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Induction of the Mdm2 gene and protein by kinase signaling pathways is repressed by the pVHL tumor suppressor

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The tumor suppressor von Hippel–Lindau, pVHL, is a multifaceted protein. One function is to dock to the hypoxia-inducible transcription factor (HIF) and recruit a larger protein complex that destabilizes HIF via ubiquitination, preventing angiogenesis and tumor development. pVHL also binds to the tumor suppressor p53 to activate specific p53 target genes. The oncogene Mdm2 impairs the formation of the p53–pVHL complex and activation of downstream genes by conjugating nedd8 to pVHL. While Mdm2 can impact p53 and pVHL, how pVHL may impact Mdm2 is unclear. Like p53 somatic mutations, point mutations are evident in pVHL that are common in renal clear cell carcinomas (RCC). In patients with RCC, Mdm2 levels are elevated, and we examined whether there was a relationship between Mdm2 and pVHL. TCGA and DepMap analysis revealed that *mdm2* gene expression was elevated in RCC with *vhl* point mutations or copy number loss. In pVHL reconstituted or deleted isogenetically match RCC or MEF cell lines, Mdm2 was decreased in the presence of pVHL. Furthermore, through analysis using genetic and pharmacological approaches, we show that pVHL represses Mdm2 gene expression by blocking the MAPK–Ets signaling pathway and blocks Akt-mediated phosphorylation and stabilization of Mdm2. Mdm2 inhibition results in an increase in the p53–p21 pathway to impede cell growth. This finding shows how pVHL can indirectly impact the function of Mdm2 by regulating signaling pathways to restrict cell growth.

Mdm2 | pVHL | p53

Significance

The tumor suppressor pVHL can augment or repress pathways to activate or repress transcription factors. This regulation is important for controlling the microenvironment, proliferation, and cell fate. We show that pVHL can repress Mapk/Erk and Akt pathways to regulate the oncogene Mdm2 gene expression and protein levels, respectively. The data also show that Mdm2 protein levels are not regulated by autoubiquitination but by pVHL blocking Akt signaling and the recruitment of the E2 ubiquitin enzyme. The loss of pVHL in renal cell carcinoma and other cancers shows how, under nongenotoxic stress conditions, Mdm2 gene and protein levels are elevated to promote tumor progression.

Cancer cells selectively target one or many tumor suppressors during tumorigenesis, which can have pleiotropic effects on pro and antiproliferative signaling pathways. The tumor suppressor von Hippel–Lindau (pVHL) is associated with a familiar predisposition that can lead to neoplastic development, but somatic mutations are also evident in human cancers. pVHL is a small docking protein that can integrate into a multi-subunit complex with elongin C, elongin B, Cul-2, and Rbx1, known as VBC (1). pVHL will bind to the transcription factor hypoxia-inducible factor (HIF1α, HIF2α, and HIF3α) dependent on prolyl hydroxylation under normoxic conditions, leading to the recruitment of VBC complex and ubiquitin-mediated degradation (1–5). Loss of pVHL and stabilization of HIF1α are important molecular events in some cancers (1). More recently, pVHL was reported to function independently of the VBC complex to negatively regulate progrowth signaling. Notably, pVHL can bind to the tumor suppressor p53 and augment the activation of specific target genes that impose cell cycle arrest and block angiogenesis (6, 7).

The tumor suppressor p53 protein, in response to DNA damage signaling pathways, can bind to DNA binding elements in the P2 promoter of Mdm2 (8–10). Elevated Mdm2 can downregulate p53 in an autoregulatory feedback loop at the conclusion of DNA repair (11). Previous work showed that in response to genotoxic stress, the pVHL–p53 complex is important for inducing cell cycle arrest through induction of the tumor suppressor p21. Independent of p53, cell surface receptors can activate numerous transcription factors, some of which can induce the Mdm2 gene expression (12–15). Mdm2 function is regulated by posttranslation modifications, whereby genotoxic stress-activated pathways lead to the recruitment of ubiquitin complex while cell surface receptor pathway recruits a nedd8 complex. Mdm2 will mediate the conjugation of nedd8 to p53 and pVHL to alter their respective tumor suppressor activity (7, 16). While the interplay between p53 and Mdm2 is mature, understanding how pVHL may influence Mdm2 is unclear.

To gain insight into how pVHL may influence Mdm2, we examined the TCGA and found that mutations and copy number variation in pVHL correlated with an increase in the mRNA of Mdm2. Further analysis showed that wild-type VHL repressed Mdm2 gene expression through the second promoter-Ets binding elements, dependent on MAPK signaling. Further, Mdm2 protein was decreased by pVHL by repressing the Akt kinase.

