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p53 Stabilization and Transactivation by a von Hippel-Lindau Protein

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Summary

von Hippel-Lindau (VHL) disease is a rare autosomal dominant cancer syndrome. Although hypoxia-inducible factor-α (HIFα) is a well-documented substrate of von Hippel-Lindau tumor suppressor protein (pVHL), it remains unclear whether the dysregulation of HIF is sufficient to account for de novo tumorigenesis in VHL-deleted cells. Here we found that pVHL directly associates with and stabilizes p53 by suppressing Mdm2-mediated ubiquitination and nuclear export of p53. Moreover, upon genotoxic stress, pVHL invoked an interaction between p53 and p300 and the acetylation of p53, which ultimately led to an increase in p53 transcriptional activity and p53-mediated cell cycle arrest and apoptosis. These results suggest that the tumor suppressor pVHL has an unexpected function to upregulate the tumor suppressor p53.

Introduction

Inactivation of the von Hippel-Lindau (VHL) tumor suppressor gene is linked to hereditary cancer syndromes including renal cell carcinoma (RCC) (Kaelin, 2002; Ratcliffe, 2003; Barry and Krek, 2004). pVHL, a product of *VHL* gene, which resembles SCF complex in yeast, forms a multimeric complex containing elongin C, elongin B, Cul2, and Rbx1 that degrades target proteins (Stebbins et al., 1999; Ivan et al., 2001). pVHL has two domains, the α domain that recruits elongin C/elongin B/Cul2/Rbx1 complex, and the β domain that interacts with the hydroxylated oxygen-dependent degradation (ODD) domain of hypoxia-inducible factor-1α (HIF-1α) (Ivan et al., 2001). The oxygen-sensing HIF-prolyl-hydroxylases hydroxylate the proline residue at the conserved sequence (LXXLAP) within an ODD domain of HIF-1α, leading to its association with pVHL and the subsequent ubiquitination and rapid degradation by the 26S proteosomes (Maxwell et al., 1999; Semenza, 2001; Kaelin, 2005). Under hypoxia or in the absence of VHL, the α subunit of HIF translocates into the nucleus and forms a heterodimer with the β subunit, which binds to HIF responsive element (HRE) and transactivates target genes for energy metabolism, erythropoiesis, and angiogenesis.

Although HIF obviously enhances the blood vessel formation around tumors (Semenza, 2003), there is evidence that the dysregulation of HIF is insufficient to account for de novo tumorigenesis in VHL-deleted cells (Kondo et al., 2002; Maranchie et al., 2002; Mack et al., 2003; Zimmer et al., 2004), suggesting that pVHL may have HIF-independent tumor suppressor functions.

RCC is the predominant form of kidney cancer, and approximately 80% of sporadic RCC are due to the biallelic inactivation of the VHL gene (Kaelin, 2002; Ratcliffe, 2003; Barry and Krek, 2004). In fact, the inactivation of pVHL has been linked to the dysregulation of proteins related to the cell cycle and apoptosis, some of which are known p53 target genes (Pause et al., 1998; Schoenfeld et al., 2000; Bindra et al., 2002; Kim et al., 2004). Moreover, a number of RCC cell lines, including A498, have been reported to express a wild-type but inactive p53, implying that p53 in RCC is repressed (Gurova et al., 2004). Intriguingly, RCC is characterized by high resistance to radiation and chemotherapy, due in part to its lacking an apoptotic suppressing mechanism, such as the p53 tumor suppressor pathway (Lowe, 1995; Kawasaki et al., 1999). Therefore, it has been suggested that the potential tumor suppressive function of pVHL is related to the regulation of p53. However, little has been known about the precise mechanism by which pVHL regulates p53.

In this study, we elucidated the mechanism whereby pVHL stabilizes and enhances p53 transcriptional activity. p53 was found to specifically bind to the α domain of pVHL where elongin C binds. pVHL blocked the Mdm2-mediated ubiquitination and nuclear export of p53. Moreover, pVHL in cooperation with a serine kinase and acetyltransferases stabilizes p53 and enhances its tranacriptional activity, thus inducing p53 target genes and ultimately triggering p53-mediated cell cycle arrest and apoptosis. Moreover, we showed how pVHL regulates p53 during DNA damage. Together, our findings extend the tumor-suppressive function of pVHL to include the regulation of p53 in RCC tumorigenesis.

Results

pVHL Directly Associates with p53

To determine whether pVHL regulates the function of p53, we first investigated the association between p53 and pVHL by transient transfection of HEK293 cells with mammalian expression vectors for HA-p53 and Flag-pVHL. HA-p53 and Flag-pVHL were found to form a complex by coimmunoprecipitation (Figure 1A). We also confirmed their interaction by transient transfection of HEK293 cells with mammalian GST-p53 (mGST-p53) and HA-pVHL, followed by affinity precipitation of glutathione-Sepharose or immunoprecipitation of anti-HA antibody (see Figure S1 in the Supplemental Data available with this article online). When we tested whether recombinant GST-pVHL binds to ^{35}[S]-labeled p53 produced by in vitro translation, we also found that

