 $\alpha$*  loss of heterozygosity (Fig. 4) [67]. Consistent with these data [67] showing *HIF1 $\alpha$*  mRNA expression, Sato et al. [54] detected *HIF1 $\alpha$*  protein in 84 % of primary ccRCC specimens. Sato et al. [54] did not report any major deletions at chromosome 14q in their patient specimens.

A genetic polymorphism in the human *HIF1 $\alpha$*  gene, C1772T, is associated with overexpression of *HIF1 $\alpha$*  and a poorer outcome in a subgroup of breast cancer patients [70]. The same SNP, C1772T, was found at a higher frequency in ccRCC patients than in healthy subjects [71].

Collectively, the data summarized here indicate both expression of *HIF1 $\alpha$*  protein in the majority of ccRCC specimens and a pro-oncogenic role for *HIF1 $\alpha$*  in ccRCC. Therefore, the putative tumor suppressor function for *HIF1 $\alpha$*  in ccRCC [1, 67] should be reconsidered.

### *HIF1 $\alpha$* and *HIF2 $\alpha$* have specific functions in normal stem cell physiology

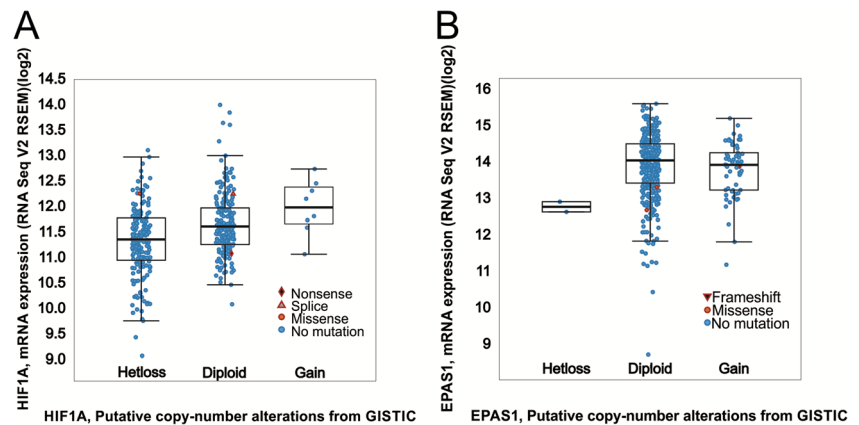
Although the HIFs are both induced under hypoxic stress conditions, the genes that *HIF1 $\alpha$*  and *HIF2 $\alpha$*  regulate can overlap or be entirely unique. Many HIF target genes are involved in mediating the change from oxidative to aerobic glycolytic metabolism [72]. HIFs not only have a major role in cancer cells by driving this Warburg effect, but indeed, *HIF1 $\alpha$*  and *HIF2 $\alpha$*  display specific effects on the functions of various types of normal stem cells. For instance, the level of *HIF1 $\alpha$*



**Fig. 3** Comparison of *HIF1 $\alpha$*  and *HIF2 $\alpha$* /(EPAS1) protein interactions. *HIF1 $\alpha$*  and *HIF2 $\alpha$*  protein interactions reported in the NCBI Gene database (<http://www.ncbi.nlm.nih.gov/gene/>) were compared. *HIF1 $\alpha$*

and *HIF2 $\alpha$*  have 90 and 74 unique interaction partners respectively, with only 31 partners common to both. This suggests that the composition and function of *HIF1 $\alpha$*  and *HIF2 $\alpha$*  complexes may differ

**Fig. 4** HIF1 $\alpha$  and HIF2 $\alpha$  mRNA levels. **a** Expression analysis of ccRCC specimens from [62]. Heterozygous loss of one tumor suppressor gene allele typically results in greatly decreased mRNA expression of the gene. In contrast, expression of HIF1 $\alpha$  generally persists within the range of diploid HIF1 $\alpha$  expression in patients harboring HIF1 $\alpha$  heterozygous loss. **b** Expression of HIF2 $\alpha$ /EPAS1 is provided for comparison



was shown to be critical for hematopoietic stem cell (HSC) maintenance in the bone marrow, in part because HIF1 $\alpha$  regulates anaerobic glycolysis in these cells [73, 74]. HIF1 $\alpha$  maintains anaerobic glycolysis in normal HSCs by activation of the transcription of pyruvate dehydrogenase kinase (PDK) isoforms 2 and 4, which prevent pyruvate from entering the TCA cycle [74]. In contrast, HIF2 $\alpha$  knockdown prevents the repopulating ability of human CD34<sup>+</sup> umbilical cord blood cells [75].