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Akt phosphorylation is key for nuclear entry of Mdm2 and increases in protein stability as blockade of this pathway pharmacologically or through PTEN diminished Mdm2 (17, 18). The loss of Akt phosphorylation also revealed that the Ring domain of Mdm2 was required for decreased protein. The loss of the Ring domain suggests that Mdm2 does not have autoubiquitination, but the recruitment of UBC5 (E2 ubiquitin enzyme) is the mediator of Mdm2 ubiquitin conjugation and destabilization. We show an interplay between how the tumor suppressor pVHL represses the oncogene Mdm2 and the loss of pVHL in patients, which correlates with higher proliferation and poor survival.

Results

Database Mining to Examine Mdm2 and pVHL Gene Analysis. TCGA analysis of renal carcinoma cells shows that the vhl gene is a loss of heterozygosity or somatic mutation, which correlates with a decrease in patent survival (Fig. 1 *A* and *B*). We also examined Mdm2 mRNA levels and RCC patient survival. Elevated levels of Mdm2 mRNA correlated with shorter survival (Fig. 1*C*). Next, we found a correlation between vhl LOH or somatic mutation and increased Mdm2 mRNA (Fig. 1 *D* and *E*). The increase in mdm2 mRNA also correlated with the proliferation marker KI67 (Fig. 1*E*). We also use the Cancer Dependency Map (DepMap) to analyze human cell lines to determine whether there was an interconnection between pVHL and regulating Mdm2 expression in RCC. The data showed that either a mutation or a deletion of one or both copies of the pVHL gene was evident in the cell lines (*SI Appendix*, Fig. S1). VHL protein levels were diminished in renal cancer cell lines (*SI Appendix*, Fig.S1*A*). Conversely, Mdm2 gene expression and protein were elevated in these cell lines (*SI Appendix*, Fig. S1*B*). These analyses suggest that there is a correlation between mutant pVHL and increase in Mdm2 gene expression and this correlates with poor survival.

pVHL Represses the Second Promoter of Mdm2. To gain insight into how pVHL might regulate Mdm2 gene expression, we used isogenically matched RCC cell lines (3). Examination of Mdm2 levels by western blot showed in O-786 and RCC4 cells, a 90 kDa band of Mdm2 was decreased in cells reconstituted with pVHL

