**Interplay Between pVHL and mTORC1 Pathways in Clear-Cell Renal Cell Carcinoma**

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**Abstract**

mTOR 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 hypoxia-inducible factor (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. *Mol Cancer Res; 9(9); 1255–65. ©2011 AACR.*

**Introduction**

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% to 85% of ccRCCs (1, 2), and mTORC1 inhibitors have been shown in 2 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 2 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; ref. 6). Other proteins are found in mTORC1, including mammalian lethal with sec-thirteen protein 8, 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). By phosphorylating S6K1 and 4E-BP1, mTORC1 stimulates protein translation. Importantly, only mTORC1 is directly inhibited by rapamycin (also called sirolimus; ref. 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; ref. 7). By phosphorylating S6K1 and 4E-BP1, mTORC1 stimulates protein translation. Importantly, only mTORC1 is directly inhibited by rapamycin (also called sirolimus; ref. 5).
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 tuberous sclerosis complex 1 and 2 (TSC1/TSC2) proteins (8). TSC2 acts as GTPase-activating protein toward a small G Protein, Ras homolog 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 without steric clashes (9).

The TSC1/TSC2 complex is inactivated in the eponymous syndrome, TSC, which is characterized by hamartomas in multiple organs (20). Although 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 characterized as having a 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 an HIF-1 target gene (10), REDD1 may be upregulated in VHL-deficient ccRCCs where it would be expected to downregulate mTORC1. Paradoxically, mTORC1 seems 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 Institutional Review Board approved tissue collection protocol and were flash frozen in liquid nitrogen and stored at −80°C. From each sample, immediately flanking sections from 2 sides perpendicular to each other were processed for hematoxylin and eosin (H&E) staining and analyzed by a pathologist (W.K.) for composition (tumor content) and polyclonal populations were selected and maintained in Hygromycin (250 μg/mL).

VHL reconstitution

786-O, A498, and Caki-2 cells were transfected using TransIT-LT1 Transfection Reagent (Mirus) 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.

siRNA transfections

siRNA oligonucleotides were from Dharmacon and Dicer-substrate siRNAs (DsiRNA) duplexes from Integrated DNA Technologies. Transfections were carried out using Lipofectamine 2000 (Invitrogen) for A498 or DharmaFECT reagent 3 (Dharmacon) for 786-0 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 catalogue numbers are listed in Supplementary Table S2.

Sequencing of TSC1, TSC2, REDD1, and PTEN

Bidirectional DNA sequencing was carried out following PCR amplification using primers designed to produce 400 to 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 SDs unless otherwise specified. P values are calculated by 2-tailed Student’s t test assuming equal variances unless otherwise indicated. Correlations were calculated using Spearman’s ρ 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 Vhlfl/fl mice (also referred to as Vhlfl/flox/flox) 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 (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 786-0 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.
Nevertheless, the VHL mutation in Caki-2 cells (c.529A > T, Supplementary Fig. S1) is likely to be pathogenic as other somatic mutations in ccRCC have been identified downstream (both missense as well as truncating; ref. 27). Another difference among the cell lines is that in 786-O and A498 HIF-2α is upregulated and HIF-1α is undetectable, whereas 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. S2). In addition, VHL expression similarly downregulated REDD1 (Fig. 2A–D, Supplementary Fig. S2). Furthermore, VHL reconstitution restored the normal regulation of HIF-α and REDD1 by hypoxia (Fig. 2A–D, Supplementary Fig. S2). 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 carried out 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-1β) as well as the corresponding HIF-α subunit [HIF-2α (786-O and A498) and HIF-1α (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

Figure 1. Acute Vhl disruption in mouse hepatocytes, which phenocopies 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 VhlF/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 (Vhl flox-rec) and unrecombined (Vhl flox) alleles is shown. B, qRT-PCR of livers from WT and VhlF/F animals injected with Ad-Cre.

Data are normalized to cyclophilin (Ppib) and represent averages with SD (n = 3–6); *, P < 0.05; **, P < 0.01 (unpaired t test). C, immunoblot of extracts from livers from WT and VhlF/F animals injected with Ad-Cre.

following VHL reconstitution (Fig. 2E, see also an independent experiment in Supplementary Fig. S3). Similarly, increased levels of ARNT and HIF-α were detected on REDD1 (Fig. 2E, Supplementary Fig. S3).

To determine whether HIF was required for REDD1 upregulation, the effects of depleting HIF-α subunits were evaluated using 2 independent siRNAs. Knockdown of either HIF-2α (in 786-O and A498) or HIF-1α (in Caki-2) downregulated REDD1 levels (Fig. 2F). Taken together these data show that VHL disruption results in an HIF-dependent direct upregulation of REDD1. Furthermore, unlike some genes that seem 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 2 previously reported, publicly available, microarray data sets (31, 32). We selected tumors with mutant VHL and evaluated REDD1 expression levels by comparison with 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 (Fig. 3A). This analysis showed that REDD1 was upregulated in VHL-mutant ccRCCs ($Q = 6 \times 10^{-12}$) and that REDD1 upregulation was indistinguishable from that of other HIF target genes (Fig. 3A).

