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Interplay between pVHL and mTORC1 pathways in clear-cell renal cell carcinoma

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Abstract

Mammalian target of rapamycin complex 1 (mTORC1) is implicated in cell growth control and is extensively regulated. We previously reported that in response to hypoxia, mTORC1 is inhibited by the protein regulated in development and DNA damage response 1 (REDD1). *REDD1* is upregulated by HIF-1, and forced *REDD1* expression is sufficient to inhibit mTORC1. REDD1-induced mTORC1 inhibition is dependent on a protein complex formed by the tuberous sclerosis complex (TSC)1 and 2 (TSC2) proteins. In clear-cell renal cell carcinoma (ccRCC), the von Hippel-Lindau (VHL) gene is frequently inactivated leading to constitutive activation of HIF-2 and/or HIF-1, which may be expected to upregulate REDD1 and inhibit mTORC1. However, mTORC1 is frequently activated in ccRCC and mTORC1 inhibitors are effective against this tumor type; a paradox herein examined. *REDD1* was upregulated in *VHL*-deficient ccRCC by in silico microarray analyses, as well as by quantitative real-time PCR, Western blot, and immunohistochemistry. *Vhl* disruption in a mouse model was sufficient to induce *Redd1*. Using ccRCC-derived cell lines, we show that REDD1 upregulation in tumors is *VHL*-dependent, and that both HIF-1 and HIF-2 are, in a cell-type dependent manner, recruited to, and essential for, *REDD1* induction. Interestingly, whereas mTORC1 is responsive to REDD1 in some tumors, strategies have evolved in others, such as mutations disrupting *TSC1*, to subvert mTORC1 inhibition by REDD1. Sequencing analyses of 77 ccRCCs for mutations in *TSC1*, *TSC2* and *REDD1*, using *PTEN* as a reference, implicate the *TSC1* gene, and possibly *REDD1*, as tumor suppressors in sporadic ccRCC. Understanding how ccRCCs become refractory to REDD1-induced mTORC1 inhibition should shed light into the development of ccRCC and may aid in patient selection for molecular targeted therapies.

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Introduction

Mammalian target of rapamycin (mTOR) complex 1 (mTORC1) is implicated in the pathogenesis of renal cell carcinoma (RCC), including clear-cell type (ccRCC). mTORC1 is thought to be activated in 60–85% of ccRCCs (1, 2) and mTORC1 inhibitors have been shown in two phase III clinical trials to delay tumor progression (3, 4). However, how mTORC1 is deregulated in ccRCCs is poorly understood.

mTOR is an atypical serine/threonine protein kinase. mTOR nucleates two different complexes, mTORC1 and mTORC2, which modify different substrates (5). mTORC1 is composed of mTOR and an adaptor protein, regulatory-associated protein of mTOR (raptor) (6). Other proteins are found in mTORC1, including mammalian lethal with sec-thirteen protein 8 (mLST8), which at least during development, is dispensable for mTORC1 function (6). mTORC1 is involved in regulating cell growth, and the two best studied substrates of mTORC1 are S6 kinase 1 (S6K1) and the eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) (7). By phosphorylating S6K1 and 4E-BP1, mTORC1 stimulates protein translation. Importantly, only mTORC1 is directly inhibited by rapamycin (also called sirolimus) (5).

mTORC1 activity is tightly controlled. Regulation of mTORC1 in response to hypoxia involves the protein regulated in development and DNA damage response 1 (REDD1; also called DNA-damage-inducible transcript 4 [DDIT4]) (8). REDD1 is an evolutionarily conserved protein with a novel fold (9) that is upregulated in response to hypoxia in most cell types (8, 10–14). Hypoxia leads to the stabilization of the α subunits of the heterodimeric (α/β) hypoxia-inducible factor (HIF)-1 and -2 transcription factors and increased expression of their target genes. HIF-1 has been shown to bind to a response element in the *REDD1* promoter and is required, at least in some cell types, for *REDD1* induction (10). REDD1 negatively regulates mTORC1 and simply overexpressing *REDD1* is sufficient to inhibit mTORC1 (8).