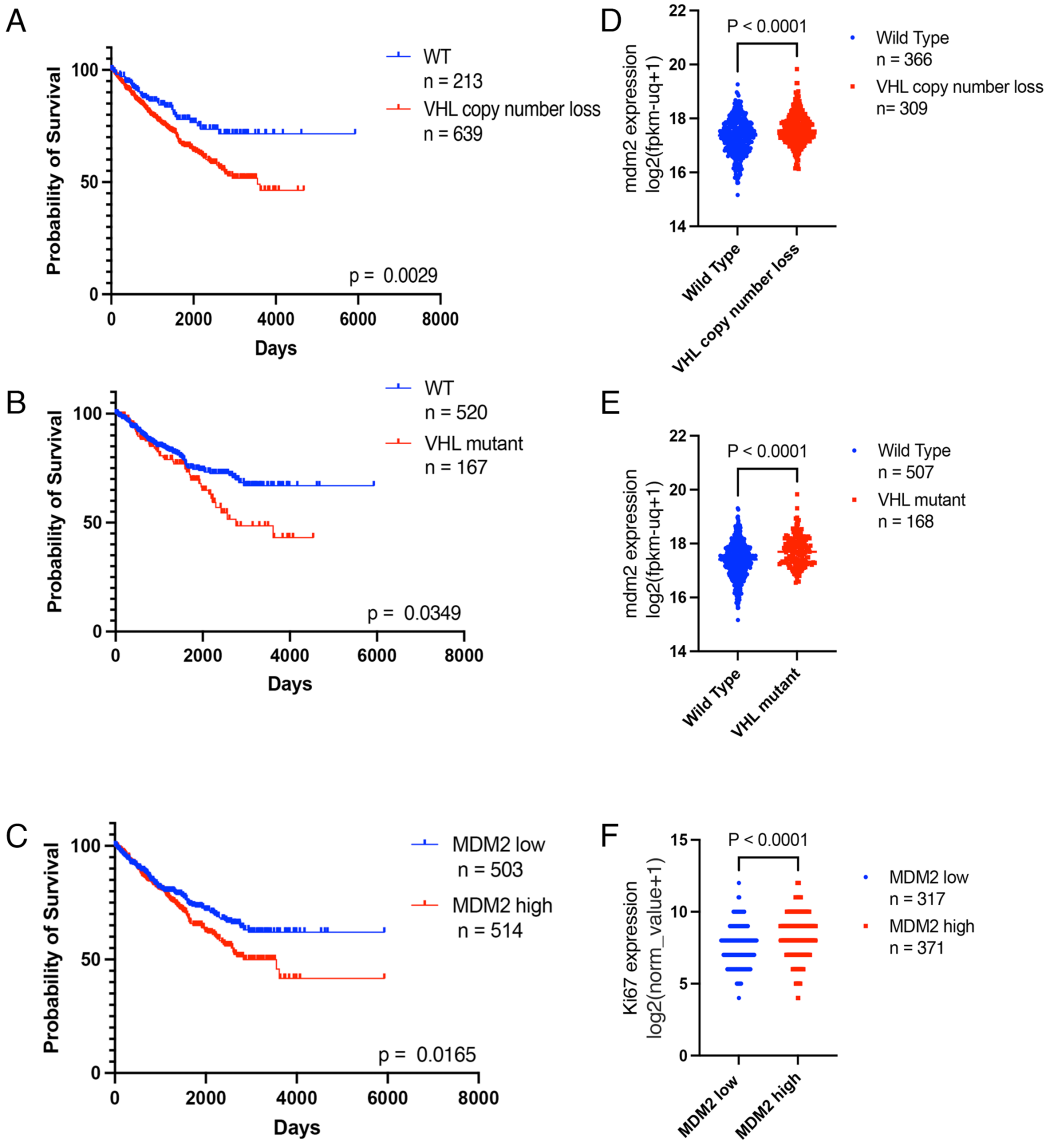

Fig. 1. TCGA analysis of pVHL and Mdm2. Kaplan–Meier survival analysis of patients with renal carcinomas (*A*) wild type and loss of VHL copy number, (*B*) wild type vs. mutant VHL, and (*C*) high and low Mdm2 expression. Log-rank Test—Mantel-Cox determined significance. (*D*) Mdm2 expression in wild type and copy number loss of VHL and (*E*) Mdm2 expression in wild type and mutant VHL. (*F*) High and low expression of Mdm2 and Ki67 expression. Welch's *t* test determined significance.

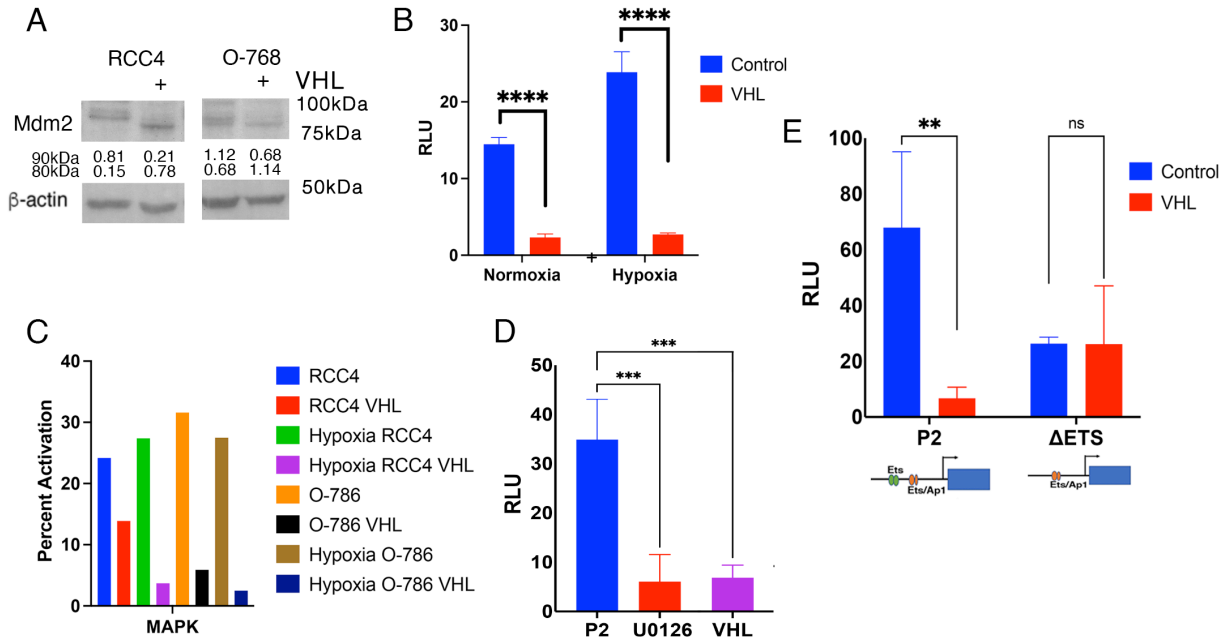

Fig. 2. Mapk induces Mdm2 gene expression in RCC. (*A*) Western blot of Mdm2 and β-Actin from control and pVHL reconstituted O-786 and RCC4 cellular extracts. 90 and 80 kDa Mdm2 bands were determined as a ratio of density of Mdm2 isoform to βActin. (*B*) Relative gene expression measured by Mdm2 P2 promoter linked to luciferase transiently transfected with RSV-βgal in O-786 control and VHL cells. Relative luciferase was calculated as the ratio of luciferase to β-gal activity from three replicate transfections. SD was calculated, and the Student *t* test determined significance. (*C*) Flow cytometry of active Mapk in control and pVHL RCC and O-786 cell under hypoxia (1% O_2) and normoxia (21% O_2). (*D*) Mdm2 P2 reporter assay treated with U0126 or inhibitor or transient expression of VHL. Analysis was performed as described in *B*. (*E*) O-786 cells (control and VHL) were transiently transfected with RSV-βGal, and the P2 luciferase reporter or the P2 luciferase reported with mutated Ets DNA binding elements (ΔETS) in triplicate for each cell line. Reporter data were analyzed as in *B*.