HIF1 $\alpha$ <sup>-/-</sup> mouse embryos die by E11 and display major defects in cardiovascular development, neural tube malformations, and massive cell death within mesenchymal cell populations [76]. HIF2 $\alpha$ <sup>-/-</sup> mouse embryos die by E16.5 from reduced levels of noradrenaline and a slow heart rate (bradycardia) [77]. In contrast to the HIF1 $\alpha$ <sup>-/-</sup> embryos, HIF2 $\alpha$ <sup>-/-</sup> embryos have no morphological defects and display normal vascular development, which indicates that HIF2 $\alpha$  does not play an essential role in vasculogenesis or angiogenesis during development [77]. ARNT<sup>-/-</sup> mice die in utero between E9.5 and E10.5, and display neural tube closure defects, forebrain hypoplasia, delayed rotation of the embryo, placental hemorrhaging, and visceral arch abnormalities [78]. Indeed, these knockout mice display the most extensive defects because without ARNT, both HIF1 $\alpha$  and HIF2 $\alpha$  are inactive [78].

### Specific functions of HIF1 $\alpha$ in carcinogenesis, tumor progression, and the generation of cancer stem cells

In addition to the specific effects of HIF1 $\alpha$  and HIF2 $\alpha$  in various types of normal stem cells, HIF1 $\alpha$  can mediate changes associated with processes that occur both during carcinogenesis and in cancer progression. For instance, HIF1 $\alpha$  mediates the activation of histone deacetylase 3 (HDAC3) in both epithelial and mesenchymal cells, ultimately resulting in epithelial-mesenchymal transition associated with both repression of the gene E-cadherin and loss of polarity of epithelial cells [79]. Another example is the regulation of HIF1 $\alpha$

transcription by Sirt6; Sirt6, a member of the sirtuin family of proteins, can act as a tumor suppressor via its role in the transcriptional repression of the HIF1 $\alpha$  gene. In the absence of Sirt6, specifically, HIF1 $\alpha$  is transcriptionally activated to a high level and this directly leads to neoplastic transformation without any additional, secondary mutations in the cells [80, 81]. The transcription factor YY1 is involved in stabilizing HIF1 $\alpha$  under hypoxic conditions in tumors, and inhibition of YY1 suppressed the proliferation of metastatic cancer cells, potentially via a reduction in HIF1 $\alpha$  levels [82]. While in many types of cells HIF1 $\alpha$  protein associates with polymerized microtubules to traffic to the nucleus, the connection between microtubules and HIF1 $\alpha$  is lost in RCC [83]. As a result, microtubule-targeting drugs do not impair HIF1 $\alpha$  nuclear activity and transcription in RCC, which can potentially explain the lack of clinical activity of microtubule inhibitors in RCC patients.

Another example of the specific association of HIF1 $\alpha$  and oncogenesis is from a recent study by Xiang et al. [84], which showed that the drug ganetespib, an inhibitor of heat shock protein 90, lowered the level of HIF1 $\alpha$ , but not HIF2 $\alpha$  protein, and also inhibited the subsequent expression of the HIF1 $\alpha$  target genes involved in tumor progression in a mouse model of triple-negative breast cancer. A HIF1 $\alpha$  high expression signature is found in human triple-negative breast cancer [85] and in human head and neck squamous cell carcinomas [86]. In another intriguing study, Conley et al. [87] demonstrated that anti-angiogenic drugs such as sunitinib and bevacizumab can increase the numbers of cancer stem cells in a human breast cancer xenograft model by generating hypoxic conditions. These hypoxic conditions lead to a massive increase in expression of HIF1 $\alpha$  in the ALDEFLUOR<sup>TM</sup> + cancer stem cell population, whereas HIF2 $\alpha$  is not detectable in this population. Furthermore, a major pathway of breast cancer stem cell self-renewal, the Wnt/ $\beta$ -catenin signaling pathway, was reported to be activated by HIF1 $\alpha$  in embryonic stem cells cultured under hypoxic conditions [88]. In glioma cells, knockdown of HIF1 $\alpha$  impairs the ability of these glioma cells to form tumor spheres [89]. In glioma and