REDD1-induced mTORC1 inhibition requires the complex formed by the proteins tuberous sclerosis complex 1 and 2 (TSC1/TSC2) (8). TSC2 acts as GTPase-activating protein (GAP) towards a small G protein, Ras homologue enriched in brain (Rheb), which plays an important role in mTORC1 activation (15). Disruption of TSC1/TSC2 blocks mTORC1 inhibition by REDD1 (8, 16, 17). How REDD1 functions remains to be elucidated. Previously, the TSC2 protein was found to bind 14-3-3 proteins (18) and REDD1 has been proposed to act by directly binding to and sequestering 14-3-3 proteins away from TSC2 (19). However, critical residues in the putative 14-3-3 binding motif in REDD1 are not conserved (9). In addition, the presumed motif does not conform to any 14-3-3 binding motif known and cannot be docked onto 14-3-3 β without steric clashes (9).

The TSC1/TSC2 complex is inactivated in the eponymic syndrome, tuberous sclerosis complex (TSC), which is characterized by hamartomas in multiple organs (20). While TSC patients exhibit an increased predisposition to develop RCC, which tends to occur at an earlier age than in the general population (20), mutations in *TSC1* or *TSC2* have not been found in sporadic ccRCC (21).

ccRCCs are characteristically associated with disruption of the tumor suppressor gene von Hippel-Lindau (*VHL*), which is located in 3p25–26, and which, in recent studies, has been found inactivated in up to 90% of sporadic tumors (22). The *VHL* gene encodes a protein (pVHL) that functions as the substrate recognition subunit of an E3 ubiquitin ligase complex that targets, among others, the α subunits of HIF-1 and HIF-2 for degradation (23). *VHL* disruption results in constitutive activation of HIF-2 (and/or HIF-1) in tumors, and increased expression of their target genes (24).

Because *REDD1* is a HIF-1 target gene (10), *REDD1* may be upregulated in *VHL*-deficient ccRCCs where it would be expected to downregulate mTORC1. Paradoxically, mTORC1 appears to be broadly activated in ccRCC (1, 2) and mTORC1 inhibitors are effective against this disease (3, 4). Herein, this paradox was examined.

Materials and Methods

Tissue processing

Tumor and normal renal cortex samples were collected from surgical specimens following informed consent of an IRB-approved tissue collection protocol and were flash frozen in liquid nitrogen and stored at -80°C . From each sample, immediately flanking sections from two sides perpendicular to each other were processed for H&E staining and analyzed by a pathologist (W.K.) for composition (tumor content) and quality (hemorrhage, necrosis). Tissues were selected and processed according to Peña-Llopis et al., in preparation.

VHL reconstitution

786-O, A498, and Caki-2 cells were transfected using TransIT®-LT1 Transfection Reagent (Mirus, Madison, WI) with pcDNA3.1/Hygro/HA-VHL (laboratory database ID #586) or empty vector (pcDNA3.1/Hygro; ID #338) and polyclonal populations were selected and maintained in Hygromycin (250 $\mu\text{g/ml}$).

siRNA transfections

siRNA oligonucleotides were from Dharmacon and Dicer-substrate siRNAs (DsiRNA) duplexes from IDT (Coralville, IA). Transfections were performed using Lipofectamine 2000 (Invitrogen) for A498 or DharmaFECT reagent 3 (Dharmacon, Lafayette, CO) for 786-O and Caki-2 cells according to manufacturer instructions using 220 pmol of siRNA and 20 pmol of DsiRNA per well of a 6-well plate. Sequences or catalog numbers are listed in Supplementary Table 2.

Sequencing of *TSC1*, *TSC2*, *REDD1* and *PTEN*

Bidirectional DNA sequencing was performed following PCR amplification using primers designed to produce 400–600 bp amplicons and to include at least 50 bp from intron/exon boundaries. Unless otherwise indicated, only bidirectionally observed somatic mutations are reported.

Statistics

All data are presented as means with standard deviations unless otherwise specified. *P*-values are calculated by two tailed Student's *t* test assuming equal variances unless otherwise indicated. Correlations were calculated using Spearman's *rho* in SPSS Statistics 17.0.

For additional information, see Supplementary Material.