(Fig. 2*A*). The lower band of Mdm2 migrated at roughly 80 kDa and increased in the presence of pVHL. We cloned this form and sequenced the cDNA from these cells with reconstituted pVHL and found this was the F-isoform of Mdm2 (*SI Appendix*, Fig. S1). The F-isoform of Mdm2 can be generated by an alternative splice of Mdm2 when the mRNA is generated from the P1 promoter of Mdm2 in human cells (19). This is specific to human cells as the F isoform of Mdm2 is missing the p53 interaction domain and transcripts generated from the P1 promoter. However, it should be noted that in mice, full-length Mdm2 can be generated from either the P1 or P2 promoter of Mdm2 (10). These data suggested that pVHL influences the Mdm2 P2 promoter, and to test this supposition, the P2-luciferase reporter construct and RSV-βgal were transiently transfected into control, and pVHL reconstituted O-786 cells. The data show a significant reduction in the Mdm2 promoter activity in the presence of pVHL, which was evident under normoxia (21% oxygen) or hypoxia (1% oxygen) (Fig. 2*B*). To verify a response to the loss of pVHL and hypoxia, we examined HIF2α in control and pVHL O-786 cells. Western blot for HIF2α (as these cells do not express HIF1α) shows an increase in HIF2α without pVHL and a further increase under hypoxia (*SI Appendix*, Fig. S2*D*). pVHL can block PKC activity, an upstream activator of the Mapk pathway. We examined Mapk activation in control and pVHL RCC4 and O-786 cells under normoxic or hypoxic conditions by flow cytometry. The data in Fig. 2*C* shows that pVHL repressed Mapk activation (*SI Appendix*, Fig. S2*A*). To examine the impact of Mapk on the Mdm2 gene, the P2 promoter was transiently transfected in 293T cells and then treated with the MEK inhibitor (U0126) and pVHL (as a control). The P2 reporter data showed that blockade of Mapk activation suppressed Mdm2 promoter activity (Fig. 2*D*). The P2 promoter has Ets DNA binding elements (13), and we use control and Ets factor DNA binding element deleted P2 promoter to determine whether this DNA binding element was sensitive to pVHL. Transient expression of these reporter constructs in 293T cells showed that the deleted Ets binding elements were not influenced by pVHL (Fig. 2*E*). These data indicate that pVHL indirectly affects the gene expression of Mdm2 by blocking the MAPK–Ets pathway.

pVHL Causes the Destabilization of Akt and Mdm2. It has been reported that pVHL can destabilize the pleiotropic kinase Akt, which is responsible for numerous cellular processes (20). One downstream target of Akt is Mdm2, which promotes Mdm2 nuclear localization and increases Mdm2 stability (17). To confirm that Akt activity was diminished in the presence of pVHL, we use flow cytometry in Rcc4 and O-786 control and pVHL cells to analyze active Akt (20). Indeed, our data confirm that active Akt was decreased in pVHL cells relative to control (Fig. 3 *A* and *F* and *SI Appendix*, Fig. S2 *A* and *B*). To determine whether pVHL regulated Mdm2 through Akt down-regulation, 293T cells were transiently transfected with Ha-tagged constitutively active Akt (Ha-CaAkt) alone or with Ha-tagged pVHL. Western blot analysis showed that endogenous Mdm2 was increased by Ha-CaAKT (Fig. 3*B* and *SI Appendix*, Fig. S3*A*). In the presence of Ha-pVHL both Ha-CaAkt, endogenous Mdm2 was decreased (Fig. 3*B* and *SI Appendix*, Fig. S3*A*). To ensure that Ha-CaAkt did not regulate the endogenous promoter of Mdm2, we transiently expressed Ha-tagged Mdm2 with or without Ha-CaAkt and Ha-pVHL in 293T cells. Western blot showed that exogenous Mdm2 was increased by Ha-CaAkt, and in the presence of Ha-pVHL, both Ha-CaAkt and Ha-Mdm2 were decreased (Fig. 3*C* and *SI Appendix*, Fig. S3*B*). These data show that the loss of active Akt by pVHL resulted in Mdm2 being destabilized. To verify that Akt phosphorylation was required to stabilize Mdm2, we use S166DS186D phosphomimic of Mdm2. The S166DS186D construct was transiently transfected alone or coexpressed with Ha-pVHL and Ha-CaAKT. The data show that pVHL did not alter S166DS186D Mdm2 (Fig. 3*D*