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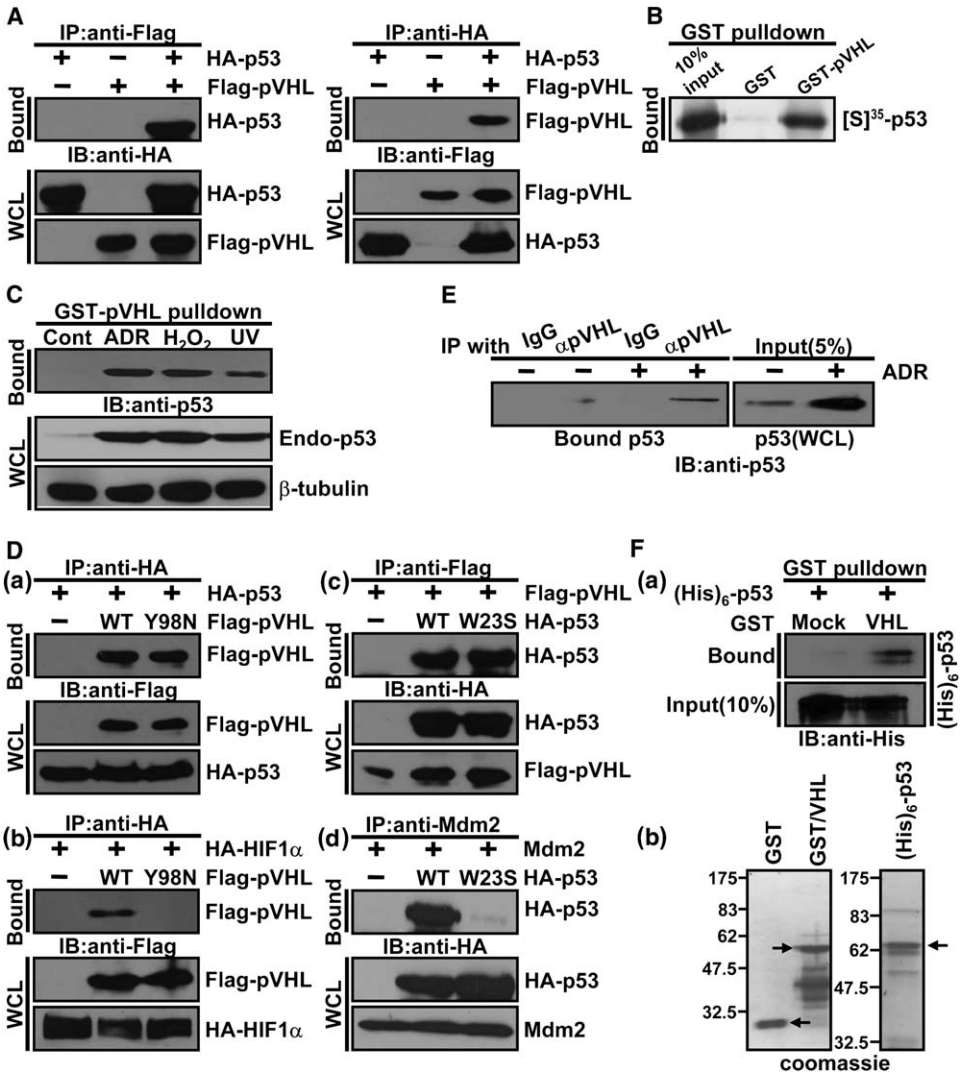

Figure 1. pVHL Directly Binds to p53

(A) pVHL associates with p53 in vivo. HEK293 cells were transiently transfected with the mammalian expression vectors Flag-pVHL (pcDNA3-Flag-VHL) and/or HA-p53 (pcDNA3-HA-p53). Cell lysates were immunoprecipitated with anti-Flag (left) or anti-HA antibody (right) and immunoblotted with anti-HA or anti-Flag antibody, respectively.

(B) Bacterially purified GST-pVHL was incubated with ^{35}[S] -p53 in vitro transcription/translation product in the presence of glutathione-Sepharose. Washed precipitates were subjected to SDS-PAGE, and ^{35}[S]-p53 was detected by autoradiography.

(C) Lysates from MCF7 cells treated with different DNA damaging agents (adriamycin [ADR], 0.2 μg/ml; UV, 25 J/m^2; H_2O_2, 1 mM) were subjected to GST-pVHL pull-down followed by immunoblotting with anti-p53 (DO-1) antibody.

(D) p53-pVHL binding does not require their cognate partners, Mdm2 and HIF-1α. Flag-pVHL (wt) or Flag-pVHL (Y98N) mutant was transfected into HEK293 cells with (Da) HA-p53 or (Db) HA-HIF-1α. In both cases, cell lysates were immunoprecipitated with anti-HA antibody and then probed with anti-Flag antibody. Conversely, HA-p53 (wt) or HA-p53 (W23S) mutant was transfected with (Dc) Flag-pVHL or (Dd) Mdm2. Cell lysates were immunoprecipitated with (Dc) anti-Flag antibody or (Dd) anti-Mdm2 and then probed with anti-HA antibody.

(E) An endogenous interaction between pVHL and p53. MCF7 cell lysates (adriamycin, 0.4 μg/ml) were immunoprecipitated with immobilized anti-pVHL-protein G beads and immunoblotted with anti-p53 (#9282) polyclonal antibody.

(F) A direct interaction between recombinant GST-pVHL and $(His)_6$-p53 proteins. Purified proteins were incubated for 2 hr and pulled down with glutathione-Sepharose. Precipitates were probed with anti-His antibody. Protein purities were confirmed by Coomassie blue staining.