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

![Figure 3](Image)

**Figure 3.** REDD1 is upregulated in VHL-deficient ccRCCs. A, heatmap representation of REDD1 expression levels compared with other hypoxia-inducible genes and a panel of other genes (bottom) in ccRCCs with VHL mutations compared with normal kidney samples from GSE17895; genes are ranked by FDR $q$ of ccRCC versus kidney expression values. B, heatmap representation of gene expression values for the same genes as in A from GSE14994 in tumors with VHL mutations or VHL gene methylation (VHL deficient) versus normal renal cortices or a set of tumors with no evidence of VHL mutation or methylation. In both A and B, bold FDR $Q$ values are statistically significant; in red and blue are the fold change (FC) values for genes that are upregulated or downregulated, respectively.
existed in REDD1 levels in ccRCCs with presumed wild-type VHL (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 with matched normal renal cortices from the same patients. As a reference, quantitative real-time PCR (qRT-PCR) was carried out for several HIF targets. By comparison with the corresponding normal cortices, CAIX and VEGFA expression was upregulated in tumors (P = 5.5 × 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 × 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. S4). 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 S1).

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% to 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-S6S240/244, which is thought to be more specific for mTORC1 (37) and phospho-S6S235/236 (P < 0.001), and some level of S6S240/244 phosphorylation was observed in 80% of tumors (Supplementary Table S1). Not unexpectedly, given that the majority of tumors stained positively for phospho-S6 and REDD1, there was a significant overlap (Supplementary Table S1).

**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
mutations are observed in TSC2 in 786-O cells, which are seems to be uncoupled from both REDD1 and TSC1/TSC2 complex. By contrast, mTORC1 activity 2 cells, mTORC1 activity is restrained by REDD1 and the cells (Fig. 4C). These data show that in both A498 and Caki-in TSC2-depleted A498 and Caki-2 cells but not in 786-O REDD1 knockdown, S6 phosphorylation was upregulated the effects of TSC2 depletion. Similar to the effects of mTORC1 regulation by REDD1 (8, 16, 17), we examined readouts in 786-O cells (Fig. 4A and data not shown).

Figure 4. Cell type-dependent regulation of mTORC1 by REDD1 and the TSC1/TSC2 complex. A, Western blot and (B) m’GTP affinity chromatography 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.

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 seems 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 posttranslationally 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 and colleagues, unpublished data) 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 splicing 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 7SC1Dexm3′ cDNA was generated by site-directed mutagenesis and was introduced into immortalized Tie1-deficient human embryo fibroblasts (MEF). In contrast to Tie1-/- MEFs reconstituted with wild-type human TSC1, TSC1Dexm3′-reconstituted MEFs expressed very low protein levels (Fig. 5D). This was not because of low TSC1Dexm3′ mRNA expression (Fig 5E and F), and experiments with a proteasome inhibitor suggested that the TSC1Dexm3′ protein was unstable (Fig. 5G). Because TSC1 functions to stabilize TSC2, not surprisingly, TSC2 protein levels were substantially reduced in TSC1Dexm3′-reconstituted MEFs (Figs 5D and G).

Next, we tested the functional consequences of TSC1Dexm3′ expression on mTORC1. Serum starvation, which downregulated mTORC1 activity in wild-type TSC1-expressing cells, failed to downregulate mTORC1 in TSC1Dexm3′-reconstituted cells (Fig. 5H). Finally, to determine whether TSC1Dexm3′ would uncouple mTORC1 from REDD1, we evaluated the response of TSC1Dexm3′-expressing cells to hypoxia, which results in a Redd1-dependent inhibition of mTORC1 (8). As shown in Figure 5I, despite substantial upregulation of Redd1 in response to hypoxia, TSC1Dexm3′-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 carried out 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, 3 additional mutations in
TSC1 were identified (Table 1). Matched normal samples were available for 2 tumors, which permitted confirmation that the mutations were somatically acquired. These mutations were both predicted to be truncating, and because 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 with 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 seem 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 because of 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, because the C-terminus of REDD1 is essential for its function (9), the mutation would be predicted to be inactivating.

Although no correlation was found between mutations and mTORC1 activation in tumors (Supplementary Table S1), 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, 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 VHL patients (data not shown). REDD1 upregulation in ccRCC requires HIF, and despite that differences exist in ccRCC depending upon whether HIF-1α or HIF-2α 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 TSCI splice site mutant. Three additional TSCI mutations were identified in the 77 tumors, including 2 truncating mutations that were confirmed to be somatically acquired. To our knowledge, this is the first report implicating TSCI 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.

In contrast to TSCI, 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 TSCI such that regional deletions may result in the simultaneous loss of the remaining wild-type copy for the 2 (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% to 85% of ccRCCs (1, 2). Although these studies were based on phospho-S6 translation initiation factor 4E-binding protein 1 (47), it is thought that they are less specific than phospho-S6 (S235/236) and phospho-S6 (S240/244) (37), we found that approximately 80% of ccRCC were positive for phospho-S6 (S235); S236), 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. Although 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 (32, 46). Furthermore, tumor-associated mTOR mutants have been found to lead to mTORC1 activation and diminished mTORC1 inhibition by hypoxia (47). 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, 48), 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 respond to its inhibition and this is supported by a small retrospective correlational study (49). Although 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 nuclear 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. Although 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. Although 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.

Disclosure of Potential Conflicts of Interest
REDD1 monoclonal antibody reported herein is licensed to Bethyl laboratories.

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References

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