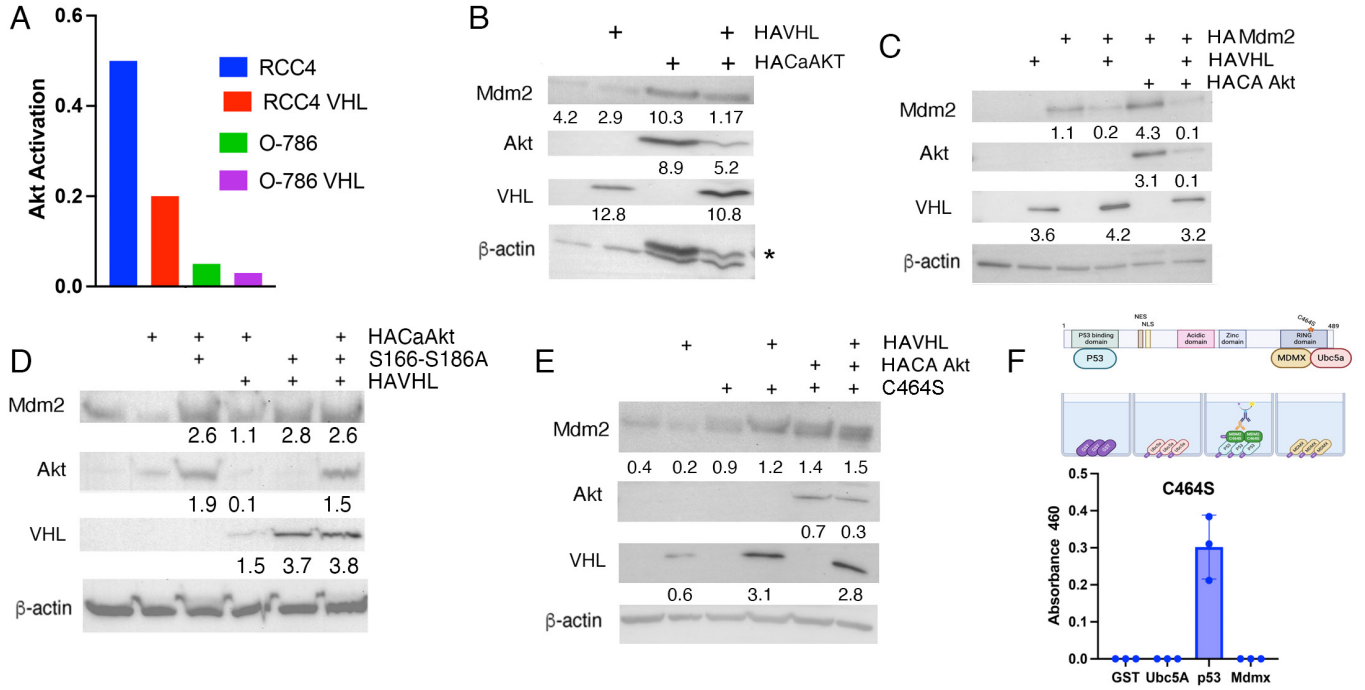

Fig. 3. pVHL regulates Akt-Mdm2 levels. (*A*) Flow cytometry of active Akt in control and VHL RCC4 and O-786 cells. (*B*) Western blot of endogenous Mdm2, and HA detected Ha-CaAkt, Ha-VHL transiently expressed in 293T cells, and β-actin from cellular extracts. * denotes residual Ha-CaAkt in the β-actin blot. (*C*) Western blot of exogenous Mdm2, Akt, and pVHL, Ha antibody was used to detect (Mdm2, CaAkt, and pVHL), and β-actin from 293T extracts transiently transfected with Mdm2, Ha-CaAkt, and Ha-pVHL. (*D*) Western blot Mdm2, Ha-Tag (Ha-pVHL and Ha-CaAkt), and β–actin from 293T extract transiently transfected with Mdm2 S166DS186D (2XSD), pVHL. (*E*) Western blot of Mdm2, Ha-Tag (Ha-CaAkt, Ha-pVHL), and β-actin form 293T cellular extracts transiently expressing pVHL, C464S mutant of Mdm2 and Ha-CaAkt. Western blots were quantitated by the intensity of the band relative to βActin. (*F*) Illustration of the experimental procedure. A linear structure of Mdm2 and the domains and the proteins (p53, Mdmx) known to bind these domains. The graph represents C464S binding to GST, Ubc5a, p53, or Mdmx using a modified ELISA method, performed in triplicate for each protein, and the SD was determined from the mean.

and *SI Appendix*, Fig. S3*D*). These data show that pVHL prevents the activation of Akt is an indirect mechanism to decrease Mdm2 protein levels.