Results

REDD1 regulation by pVHL

Recently, we reported that intravenous administration of adenovirus-Cre (Ad-Cre) to *Vhl*^{F/F} mice (also referred to as *Vhl*^{flloxP/flloxP}) led to *Vhl* inactivation and constitutive Hif activation in hepatocytes phenocopying the lipid accumulation observed in ccRCC (25). Using this system, we examined whether *Vhl* loss was sufficient to induce *Redd1*. *Vhl* disruption (see Fig. 1A) led to Hif activation (as determined by the upregulation of the Hif target gene *Glut1*; Fig. 1B) and *Redd1* induction, which was observed at the mRNA (Fig. 1B) and protein levels (Fig. 1C). Thus, *Vhl* loss is sufficient to induce *Redd1* expression.

To examine REDD1 regulation by pVHL in ccRCC, we selected a panel of ccRCC-derived cell lines (786-O, A498 and Caki-2). These cell lines have undergone loss of heterozygosity (LOH) at the *VHL* locus and contain a single mutant *VHL* allele (26). However, whereas pVHL function is completely disrupted in 786-O and A498 cells, which harbor truncating mutations upstream of the α -domain, which contains the elongin C binding motif, the α -domain is only partially truncated in Caki-2 cells (26). Nevertheless, the *VHL* mutation in Caki-2 cells (c.529A>T, Supplementary Fig. 1) is likely to be pathogenic as other somatic mutations in ccRCC have been identified downstream (both missense as well as truncating) (27). Another difference among the cell lines is that whereas in 786-O and A498 HIF-2 α is upregulated and HIF-1 α is undetectable, in Caki-2 cells, the reciprocal pattern is observed.

To determine whether REDD1 was upregulated in ccRCC as a consequence of *VHL* loss, we examined the effects of stable reconstitution with wild-type *VHL*. Ectopic *VHL* was expressed at different levels across the cell lines (Fig. 2A and data not shown), and as expected, the levels were lower than in previously selected monoclonal populations of reconstituted 786-O cells (28). Nevertheless, *VHL* reconstitution uniformly downregulated the levels of HIF- α and its target Glut-1 (Fig. 2A–D, Supplementary Fig. 2). In addition, *VHL* expression similarly downregulated REDD1 (Fig. 2A–D, Supplementary Fig. 2). Furthermore, *VHL* reconstitution restored the normal regulation of HIF- α and REDD1 by hypoxia (Fig. 2A–D, Supplementary Fig. 2). In keeping with the idea that endogenous mutant *VHL* in Caki-2 cells retains some functionality, baseline REDD1 levels were lower in these cells (Fig. 2A). Furthermore, the effects of *VHL* reconstitution on REDD1 induction by hypoxia in Caki-2 cells were incremental (Fig. 2D). Taken together these data show that REDD1 is induced in ccRCC cell lines as a consequence of *VHL* disruption and that REDD1 levels and its normal regulation can be restored by *VHL* reconstitution.

HIF- α -dependent regulation of REDD1 in ccRCC

To unequivocally determine whether REDD1 regulation by pVHL involved HIF and whether both HIF-1 and HIF-2 were implicated, we performed both chromatin immunoprecipitation (ChIP) as well as knockdown experiments. ChIP was conducted using antibodies against the aryl hydrocarbon receptor nuclear translocator (ARNT, also called HIF-1 β) as well as the corresponding HIF- α subunit (HIF-2 α [786-O and A498] and HIF-1 α [Caki-2]). *VHL*-reconstituted cell lines were used as a control. As a reference, the amount of HIF bound to the HIF target genes phosphoglycerate kinase (*PGK1*) and lactate dehydrogenase A (*LDHA*) was measured.

As expected, ARNT and HIF- α were observed bound to *PGK1* and *LDHA* sequences, and the amount decreased following *VHL* reconstitution (Fig. 2E, see also an independent experiment in Supplementary Fig. 3). Similarly, increased levels of ARNT and HIF- α were detected on *REDD1* (Fig. 2E, Supplementary Fig. 3).

To determine whether HIF was required for REDD1 upregulation, the effects of depleting HIF- α subunits were evaluated using two independent siRNAs. Knockdown of either HIF-2 α (in 786-O and A498) or HIF-1 α (in Caki-2) downregulated REDD1 levels (Fig. 2F). Taken together these data show that *VHL* disruption results in a HIF-dependent direct upregulation of REDD1. Furthermore, unlike some genes that appear to be selectively regulated by HIF-1 or HIF-2 (29, 30), our results show that both HIF-1 and HIF-2 can mediate the upregulation of *REDD1* in ccRCC.