^{35}[S]-p53 could be pulled down along with GST-pVHL (Figure 1B). Moreover, GST-pVHL was found to associate with endogenous p53 in MCF7 cell lysates treated with different genotoxic reagents (Figure 1C). HIF-1α is known to stabilize p53 through Mdm2-bridged interaction (Chen et al., 2003). To exclude the possibility that the pVHL-p53 interaction might be mediated by either HIF-1α or Mdm2, we tested the interaction between p53 and the Y98N mutant of pVHL, which is unable to interact with HIF-1α (Figure 1Db). We found that p53 still bound to the pVHL (Y98N) mutant (Figure 1Da). Conversely, when we examined the interaction between pVHL and p53 (W23S) mutant, which is unable to interact with Mdm2 (Figure 1Dd), we observed that pVHL bound to the p53 (W23S) mutant (Figure 1Dc). We next confirmed the interaction between endogenous pVHL and p53. To avoid the overlap of endogenous p53 with the IgG heavy chain on immunoblot, we chemically

crosslinked pVHL antibody with protein G beads and then immunoprecipitated endogenous pVHL from MCF7 cell lysates treated with/without adriamycin. We observed that immunoprecipitates with anti-pVHL antibody contained endogenous p53 (Figure 1E). To verify that the interaction between pVHL and p53 is direct, we incubated bacterially purified GST-pVHL and $(His)_6$-p53 and pulled down GST-pVHL with glutathione-Sepharose. When the precipitates were probed with anti-His antibody, $(His)_6$-p53 was detected, suggesting a direct interaction between recombinant GST-pVHL and $(His)_6$-p53 (Figure 1F).

p53 Binds to the α Domain of pVHL and Competes with Elongin C

We then generated GST-fused recombinant full-length, α domain (amino acids 155–213), or β domain (amino acids 1–154) of pVHL and incubated these recombinant truncated pVHL mutants with lysates prepared from cells overexpressing HA-p53. It has been shown that the β domain of pVHL is specifically associated with HIF-1α (Stebbins et al., 1999; Ohh et al., 2000). On the other hand, the α domain of pVHL had an ability to associate with p53 (Figure 2A), which suggests that pVHL directly associates with p53 in a HIF-1α-independent manner (Figure 1D). To further narrow down the binding region of p53 on pVHL, we generated pVHL truncated mutants by deleting every ten amino acids from C terminus and then examining the interaction of these mutants with mGST-p53 (Figure 2B). Most of the truncated pVHL mutants, including pVHL (1–164), associated with mGST-p53. However, the truncated pVHL (1–154) mutant lost the ability to interact with mGST-p53, indicating that the ten amino acids (154–163) are important for the p53-pVHL interaction. However, this ten amino acid fragment alone was insufficient for pVHL binding to p53 (Figure S2). It is noteworthy that this ten amino acid region overlaps with the established elongin C binding region (Stebbins et al., 1999). Thus, we speculate that p53 competes with elongin C for pVHL binding. As expected, when elongin C was transiently overexpressed, it blocked the pVHL-p53 interaction in a dose-dependent manner (Figure 2C).

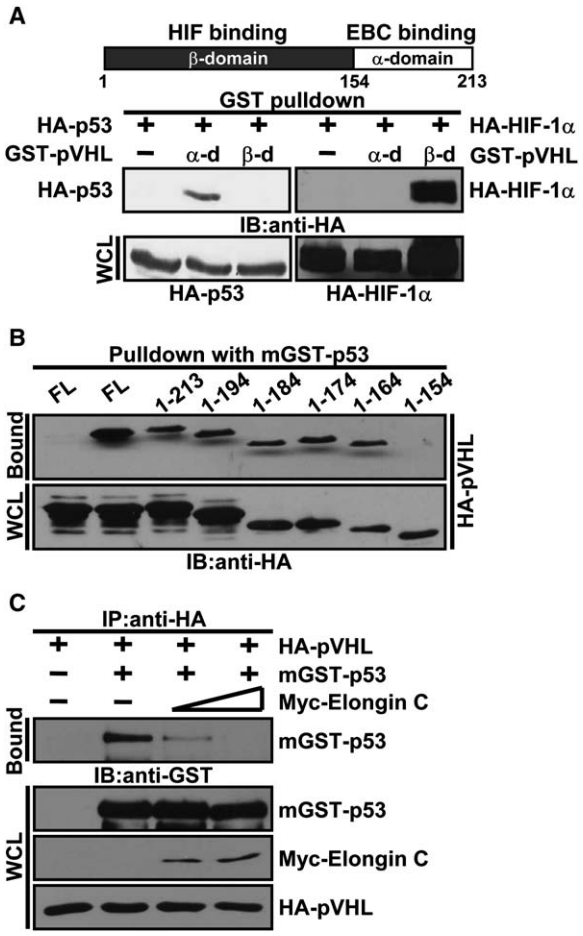

Figure 2. pVHL Binds to the α Domain of pVHL and Competes with Elongin C

(A) p53 binds to the α domain of VHL. The GST-α domain of pVHL (155–213) or the GST-β domain of pVHL (1–154) was incubated with p53-overexpressing cell lysates and precipitated with glutathione-Sepharose.

(B) p53 binds to the amino acids of 154–163 on the α domain of pVHL. HEK293 cells were transiently transfected with mammalian expression vector mGST-p53 along with serial deletion mutants of HA-tagged pVHL. Cell lysates were precipitated with glutathione-Sepharose and probed with anti-HA antibody.