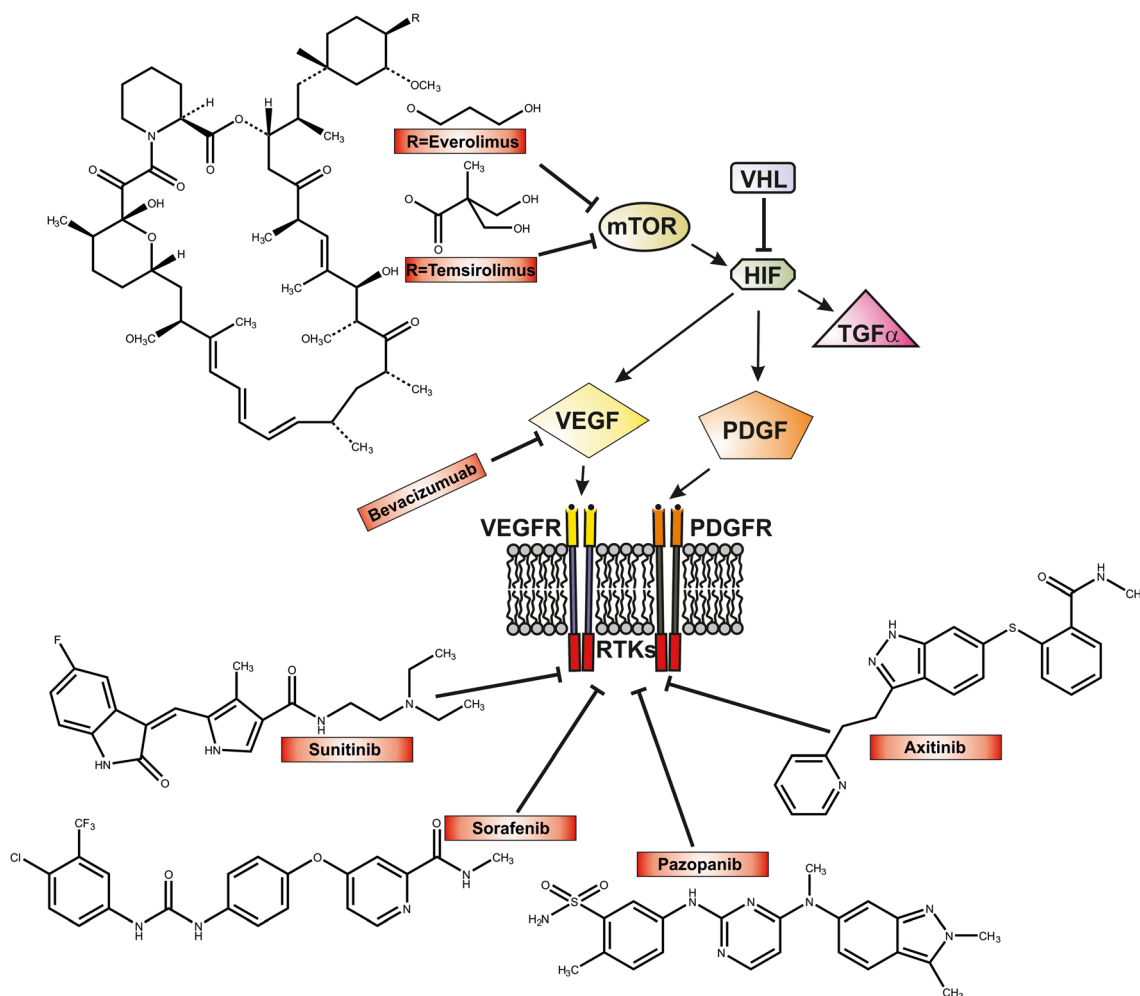
glioblastoma, the expression of both HIF1 $\alpha$  and HIF2 $\alpha$  stimulates cancer stem cell growth ([90, 91]; for review [92]). Thus, large numbers of studies have specifically implicated HIF1 $\alpha$  in carcinogenesis, tumor cell proliferation, cancer progression, and in the generation of larger cancer stem cell populations.

### Clinical data and new treatment strategies for ccRCC

As discussed earlier, the major drugs currently used to treat late stage ccRCC are temsirolimus, everolimus, bevacizumab, and the tyrosine kinase inhibitors that act as VGFR (Fig. 5). mTOR consists of two enzymatically active complexes, mTOR complex 1 (mTORC1) and mTORC2 [14]. Activation of mTOR complexes leads to the stimulation of ribosomal translation of various mRNAs, including the translation of HIF1 $\alpha$  message, which is governed by mTORC1, and the translation of HIF2 $\alpha$  mRNA, which is regulated by mTORC2 [93]. Temsirolimus competitively inhibits mTORC1 kinase,

indicating that HIF1 $\alpha$  translation should be reduced to a greater extent than HIF2 $\alpha$  translation by this drug (Fig. 5).