REDD1 is highly expressed in *VHL*-deficient ccRCC tumors

Next we evaluated whether *REDD1* expression was upregulated in ccRCC tumors. For these experiments we analyzed two previously reported, publicly available, microarray datasets (31, 47). We selected tumors with mutant *VHL* and evaluated *REDD1* expression levels by comparison to normal kidney samples. The pattern of *REDD1* expression was correlated to that of a cohort of hypoxia-inducible genes, largely HIF-1 and HIF-2 targets (30, 33–35), found to be upregulated in ccRCC. In addition as a reference, a second gene cohort was selected of genes implicated in ccRCC pathogenesis that for the most part are not regulated by HIF or upregulated in ccRCC (see Fig. 3A). This analysis showed that *REDD1* was upregulated in *VHL*-mutant ccRCCs ($q=6 \cdot 10^{-12}$) and that *REDD1* upregulation was undistinguishable from that of other HIF target genes (Fig. 3A).

In an independent tumor cohort, we compared tumors harboring *VHL* mutations or *VHL* gene methylation, to normal renal cortices (from where ccRCC is thought to arise) as well as to ccRCCs with presumed intact *VHL* function. This analysis showed that *REDD1* was upregulated in *VHL*-deficient ccRCCs, by comparison to normal cortices, and that substantially more heterogeneity existed in *REDD1* levels in ccRCCs with presumed wild-type *VHL* status (Fig. 3B).

To further establish that *REDD1* was upregulated in *VHL*-deficient ccRCC, we evaluated the expression of *REDD1* in a cohort of fresh-frozen ccRCC tumors containing *VHL* mutations and compared the levels to matched normal renal cortices from the same patients. As a reference, quantitative real-time PCR (qRT-PCR) was performed for several HIF targets. By comparison to the corresponding normal cortices, *CAIX* and *VEGFA* expression was upregulated in tumors ($P = 5.5 \times 10^{-5}$ and 1.2×10^{-3} , respectively, paired t test, Fig. 3C). Similarly, *REDD1* was upregulated in *VHL*-deficient ccRCCs with an overall increase in expression of approximately 3.3-fold ($P = 3.4 \times 10^{-3}$, paired t test, Fig. 3D).

We evaluated REDD1 protein levels in tumor lysates and tissue sections using a monoclonal anti-REDD1 antibody we generated. This antibody recognized REDD1 both by western blot and immunoprecipitation and appeared to be highly specific (Supplementary Fig. 4). REDD1 protein levels were consistently upregulated in *VHL*-deficient ccRCCs (Fig. 3E). In normal kidneys, REDD1 was found to be expressed in normal renal tubular cells (Fig. 3F), and these results were consistent with observations in hypoxic mice (36). Overall, the pattern of staining appeared to be diffusely cytoplasmic (Fig. 3F). In tumors, REDD1 levels were substantially elevated (Fig. 3F). Next, we examined REDD1 levels by IHC in an unselected cohort of 78 ccRCCs. REDD1 was highly expressed in the vast majority of tumors (Supplementary Table 1).

We previously reported that REDD1 is a critical negative regulator of mTORC1, and that *REDD1* overexpression was sufficient to inhibit mTORC1 (8). However, mTORC1 has been reported to be active in 60–85% of ccRCCs (1, 2). We examined the same tumor cohort for mTORC1 activation. As a readout, we evaluated the phosphorylation of S6 at S235/236 and S240/244. A statistically significant correlation was found between phospho-S6^{S240/244}, which is thought to be more specific for mTORC1(37), and phospho-S6^{S235/236} ($p<0.001$),

and some level of S6^{S240/244} phosphorylation was observed in 80% of tumors (Supplementary Table 1). Not unexpectedly, given that the majority of tumors stained positively for phospho-S6 and REDD1, there was significant overlap (Supplementary Table 1).

mTORC1 regulation by REDD1 in ccRCC

To begin to explore this paradox, we asked whether REDD1 was involved in regulating mTORC1 in ccRCC. Should REDD1 be regulating mTORC1 in ccRCC cell lines, REDD1 knockdown should increase mTORC1 activity. For these experiments, cell lines were grown in 1% serum, which more likely mimics the growth factor milieu of tumors. In both A498 and Caki-2 cells, REDD1 knockdown increased S6 phosphorylation (Fig. 4A) and reduced 4E-BP1 binding to eIF4E (Fig. 4B). Thus, REDD1 normally inhibits mTORC1 in these cells. By contrast, REDD1 knockdown had practically no effect on mTORC1 readouts in 786-O cells (Fig. 4A and data not shown).