(C) p53 competes with elongin C for binding to pVHL. HEK293 cells were transfected with HA-pVHL and mGST-p53 in the increasing amount of myc-elongin C. Cell lysates were immunoprecipitated with anti-HA, and bound p53 was detected with anti-GST antibody.

pVHL Blocks the Mdm2-Mediated Ubiquitination and Nuclear Export of p53

Since p53 binds to the α domain of pVHL and competes with elongin C, it is unlikely that p53 is degraded by pVHL-elongin B/C complex. To examine the effect of pVHL on the stability of p53, we transfected H1299 cells with increasing amounts of pVHL along with $(Myc)_6$-p53. After normalization of p53 in cell lysates, we detected the Ser-15 phosphorylation of p53 in pVHL-transfected cells (Figure 3A). Based on the previous report that ATM is a well-known kinase to phosphorylate Ser-15 in p53 (Banin et al., 1998; Canman et al., 1998), we examined whether pVHL associates with ATM. When HEK293 cells were transfected with expression plasmids encoding Flag-ATM and/or HA-pVHL, pVHL was found to interact with ATM by coimmunoprecipitation (Figure 3B, left). Moreover, pVHL could interact with ATM in p53-deficient H1299 cells (Figure 3B, right), indicating that pVHL interacts with ATM independent of p53. To investigate whether pVHL affects the stability of p53, we transiently transfected H1299 cells with different combinations of mammalian expression vectors for p53, ubiquitin, Mdm2, and/or pVHL (Figure 3C). After 24 hr of incubation, we pretreated cells with 10 μM of MG132 for 16 hr, immunoprecipitated cell lysates with anti-p53 antibody, and probed samples with anti-ubiquitin antibody. The coexpression of ubiquitin and Mdm2 triggered significant p53-ubiquitination, whereas increasing amounts of pVHL (wt) significantly reduced the ubiquitination. Moreover, pVHL (Y98N) mutant also reduced the ubiquitination of p53, indicating that pVHL is capable of stabilizing p53 by blocking the Mdm2-mediated ubiquitination of p53 in a HIF-independent manner. To examine whether pVHL affects p53 ubiquitination at an endogenous level, HCT116 ($p53^{+/+}$) cells

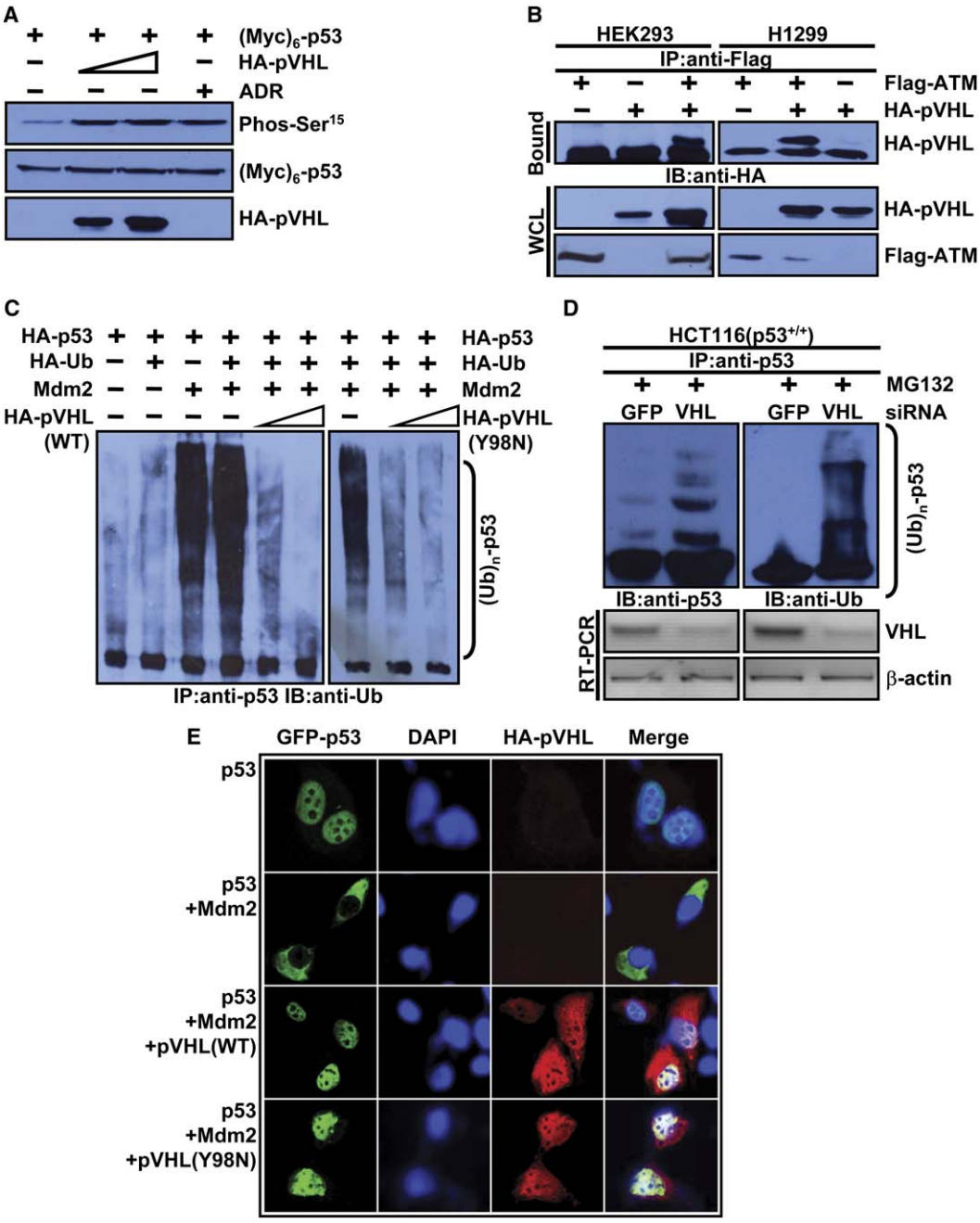

Figure 3. pVHL Associates with ATM and Blocks the Mdm2-Mediated Ubiquitination and Nuclear Export of p53