Researchers are investigating strategies to target other HIF1 $\alpha$  target genes for treatment of HIF overexpressing tumors, such as ccRCC, rather than blocking tumor angiogenesis, which is and has been the focus for drug development [10]. For example, IL-11, a member of the IL-6 family of cytokines, is a HIF1 $\alpha$  target that, when silenced, significantly abrogates the ability of hypoxia to increase anchorage-independent growth and results in reduced tumor growth in xenograft models [94]. Thus, targeting inhibition of IL-11 with small molecules could be a useful approach. In work on diabetic nephropathy, the drug fasudil, a rho-kinase inhibitor, promoted HIF1 $\alpha$  degradation [95, 96]. By inhibiting expression of HIF1 $\alpha$  target genes in the kidney via reduction in HIF1 $\alpha$  expression, fasudil may also inhibit ccRCC. In a breast cancer model, the oncogene HER2/neu required HIF1 $\alpha$  for anoikis resistance, anchorage independence, and 3D culture growth. Researchers also demonstrated that in this model, ErbB2 overexpression in cells stabilized HIF1 $\alpha$  under normoxic conditions and that HIF1 $\alpha$  is a major downstream



**Fig. 5** Current FDA approved drugs used for the treatment of ccRCC and their mechanisms of action



effector of ErbB2 actions [97]. Therefore, inhibitors of ErbB2 could potentially be tested in ccRCC because such inhibitors could reduce HIF1 $\alpha$  actions downstream of ErbB2. ErbB2 is expressed in human ccRCC [98].

The protein XBP1 drives tumorigenicity in triple negative breast cancer by forming a transcription complex with HIF1 $\alpha$ , and this transcription signature is associated with a poor prognosis. Thus, inhibition of XBP1 activity could form a new treatment strategy for ccRCC, as XBP1 is moderately overexpressed in ccRCC in the Oncomine database [99].

Mitochondrial autophagy is induced by hypoxia in normal mouse embryo fibroblasts in a HIF1 $\alpha$ -dependent process that requires the expression of BNIP3, beclin1, and ATG5; mitochondrial autophagy is necessary to *prevent* increased reactive oxygen species and cell death during long-term hypoxia [100]. In ccRCC, high expression of HIF1 $\alpha$  in the presence of either hypoxia or normoxia (in the absence of VHL) could also lead to mitochondrial autophagy as a strategy for cell survival. Blocking such autophagy could be a useful strategy to promote ccRCC death by generating increased reactive oxygen species. However, STF-62247 is a drug that *stimulates* autophagy and inhibits RCC growth in xenograft models [101]. Autophagy is a complex process and the VHL protein enhances the expression of LC3C, a HIF-regulated LC3B paralog, that suppresses ccRCC growth [102]. This suppression of ccRCC growth by LC3C occurs by protection of RCC cells from the loss of LC3B-mediated autophagy [102]. Thus, targeting mitochondrial or cell autophagy programs could be a viable strategy for ccRCC treatment in the future.

## Conclusions

Clear cell renal cell carcinoma remains a major health concern despite advances over the past 10 years in understanding the genetic pathways leading to overexpression of the VEGF axis and the development of a variety of therapies that target these pathways. Our understanding of the precise roles of the HIF transcription factors, HIF1 $\alpha$  and HIF2 $\alpha$ , in the development and progression of ccRCC continues to evolve. Unrepressed expression of HIF1 $\alpha$  through VHL loss was initially proposed to be an initiating oncogenic event leading to ccRCC development. This concept was subsequently challenged by the idea that genetic loss of HIF1 $\alpha$  was a key event in ccRCC evolution, leaving HIF2 $\alpha$  as a major driver of renal cell neoplastic transformation. However, as reviewed here, a large number of studies, including transgenic mouse models, expression analyses of human tumors, and refined analyses of large data sets profiling ccRCCs recently made available call into question the dismissal by some in the field of HIF1 $\alpha$  as a critical protein necessary and possibly sufficient to initiate development of ccRCC. These and other studies also indicate that ccRCC is a highly heterogeneous disease, with both intra-

and intertumor heterogeneity. Collectively, the data reviewed here indicate that HIF1 $\alpha$  plays a key role in promoting the tumor phenotype in a major subset of ccRCCs and that therapeutic strategies aimed at targeting HIF1 $\alpha$  and the genes it affects should continue to be explored.

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