Because the TSC1/TSC2 complex is required for mTORC1 regulation by REDD1 (8, 16, 17), we examined the effects of TSC2 depletion. Similar to the effects of REDD1 knockdown, S6 phosphorylation was upregulated in TSC2-depleted A498 and Caki-2 cells, but not in 786-O cells (Fig. 4C). These data show that in both A498 and Caki-2 cells mTORC1 activity is restrained by REDD1 and the TSC1/TSC2 complex. By contrast, mTORC1 activity appears to be uncoupled from both REDD1 and TSC1/TSC2 in 786-O cells, which are *PTEN* deficient (38). No mutations are observed in *TSC1* or *TSC2* in 786-O cells (38), and we speculate that TSC1/TSC2 may be inactivated post-translationally as a result of increased Akt activity due to *PTEN* loss. As the TSC1/TSC2 complex is required for REDD1-induced mTORC1 inhibition (8, 16, 17), these results would provide an explanation for mTORC1 activation in these cells despite high REDD1 levels. Furthermore, the finding that in 786-O cells mTORC1 was uncoupled from REDD1 provided an explanation for the coexistence of high REDD1 levels with mTORC1 activation in ccRCC. However, *PTEN* mutations are rare in ccRCC (39–41), and we sought to explore this further.

TSC1 is a tumor suppressor in sporadic ccRCC

We set out to examine other components of the mTORC1 pathway. A previous study failed to identify mutations in *TSC1* or *TSC2* in ccRCC (21). However, the study involved a small number of tumors, and we were encouraged by the finding during a whole-genome sequencing study (Peña-Llopis et al. in preparation) of a somatically acquired mutation in *TSC1*, which we sought to characterize. The mutation, which was accompanied by LOH, was a splice site mutation in the exon 5 splice acceptor (IVS211-2A > T; Fig. 5A). This mutation, as determined by RT-PCR and cDNA sequencing, led to exon 5 skipping (Fig. 5B and C). Exon 5 loss, however, would be predicted not alter the reading frame. To assess the results of exon 5 skipping, a *TSC1*^{ΔEx5} cDNA was generated by site-directed mutagenesis and was introduced into immortalized *Tsc1*-deficient mouse embryo fibroblasts (MEFs). By contrast to *Tsc1*^{−/−} MEFs reconstituted with wild-type human *TSC1*, *TSC1*^{ΔEx5}-reconstituted MEFs expressed very low protein levels (Fig. 5D). This was not due to low *TSC1*^{ΔEx5} mRNA expression (Figs 5E, F) and experiments with a proteasome inhibitor suggested that the *TSC1*^{ΔEx5} protein was unstable (Fig. 5G). Since TSC1 functions to stabilize TSC2, not surprisingly, TSC2 protein levels were substantially reduced in *TSC1*^{ΔEx5}-reconstituted MEFs (Figs 5D and G).

Next we tested the functional consequences of *TSC1*^{ΔEx5} expression on mTORC1. Serum starvation, which downregulated mTORC1 activity in wild-type *TSC1* expressing cells, failed to downregulate mTORC1 in *TSC1*^{ΔEx5}-reconstituted cells (Fig. 5H). Finally, to determine whether *TSC1*^{ΔEx5} would uncouple mTORC1 from REDD1, we evaluated the

response of *TSC1*^{ΔEx5}-expressing cells to hypoxia, which results in a Redd1-dependent inhibition of mTORC1 (8). As shown in Fig. 5I, despite substantial upregulation of Redd1 in response to hypoxia, *TSC1*^{ΔEx5}-reconstituted cells, like empty vector reconstituted cells, failed to inhibit mTORC1. Taken together these data show that *TSC1* (IVS211-2A > T) results in exon 5 loss and an unstable protein leading to constitutive mTORC1 activation and its uncoupling from REDD1.