Decreased Mdm2 by pVHL Requires the Ring Domain. The S166DS186D phospho-mimetic of Mdm2 was protected from pVHL destabilization, which signals for Mdm2 nuclear localization (17, 18). It is unclear how unphosphorylated Mdm2 is degraded. The Ring domain of Mdm2 is associated with its E3 ligase activity, and to determine whether the stability of Mdm2 is dependent on the Ring domain, we transiently expressed C464S Ring mutant of Mdm2 with Ha-CaAkt and with or without Ha-pVHL. pVHL did not affect the C464S Ring domain mutant levels (Fig. 3*E* and *SI Appendix*, Fig. S3*C*). The carboxy terminus and Ring domain are necessary for binding to the UBC5, E2 ubiquitin transferase. We use an ELISA-based approach to assess the binding of Mdm2 to UBC5. Mdmx is a family member of Mdm2 and forms a complex by binding the Ring domain. p53 binds in the hydrophobic amino terminus of Mdm2. We use p53 and Mdmx as binding controls for the assay. Gst was used to control for nonspecific binding. C464S bound to p53, demonstrating that both proteins have an intact amino terminus, whereas the C464S Ring mutant did not bind Mdmx (the positive control for an intact Ring domain) or UBC5 (Fig. 3*F*). These data suggest the association of UBC5 is required for the conjugation of ubiquitin and the subsequent decrease in Mdm2 mediated by the blockade of Akt by pVHL.

VHL-p53-Mdm2 Interplay for Proliferation. Considering that Mapk and Akt are involved in the gene expression and stability of Mdm2, and pVHL can block these pathways, we examined the functional aspect of proliferation. Since the *vhl* gene is a target of mutation in renal carcinoma yet maintains wild-type p53, we examined the proliferation rate in control and pVHL reconstituted RCC4 cells. We observed that RCC4 cells with pVHL had a slower proliferation rate than control (Fig. 4*A*). To confirm these results, we used control and $vhl^{-/-}$ murine embryo fibroblast. The loss of $vhl^{-/-}$ in MEFs was more proliferative than in control cells (Fig. 4*B*) and had active Akt (20) (*SI Appendix*, Fig. S2*B*). To determine whether p53 was involved, we performed a western blot from RCC4 and MEF for p53 and showed that in the absence of pVHL, p53 levels were lower. The downstream target of p53, p21, reflected the changes in p53 as shown by western blot and p21 reporter data (Fig. 4*C*). These data show that pVHL impacts proliferation by regulating the cell cycle checkpoint regulated by p53 (Fig. 4*D*).

Discussion

Cell surface receptors activate a cascade of signaling pathways that bifurcate to reprogram the cellular machinery to induce a specific cellular response. Depending on the activated receptor, some will promote cellular proliferation by activating transcription factors. There are safeguards that temper the signaling cascade through cell cycle checkpoints. Alterations of these checkpoints are evident in mutations to cell surface receptors, downstream signaling kinases, and/or loss of checkpoint regulators such as tumor suppressors. This leads to unregulated progression through the cell cycle and rapid growth. The Mdm2 oncogene alleviates cell cycle checkpoints by regulating tumor suppressors such as p53 (11). While the interplay between Mdm2 and p53 forming an autoregulatory feedback loop in response to genotoxic stress is well established, several papers have demonstrated that Mdm2 gene expression is induced by transcription factors independent of p53.