(A) pVHL enhances the phosphorylation of Ser-15 in p53. H1299 cells were transfected with $(Myc)_6$-p53 in an increasing amount of pVHL. As a control, cells were treated with adriamycin (0.4 μg/ml) for 6 hr. After normalizing the cellular amounts of p53 using immunoblotting of $(Myc)_6$-p53, normalized cell lysates were immunoblotted with anti-phospho-p53 (S15) antibody. For the detection of pVHL, the same amounts of cell lysates (50 μg) were used and probed with anti-HA antibody.

(B) pVHL associates with ATM in the presence or absence of p53. HEK293 or H1299 cells were transiently transfected with HA-pVHL (pCR3-HA-pVHL, 0.5 μg) and/or Flag-ATM (pcDNA3-Flag-ATM, 1.5 μg). Cell lysates were immunoprecipitated with anti-flag (M2) antibody and probed with anti-HA antibody.

(C) pVHL blocks the Mdm2-mediated ubiquitination of p53. H1299 cells were transiently transfected with different combinations of expression vectors of p53, Mdm2, pVHL, and/or ubiquitin. After 24 hr of incubation, cells were pretreated with MG132 (10 μM) for a further 16 hr. Cells lysates were immunoprecipitated with anti-p53 antibody (DO-1) and probed with anti-ubiquitin antibody.

(D) The knockdown of pVHL increases p53 ubiquitination at an endogenous level. HCT116 ($p53^{+/+}$) cells were transfected with siVHL (100 pmol) for 48 hr. As control, the same amount of siGFP was treated. Si-VHL-treated cells were further incubated with 20 μM of MG132 for 6 hr. siVHL-treated HCT116 ($p53^{+/+}$) cell lysates were immunoprecipitated with anti-p53 (#9282) polyclonal antibody and then immunoblotted with either anti-p53 (DO-1) antibody or anti-ubiquitin (P401) antibody. The mRNA levels of VHL and β actin were detected using RT-PCR.

(E) pVHL blocks the Mdm2-mediated nuclear export of p53. H1299 cells were transfected with different combinations of expression vectors with GFP-p53, pVHL, and Mdm2. Expression of pVHL was stained with anti-HA monoclonal antibody and Rhodamine Red-X-conjugated anti-mouse antibody.

were treated with siVHL for 48 hr and followed by the treatment of MG132 (20 μM) for 6 hr. Cell lysates were subjected to immunoprecipitation with anti-p53 antibody and probed with either anti-p53 antibody or anti-ubiquitin antibody. The treatment of siVHL significantly reduced the pVHL expression and increased the p53-ubiquitination (Figure 3D), which indicates that pVHL contributes to the stabilization of endogenous p53.

We then examined the possibility that pVHL blocks the nuclear export of p53 by Mdm2. When we transfected H1299 cells with GFP-p53, Mdm2, and/or pVHL, we observed that GFP-p53 was entirely located in nucleus when expressed alone, but translocated into the cytoplasm when coexpressed with Mdm2 (Figure 3E). As expected, GFP-p53 redistributed to the nucleus when pVHL was expressed with Mdm2, indicating that pVHL blocks the Mdm2-mediated nuclear export of p53.

From the above results, the pVHL has the potential to stabilize p53 at the protein level. To address this possibility, we transiently transfected p53-deficient H1299 cells with the different ratios of mammalian expression vectors for HA-p53 and HA-pVHL. When excess amounts of both p53 and pVHL were ectopically expressed, p53 was stabilized regardless of the pVHL coexpressed (Figure S3, lanes 5 and 6). However, when relatively low amount of p53 was expressed in the presence of high amount of pVHL, p53 was significantly stabilized by pVHL (Figure S3, lanes 3 and 4).

pVHL Stabilizes and Transactivates p53 through Lysine Acetylation

We next determined whether pVHL affected the transactivation of p53. pVHL increased stabilization and transcriptional activity of both transfected and endogenous p53 in H1299 and MCF7 cells, respectively (Figures 4A and 4B). It has been reported that Noxa is a well-known downstream target gene of p53 (Oda et al., 2000). Therefore, we tested whether pVHL has an effect on p53-mediated transcriptional regulation by transiently expressing different combinations of pVHL and p53 together with the wild-type Noxa luciferase or two different Noxa mutant luciferase reporter genes (Noxa-Δluc and Noxa-mt-luc). As expected, pVHL synergistically increased p53-mediated transcriptional activation of the Noxa-luc reporter gene but had no effect on the two Noxa mutant reporters (Figure 4C).