Having established a precedent for an inactivating somatically acquired *TSC1* mutation in ccRCC, and while suspecting based on the previous study (21) that the *TSC1* mutation frequency in ccRCC may be low, we set out to determine whether additional *TSC1* mutations could be found in ccRCC. For these experiments, we performed PCR amplification and bidirectional capillary sequencing analyses of *TSC1* coding sequences (and splice sites) in the aforementioned cohort of 77 fresh-frozen ccRCCs specimens, including 71 tumors for which we had matched normal samples. Interestingly, three additional mutations in *TSC1* were identified (Table 1). Matched normal samples were available for two tumors, which permitted confirmation that the mutations were somatically acquired. These mutations were both predicted to be truncating, and since truncating *TSC1* mutations further downstream have been associated with TSC syndrome (42), they are most likely pathogenic.

We sought to compare the mutation frequency of *TSC1* to that of *PTEN*, which has been previously implicated in ccRCC (39, 40). As shown, in Table 1, *PTEN* sequencing of the same tumor cohort revealed a single *PTEN* mutation (p.G165V). This substitution was previously reported in association with Cowden syndrome (43) and is therefore pathogenic. Thus, *TSC1* mutations in ccRCC appear to occur at a frequency similar to, if not greater than, those in *PTEN*.

Because the TSC1/TSC2 complex may also be inactivated by mutations in *TSC2*, *TSC2* coding and splice site sequences were similarly sequenced in the tumor cohort. However, no somatically acquired mutations were found in *TSC2*. Failure to identify *TSC2* mutations was not due to suboptimal sequencing as, equivalent to *TSC1* and *PTEN*, 100% of the *TSC2* amplicons were successfully sequenced across every one of the tumor samples.

Finally, we examined *REDD1*. As for *PTEN*, a single mutation was found in *REDD1* (Table 1). This mutation was predicted to be truncating and, since the C-terminus of REDD1 is essential for its function (9), the mutation would be predicted to be inactivating.

While no correlation was found between mutations and mTORC1 activation in tumors (see Supplementary Table 1), only a few tumors had mutations, and we hypothesize that other mechanisms, genetic or epigenetic, account for mTORC1 activation despite REDD1 induction in many other samples. Conspicuously, there were no concurrent mutations in *TSC1*, *REDD1* and *PTEN*, a fact that could be explained simply by the low individual gene mutation frequency, but which may also indicate that the simultaneous occurrence of these mutations does not confer an additive advantage in ccRCC development and is therefore not selected for.

Discussion

Herein, we evaluated the interplay between pVHL and mTORC1 pathways in ccRCC. We show that REDD1 is upregulated in *VHL*-deficient ccRCC tumors, that *VHL* disruption is sufficient to upregulate REDD1, and that REDD1 upregulation in ccRCC depends on pVHL and can be mediated by either HIF-1 or HIF-2. Furthermore, our data show that whereas REDD1 is involved in restraining mTORC1 activity in some ccRCCs, in others, mechanisms have evolved to uncouple mTORC1 from REDD1 inhibition. One such

mechanism involves the disruption of the TSC1/TSC2 complex and our results implicate *TSC1* as a novel tumor suppressor gene in sporadic ccRCC.

REDD1 is broadly upregulated in *VHL*-deficient ccRCCs and *in vivo* as well as *in vitro* experiments indicate that REDD1 regulation is *VHL*-dependent. As *VHL* inactivation and HIF stabilization are thought to be among the earliest molecular events in renal tumorigenesis, it is not surprising that REDD1 can already be observed upregulated in renal cysts in von Hippel-Lindau patients (data not shown). REDD1 upregulation in ccRCC requires HIF, and despite that differences exist in ccRCC depending upon whether HIF-1 α or HIF-2 α is expressed (24), both HIF-1 and HIF-2 are involved in the regulation of REDD1, and as supported by our ChIP studies, *REDD1* is directly acted upon by both. Nonetheless, should preferential regulation by HIF-1 occur in tumors, these data could contribute to explain why HIF-1 may function as a tumor suppressor in ccRCC.

Importantly, our results show that mTORC1 is regulated by REDD1 in ccRCC in a context-dependent manner. The two extremes are illustrated by Caki-2 and 786-O cell lines. In Caki-2 cells, REDD1 is engaged in mTORC1 inhibition suggesting that REDD1 functions in a negative feedback loop to downregulate mTORC1 following *VHL* disruption. These data are consistent with the findings that acute disruption of *Vhl* in immortalized and primary MEFs has previously been shown to lead to senescence (44, 45). We conjecture that mTORC1 remains responsive to REDD1 in Caki-2 cells because, as a consequence of a partially active pVHL, the level of REDD1 upregulation is modest, such that the selective pressure to uncouple mTORC1 from REDD1 is lower than in 786-O cells. By contrast, REDD1 levels are high in 786-O cells, but mTORC1 is insensitive to it.