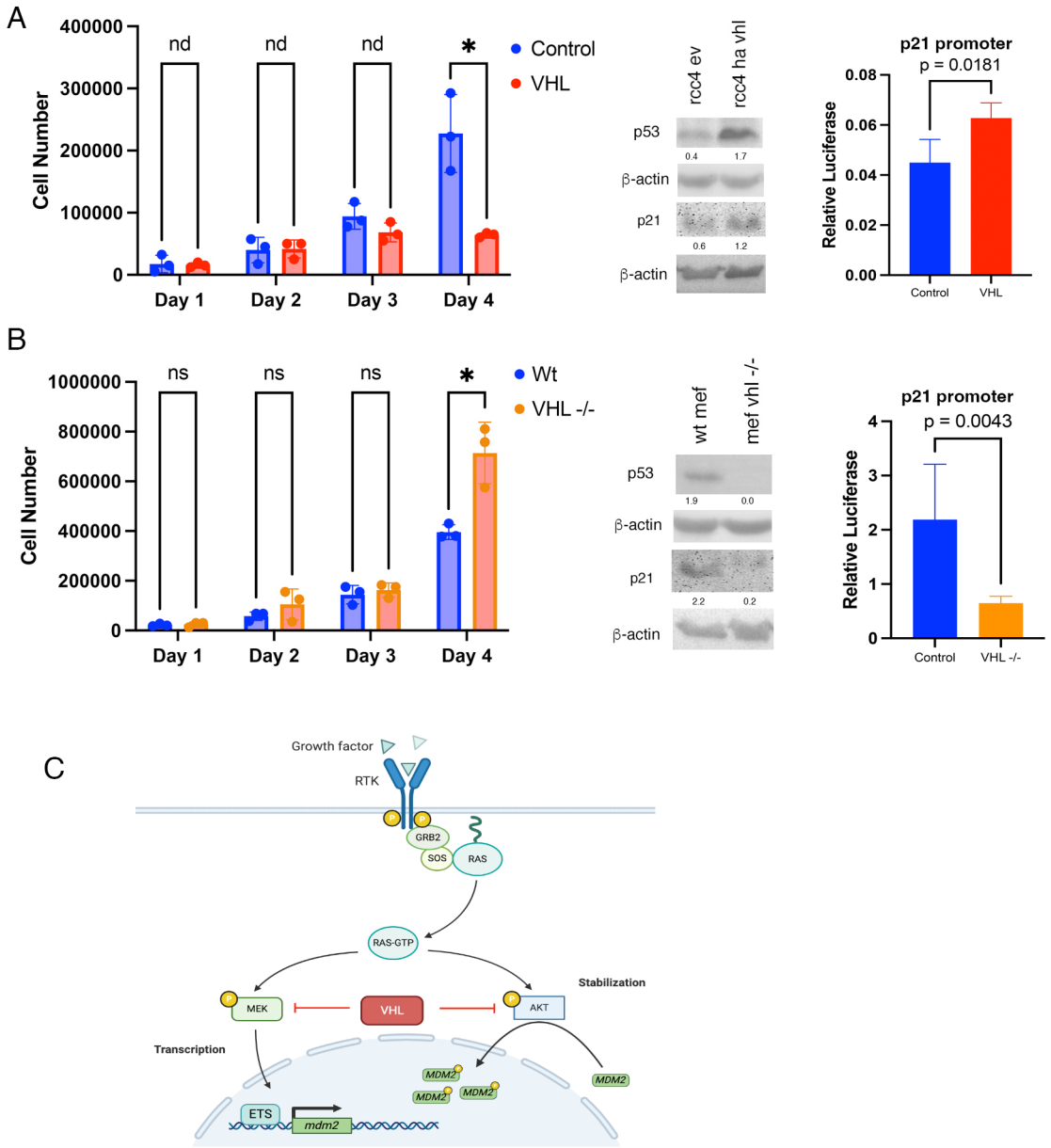

Fig. 4. Regulation of cell growth through the pVHL–p53 pathway. (*A*) RCC4 control and pVHL cell number were determined over 4 d. Western blot of p53, p21, and βActin in control and pVHL RCC4 cells. (*B*) Growth of MEF control or VHL$^{-/-}$ over 4 d. Western blot of p53, p21, and βActin from the extracts and Western blots were quantitated by the intensity of the band relative to βActin. A and B cell numbers were determined from three wells for each cell line and day. The SD was determined from the mean, and Student's *t* test showed significance. A and B graphs represent the p21 promoter linked to luciferase transiently expressed in control or VHL Rcc4 or control or *vhl*$^{-/-}$mef. Transient assays were performed in triplicate, and SD and unpaired *t* tests determined the significance. (*C*) Model of how pVHL regulates two kinase pathways that impact Mdm2 levels.

In this scenario, hyperactivated cell surface receptors or downstream signaling do not utilize the p53/Mdm2 autoregulatory feedback loop.

As expected, kinase signaling pathways must be governed to ensure error-free progression through the cell cycle, and the understanding of how tumor suppressors such as pVHL are involved has been limited until recently. pVHL will repress some of the PKC family members, yet a comprehensive analysis of all the family members has yet to be determined (21). PKC can activate numerous downstream kinase-signaling pathways, including the Mapk signaling pathway. Mapk kinase is a key signaling pathway that can activate numerous transcription factors to increase genes responsible for cell cycle progression (22). One gene induced is the Mdm2 through the activation of the Ets transcription factor (13, 23). Fig. 2 shows that pVHL can repress the Mapk–Ets–Mdm2 pathway, demonstrating an unrealized regulatory pathway whereby pVHL can indirectly influence Mdm2 gene expression. The loss of the P2 promoter showed an increase in the P1 promoter. The P1 promoter creates an isoform missing part of the p53 binding domain. The function of this isoform is not clearly understood but may have a physiological role in response to stimuli-activated pathways that shut down the P2 promoter of Mdm2.