It has been reported that p53 transactivation is regulated in part by the acetylation of lysine residues in the COOH-terminus of p53 (Gu and Roeder, 1997) and that members of the ING tumor suppressor family regulate p53 transcriptional activity via its acetylation (Garkavtsev et al., 1998; Nagashima et al., 2001). We speculated that pVHL, like ING proteins, might mediate p53 acetylation. When pVHL was coexpressed with p53 in H1299 cells, p53 was found to be significantly acetylated at lysine residues (K373, K382) in cell lysates (Figure 5A), indicating that pVHL can mediate the acetylation of p53. As the acetyltransferase coactivators (p300 and pCAF) bind to the NH_2-terminal region of p53 and catalyze the acetylation of the COOH-terminal region of p53 (Barlev et al., 2001), we examined the effect of pVHL on the p53-p300 or p53-pCAF interaction. An ectopically expressed pVHL was found to strengthen these interactions (Figures 5B and 5C). In addition, upon treatment

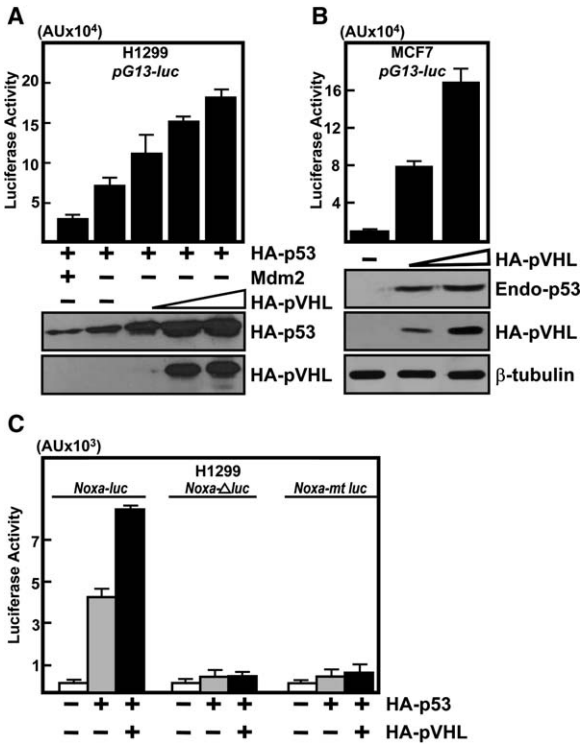

Figure 4. pVHL Stabilizes and Transactivates p53

(A and B) A gradual increase in pVHL augments the transcriptional activation of both transfected and endogenous p53. In (A), H1299 cells were transfected with pcDNA-HA-p53 along with increasing amount of pCR3-HA-VHL. In (B), MCF7 cells were transfected with increasing amount of pCR3-HA-VHL. For detecting p53 transcriptional activity, pG13-luciferase reporter genes were transfected in both (A) and (B). Luciferase activities were measured and normalized with protein contents. In both (A) and (B), means ± standard deviations for n = 3 are shown. Immunoblots represent one of three independent experiments.

(C) pVHL synergistically increases p53-mediated Noxa-luciferase activity. H1299 cells were transfected with different combinations of p53 and pVHL under the wild-type of Noxa-luc or two mutants of Noxa-luc (Noxa-Δluc and Noxa-mt-luc). Luciferase activities were measured and normalized with protein contents. Means ± SD for n = 3 are shown.

with adriamycin, endogenous p53-p300 interaction appeared to be stronger in pVHL-expressing A498/VHL stable cells than in the pVHL-deficient A498 RCC (Figure 5D). To understand the pVHL-mediated enhancement in the interaction between p53 and acetyltransferases, we investigated whether pVHL associates with p300 or pCAF in the presence or absence of p53 (Figures 5E and 5F). pVHL was found to be associated with either p300 or pCAF even in p53-deficient HCT116 or H1299 cells, indicating that pVHL has its own ability to recruit acetyltransferases without p53. This finding suggests that pVHL acts as a cofactor of p53 by recruiting acetyltransferases and mediating its acetylation.

Since pVHL can stabilize p53 by recruiting ATM and acetyltransferases, we examined the effect of pVHL on the stabilization and transactivation of two p53 mutants, a p53 (S15A) and p53 (5KR, K320R/K370R/K372R/K373R/K382R). When p53 (5KR) mutant was expressed along with or without pVHL in H1299 cells, the accumulation of p53 (5KR) was saturated, even in the