Loss of function mutations in *TSC1* activate mTORC1 and result in its uncoupling from REDD1 as shown here with the *TSC1* splice site mutant. Three additional *TSC1* mutations were identified in the 77 tumors, including two truncating mutations that were confirmed to be somatically acquired. To our knowledge, this is the first report implicating *TSC1* as a tumor suppressor in sporadic ccRCC. As for *PTEN*, one somatically acquired inactivating mutation was found in *REDD1*, suggesting that, although rarely, mutations in *REDD1* may similarly contribute to ccRCC development.

By contrast to *TSC1*, somatic mutations in *TSC2* were not identified. A potential explanation for these findings would be furnished by the existence of a second tumor suppressor gene in the proximity of *TSC1* such that regional deletions may result in the simultaneous loss of the remaining wild-type copy for the two (or more) tumor suppressor genes. In fact, precedent for this exists in ccRCC and a tumor suppressor gene, *PBRM1*, was recently identified in relative proximity to *VHL* (46).

mTORC1 has been previously reported to be activated in 60–85% of ccRCCs (1, 2). While these studies were based on phospho-S6^{S235/236} signal, which is thought to be less specific than phospho-S6^{S240/244} (37), we found that approximately 80% of ccRCC were positive for phospho-S6^{S240/244}, adding support to the notion that mTORC1 is broadly activated in this tumor type.

Given the low frequency of mutations in *TSC1* and *PTEN*, other mechanisms must exist to prevent mTORC1 inhibition by REDD1. While targeted sequencing studies have failed to identify activating mutations in *Rheb* and *RhebL1* (2), recently, mutations in the *mTOR* gene itself were identified in ccRCC (46, 47). Furthermore, tumor associated *mTOR* mutants have been found to lead to mTORC1 activation and diminished mTORC1 inhibition by hypoxia (48). Nevertheless, these mutations are also rare. It is also possible that inactivation of a single *TSC1* allele, and *TSC1* is found in 9q, a region that is deleted in approximately 20% of ccRCC (31, 32), may be sufficient to activate mTORC1 and render it unresponsive to

REDD1. In keeping with this idea, modest depletion of TSC2 appears to be sufficient to block REDD1-induced mTORC1 inhibition (8).

Understanding how mTORC1 is deregulated in ccRCC may pave the way for the identification of patients most likely to benefit from mTORC1 inhibitors. It would be expected that only tumors with active mTORC1 would respond to its inhibition and this is supported by a small retrospective correlative study (49). While the phosphorylation state of mTORC1 effector proteins may be used as the readout for mTORC1 activation, TORC1 is a critical regulator of ribosomal biogenesis and nucleolar size (50, 51) and conceivably, nucleolar dimensions could serve as a surrogate for mTORC1 activity in tumors. In RCC, nucleolar prominence is a major determinant of the prognostic Fuhrman grading scale (52). The regulation of nucleolar size by mTORC1 provides an explanation for the positive correlation previously reported between phospho-S6 and tumor grade (1). This raises the possibility that the activation state of mTORC1 may contribute to the prognostic significance of the Fuhrman grading scale. Furthermore, it is possible that nucleolar size could serve as a pharmacodynamic indicator of mTORC1 inhibition in RCC. Should nucleolar prominence be dynamically regulated by mTORC1, the exposure of patients to mTORC1 inhibitors could affect Fuhrman grading. Supporting this concern, rapamycin was previously shown to reduce nucleolar size in both mammalian and yeast cells (51).

Driver mutations in tumors may reflect a state of addiction that could be exploited therapeutically. In this context, and despite that the TSC1/TSC2 complex likely regulates other processes besides mTORC1 (53, 54), mutations in *TSC1* in ccRCC could portend a state of addiction to mTORC1. While this represents a single instance, it is noteworthy that the index patient who had the original *TSC1* splice site mutation we evaluated had an extraordinary response to everolimus in the second line. Whereas the median progression-free interval on everolimus in the second line in the pivotal phase III clinical trial was 4 months (4), the patient remained on everolimus without progression for 13 months, and this was despite progression to sunitinib in 3 months.