It was recently reported that pVHL could block and inhibit Akt (PKB) (20). We and others have reported that Mdm2 stability is controlled by posttranslation modifications changing the cellular compartment or the E3 ligase activity (18, 24, 25). Mdm2 is shuttled from the cytoplasm to the nucleus through the phosphorylation of serines in the nuclear localization domain by Akt. Blockade of this pathway by the tumor suppressor PTEN, through the dephosphorylation of phosphatidyl-inositol 3,4,5 phosphate, prevents the activation of Akt (18, 26). The loss of activated Akt

renders Mdm2 to the cytoplasm and is unstable. The data in Fig. 3 show that there is yet another level of regulation whereby pVHL can lead to the loss of Mdm2 by repressing Akt activation. By circumventing the pVHL–Akt pathway using the S166DS186D phosphomimetic of Mdm2, we show that Mdm2 is stable with Akt loss mediated by pVHL. Further, disruption of the Ring domain of Mdm2 protected Mdm2 from pVHL-mediated destabilization. These data suggest that the binding of UBC5 E2 for ubiquitin to Mdm2 is necessary to mediate the loss of Mdm2. Considering that ring domains don't have intrinsic ubiquitin ligase activity, this suggests that Mdm2 is not autoubiquitinating activity, but UBC5 is conjugating ubiquitin to Mdm2, signaling for proteasomal degradation.

The perturbations in signaling pathways through loss of pVHL lead to proliferation and other functions in renal cell carcinomas (RCC), and this pathway may be at play in other tumors that exhibit alteration in pVHL. Most RCC tumors maintain inactive but wild-type p53 and we show that p53 can be activated to engage cell cycle checkpoints through p21 expression (Fig. 4*C*). This is not a complete proliferation block but slows the growth rate. One explanation is that cell surface receptors can activate Akt, Mapk, and c-Src upstream of these kinases. Interestingly, we demonstrated that Mdm2 could be converted to a neddylating E3 ligase by recruiting UBC12 in response to c-Src phosphorylating Mdm2 at tyrosines 281 and 302 (16). Converting Mdm2 to a neddylating E3 ligase to conjugated nedd8 to p53 and pVHL, Nedd8-p53 stabilized p53 but was inactive, and conjugation of nedd8 to pVHL prevented the p53–pVHL complex formation and the induction of p53 target gene expression (7, 16). The convergence of several kinase signaling pathways feeding into Mdm2 suggests that a series of feedback loops tightly regulate the Mdm2 gene expression and protein stability to govern cell proliferation. These data show how full-length Mdm2 is regulated by growth factor signaling pathways, and it will be interesting to understand how these same pathways influence the expression and activity of the numerous isoforms of Mdm2 that contribute to the growth and transformation of normal cells (27).

Materials and Methods

Bioinformatics Analysis and Illustrations. TCGA and DepMap were used to investigate gene expression of Mdm2 and pVHL and protein levels when available (28, 29). TCGA gene expression analysis was performed to examine mutants of VHL and *mdm2* gene expression. Statistical analysis was performed from databases and western blots bands were quantitated using ImageJ. All illustrations were generated in Biorender.

Cell Culture. Rcc4, 293T, murine embryo fibroblast cells, and O-786 cells were grown in DMEM or RPMI supplemented with 8% FBS, glutathione, and pen/strep in a humidified incubator at 37 °C with 5% CO_2. As previously reported, hypoxic experiments were conducted at 1% oxygen in a hypoxic chamber (7). Proliferation assays were performed by plating 50,000 cells in a 24-well plate in triplicate. One day after plating, cells were counted at day 0. The experiment was done three times. Phospho-Akt (S473) and phospho-Mapk (T202/Y204/T185/T187) were analyzed using the Muse PI3K/Mapk dual pathway activation kit. Transient transfections were performed using PEI to induce plasmids for analysis by western blots and luciferase (7). As previously described, luciferase assay, replicates, SD, and significance were determined (30).

Western Blot and Analysis of Protein–Protein Interaction. Western blots were conducted using antibodies to Mdm2 (2A10,4B2, 2A9 Calbiochem), β-Actin (AC15), pVHL(VHL40), and HA(F7) from Santa Cruz biotechnology, and HIF2α (264221ap) from Proteintech. For protein–protein interaction analysis, His-p53, GST-VHL, His-Mdm2, Gst-Mdmx, GST-Ubc5, and GST were produced in bacteria. The proteins were purified by Ni-agarose or Glutathione columns using the Akta chromatography system, and all proteins were dialyzed in PBS with 30% glycerol, aliquoted, and stored at −80 °C. Protein interaction was conducted by binding the proteins to a 96-Well plate, blocking with BSA, and adding the C464S mutant. After washing four times in PBS, the 2A10 antibody was incubated, followed by washing and then anti-mouse HRP. After washing, the TMB substrate (Promega) was added; when color developed, it was neutralized and then quantified on a plate reader. All protein interactions were analyzed in triplicate and repeated twice.

Data, Materials, and Software Availability. All study data are included in the article and/or *SI Appendix*.

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