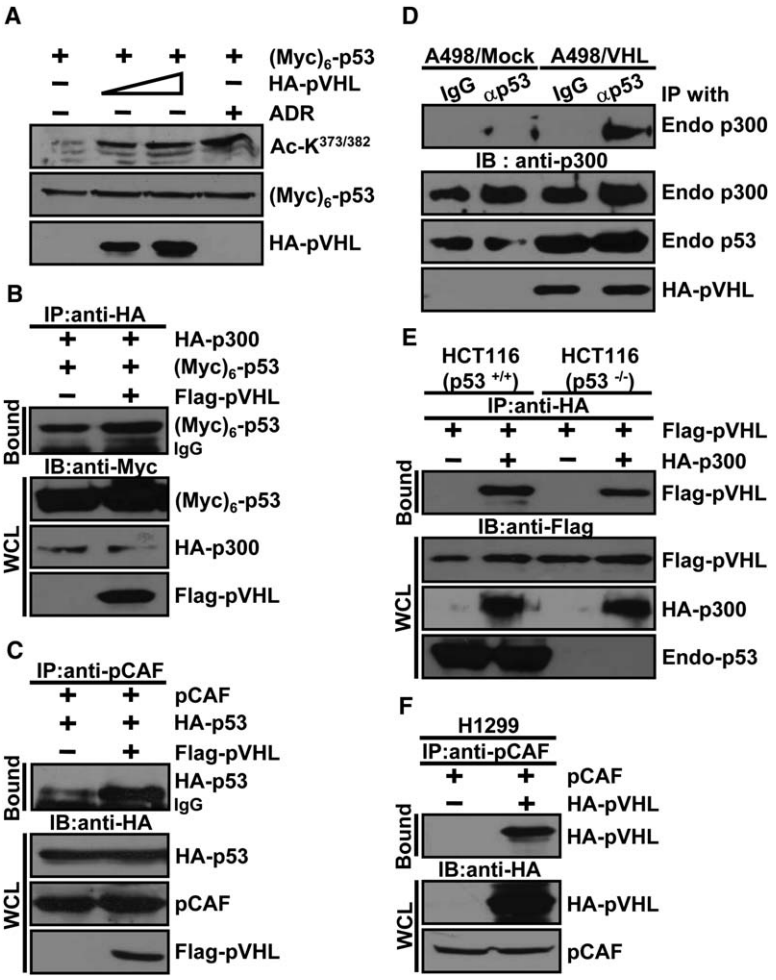

Figure 5. pVHL Acetylates and Stabilizes p53 in Cooperation with Acetyltransferases

(A) pVHL specifically increases the acetylation of p53. H1299 cells were transiently transfected with p53 along with pVHL. As a control, cells were treated with adriamycin (0.4 μg/ml) for 6 hr. After normalizing the cellular amounts of p53 using immunoblotting of $(Myc)_6$-p53, normalized cell lysates were immunoblotted with anti-acetyl-p53 (K373/382) antibody. For the detection of pVHL, the same amounts of cell lysates (50 μg) were used and probed with anti-HA antibody.

(B) pVHL augments the p53-p300 interaction. H1299 cells were transfected with different combinations of excess amount of mammalian expression vectors (each 1 μg) for HA-p300, $(Myc)_6$-p53 in the absence or presence of Flag-pVHL. Cell lysates were immunoprecipitated with anti-HA antibody, and precipitated p53 was detected with anti-myc antibody.

(C) pVHL augments the p53-pCAF interaction. H1299 cells were transfected with excess amount of mammalian expression vectors (each 1 μg) for pCAF and HA-p53 in the absence or presence of Flag-pVHL. As in (B), cell lysates were immunoprecipitated with anti-pCAF antibody, and precipitated p53 was detected with anti-HA antibody.

(D) Reintroduction of VHL into A498/Mock RCC cells strengthens the endogenous p53-p300 interaction. Both A498/Mock and A98/VHL were treated with adriamycin (0.4 μg/ml) for 16 hr. Immunoprecipitation was performed with anti-p53 (#9282) antibody and immunoblotting with anti-p300 antibody.

(E) pVHL interacts with p300 in the absence of p53. Either HCT116 ($p53^{+/+}$) or HCT116 ($p53^{-/-}$) cells were transfected with HA-p300 (pCMV-HA-p300, 1 μg) with or without Flag-pVHL (pcDNA3-Flag-pVHL, 1 μg). Cell lysates were immunoprecipitated with anti-HA antibody and probed with anti-Flag antibody.

(F) pVHL interacts with pCAF in the absence of p53. H1299 cells were transfected with pCAF along with or without HA-pVHL. Cell lysates were immunoprecipitated with anti-pCAF antibody and probed with anti-HA antibody.

absence of pVHL (Figure S4). Similarly, pVHL had no significant effect on the transactivation of p53 (5KR) (Figure S4). It was known that the acetylation of six lysines (K370, K372, K373, K381, and K382 for p300/CBP, K320 for pCAF) in p53 is important for p53 stabilization (Liu et al., 1999; Brooks and Gu, 2003). In this study, the mutations of five lysine residues in p53 (K320, K370, K372, K373, and K382) led to significant accumulation of p53. However, the p53 (S15A) mutant was still stabilized and transactivated by pVHL, implying that pVHL may have potential for blocking different types of ubiquitin E3 ligases involved in p53 degradation. Besides Mdm2, other ubiquitin E3 ligases—COP1 and Pirh2—have been reported to regulate p53 degradation in an Mdm2-independent manner (Leng et al., 2003; Dornan et al., 2004). pVHL binding region on p53 was identified to be the central DNA binding domain (DBD) (aa 160–290) (Figure S5). Pirh2, especially, was reported to bind to the central region of p53, which appears to overlap pVHL binding region (Leng et al., 2003). Thus, it will be interesting to determine whether pVHL competes with Pirh2 for regulating the stability of p53.

pVHL Is Involved in p53-Mediated Cell Cycle Arrest and Apoptosis upon DNA Damage

To examine whether pVHL induces p53-targeted downstream genes at the transcriptional level, we first transiently transfected MCF7 cells with different combinations of p53, pVHL, and/or p300 along with p21 promoter- or Bax promoter-driven luciferase reporter gene (Figure 6). As expected, pVHL enhanced the transcriptional activity of p53 in both p21 and Bax promoters (Figures 6A and 6C). Moreover, pVHL, in cooperation with p300, further increased p53-mediated transcriptional activity (Figures 6B and 6D).