In summary, this work begins to unravel the complexity of signaling pathways linking pVHL and mTORC1 in ccRCC and suggests that mechanisms have evolved in tumors to escape growth suppressive signals resulting from *VHL* loss and REDD1 upregulation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Acute *Vhl* disruption in mouse hepatocytes, which phenocopy important aspects of *VHL* loss in renal carcinoma cells in humans, is sufficient to upregulate *Redd1*. A, PCR of genomic DNA extracted from wild-type (WT) and *Vhl^{F/F}* livers after the indicated number of days following intravenous administration of Ad-Cre; as a control, a 1:1 mixture of DNA containing fully recombined *Vhl^F* (*Vhl^{Frec}*) and unrecombined (*Vhl^F*) alleles is shown. B, qRT-PCR of livers from WT and *Vhl^{F/F}* animals injected with Ad-Cre. Data are normalized to cyclophilin (*Ppib*) and represent averages with standard deviation ($n = 3-6$); * $p < 0.05$, ** $p < 0.01$ (unpaired *t*-test). C, immunoblot of extracts from livers from WT and *Vhl^{F/F}* animals injected with Ad-Cre.

Figure 2.

HIF- and pVHL-dependent regulation of REDD1 in ccRCC. A–D, immunoblot of protein extracts (heat-denatured or not [Glut-1]) from ccRCC cell lines reconstituted with *HA-VHL* (*VHL*) or an empty vector (EV), compared to monoclonal populations of 786-O cells reconstituted with *HA-VHL* (WT8) or an EV (pRC3), and exposed to hypoxia (where indicated) for the stated number of hours. E, ChIP using antibodies for ARNT and HIF- α (HIF-2 α for both 786-O and A498; and HIF-1 α for Caki-2) vs. rabbit IgG control in *VHL*- or *EV*-stably transfected cell lines evaluated for the indicated genes or a region far upstream of *VEGF* used as a control. F, immunoblot of ccRCC cell lines transfected with two independent siRNAs targeting HIF-2 α (786-O, A498) or HIF-1 α (Caki-2) vs. scrambled (Sc) control.

Figure 4.

Cell type-dependent regulation of mTORC1 by REDD1 and the TSC1/TSC2 complex. A, western blot and m⁷GTP affinity chromatography (B) of the indicated cell lines treated with siRNAs targeting REDD1 versus a scrambled control (Sc). C, western blot of cells grown as in (A) but treated with siRNA against TSC2.

Figure 5.

Characterization of a somatic *TSC1* mutation from a ccRCC tumor. A, sequence chromatograms of exon 5 splice acceptor in normal (N) and tumor (T) (arrow indicates mutation). B, RT-PCR using primers in the indicated exons of normal (N) and tumor (T) samples compared to those from other patients and to ccRCC cell lines. C, cDNA sequence chromatograms of normal (N) and tumor (T) samples. D, immunoblot of immortalized *Tsc1*^{-/-} MEFs (compared to immortalized *Tsc1*^{+/+} MEFs) reconstituted with empty vector (EV), wild-type human *TSC1* (WT) or human *TSC1* deficient for exon 5 (Δ Ex5). RT-PCR (E) and qRT-PCR (F) using primers in the indicated exons of *Tsc1*^{-/-} MEFs reconstituted as indicated (data are means $\pm$ SEM; $n = 2$). G-I, immunoblot of MEFs of the indicated genotypes exposed to MG132 (G), serum withdrawal (H), and hypoxia (I) for the indicated number of hours.

Table 1

Somatic mutations in ccRCC tumors

ID	Gene	cDNA	Protein
78	<i>REDD1</i>	c.550delC	Fs
9	<i>TSC1</i>	c.2459dupA	Fs
3246	<i>TSC1</i>	c.1546C>T	p.Q516X
4562	<i>TSC1</i>	IVS211-2A>T [†]	Sp
5533	<i>TSC1</i>	c.1342C>T [*]	p.P448S
4363	<i>PTEN</i>	c.494G>T	p.G165V

Mutations within non-coding areas are prefixed with “IVS” and their position is identified by the number of nucleotides away from the closest coding sequence.

[†] mutation in index patient’s tumor;

^{*} no normal tissue available; Fs, frame shift mutation; Sp, splice site mutation; ID, tumor identifier.