We next examined the effect of pVHL on p53 transactivation. When pVHL was knocked down by the treatment of siVHL in both MCF7 and HCT116 ($p53^{+/+}$) cells, both stabilization of p53 and induction of its target genes (p21, Bax) were reduced upon adriamycin treatment (Figure 7A), indicating that pVHL is involved in the stabilization of endogenous p53. We then investigated the pVHL-mediated stabilization of p53 and induction of p53-targeting genes in A498/Mock cells and A498/VHL stable cells (Figure 7B). Upon adriamycin treatment, p53 was found to be significantly more stable

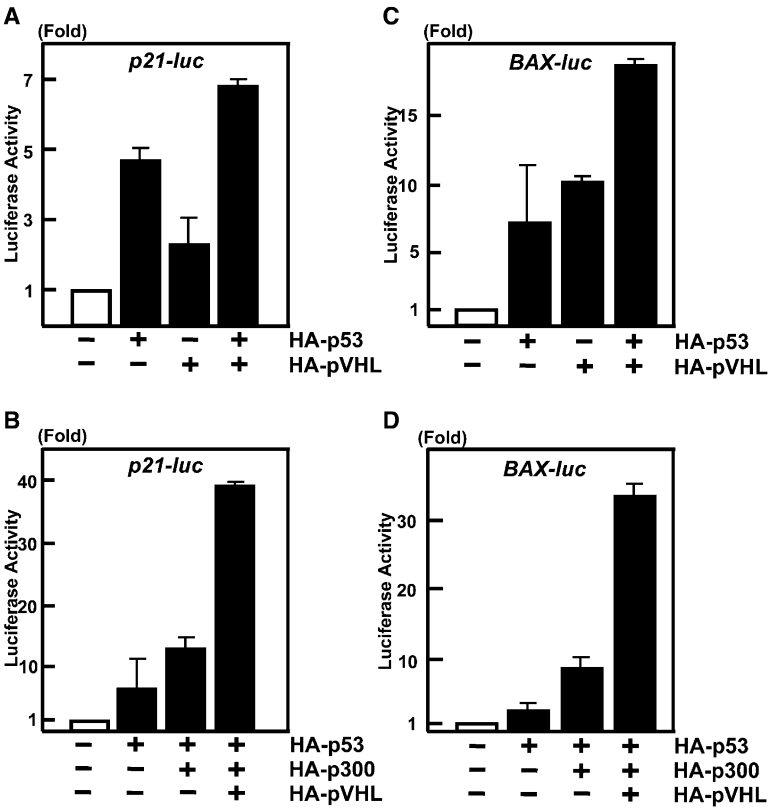

Figure 6. pVHL Synergistically Enhances p53 Transcriptional Activity along with p300

MCF7 cells were transiently transfected with different combinations of expression vectors for p53, pVHL, and or p300 along with (A and B) p21-promoter- or (C and D) Bax-promoter-driven luciferase reporter gene. Luciferase activities were normalized with protein contents. In (A)–(D), means ± standard deviations for n = 3 are shown.

in A498/VHL than in A498/Mock cells. Moreover, p21 and Bax were also significantly induced in parallel with p53 accumulation (Figure 7B).

To examine whether pVHL is involved in p53-mediated G1 arrest, we synchronized both A498/Mock and A498/VHL cells at G0 state by serum starvation and stimulated both cells to reenter the cell cycle by adding serum for 12 hr, followed by exposure of cells to adriamycin for various times (Figure 7C). Serum starvation prior to adriamycin exposure avoids arrest responses in multiple phases of the cell cycle, some of which are p53 indepenent (Attardi et al., 2000). Interestingly, A498/Mock cells failed to arrest at G1 phase, even in the presence of adriamycin, while most A498/VHL cells were significantly arrested at the G1 phase (Figure 7C).

We also investigated the effect of pVHL on p53-mediated apoptosis upon adriamycin treatment. We treated both unsynchronized A498/Mock and A498/VHL cells with adriamycin and analyzed the sub-G1 cell population at different times by FACS. As expected, the A498/VHL cells were more sensitive to adriamycin-induced apoptosis than A498/Mock cells (Figure 7D), which was consistent with previous data that pVHL increased p53-downstream genes involved in apoptosis, such as Bax and Noxa (Figures 4C, 6C, 6D, 7A, and 7B).

Discussion

In this study, we demonstrate that pVHL enhances p53 stabilization and transactivation. The significance of this finding lies at the newly defined function of pVHL in the activation of p53, especially through its phosphorylation and acetylation upon DNA damage.

It is known that p53 can be stabilized in response to DNA damage through two modifications: the phosphorylation at the N terminus and acetylation at the C terminus (Brooks and Gu, 2003). We found that pVHL associates with p53 through its α domain where elongin C binds. The β domain, a substrate-recognition domain, has been emphasized for tumor formation (Maranchie et al., 2002). However, it was recently reported that pVHL containing β domain mutations that disrupt pVHL/HIF-α interaction does not suppress tumor formation (Bonicalzi et al., 2001), indicating that α domain is also critical for the tumorigenesis of VHL-mutated RCC cells. Thus, p53 binding to the α domain of pVHL raises the possibility that p53 could be dysregulated in VHL-deficient RCC cells. In addition, pVHL was found to associate with ATM and to increase the Ser-15 phosphorylation of p53, which was further confirmed by the finding that pVHL blocks the Mdm2-mediated degradation and nuclear export of p53. pVHL was found to bind to the DBD of p53 (Figure S5). Based on the fact that Mdm2 binds to the NH_2-terminus of p53, pVHL is unlikely to directly block the Mdm2-p53 interaction. Instead, pVHL is likely to indirectly block Mdm2-mediated degradation of p53 by recruiting ATM and mediating ATM-dependent Ser-15 phosphorylation of p53. Even though p53 has been known to directly associate with ATM (Khanna et al., 1998), the finding that pVHL itself has an ability to associate with both ATM and p53 indicates that pVHL probably functions to enhance the ATM-p53 connection. Based on the observation that p53 stabilization is drastically reduced in VHL-deficient RCC cells in response to genotoxic stress (Figure 7B), we suggest that pVHL serves as an important

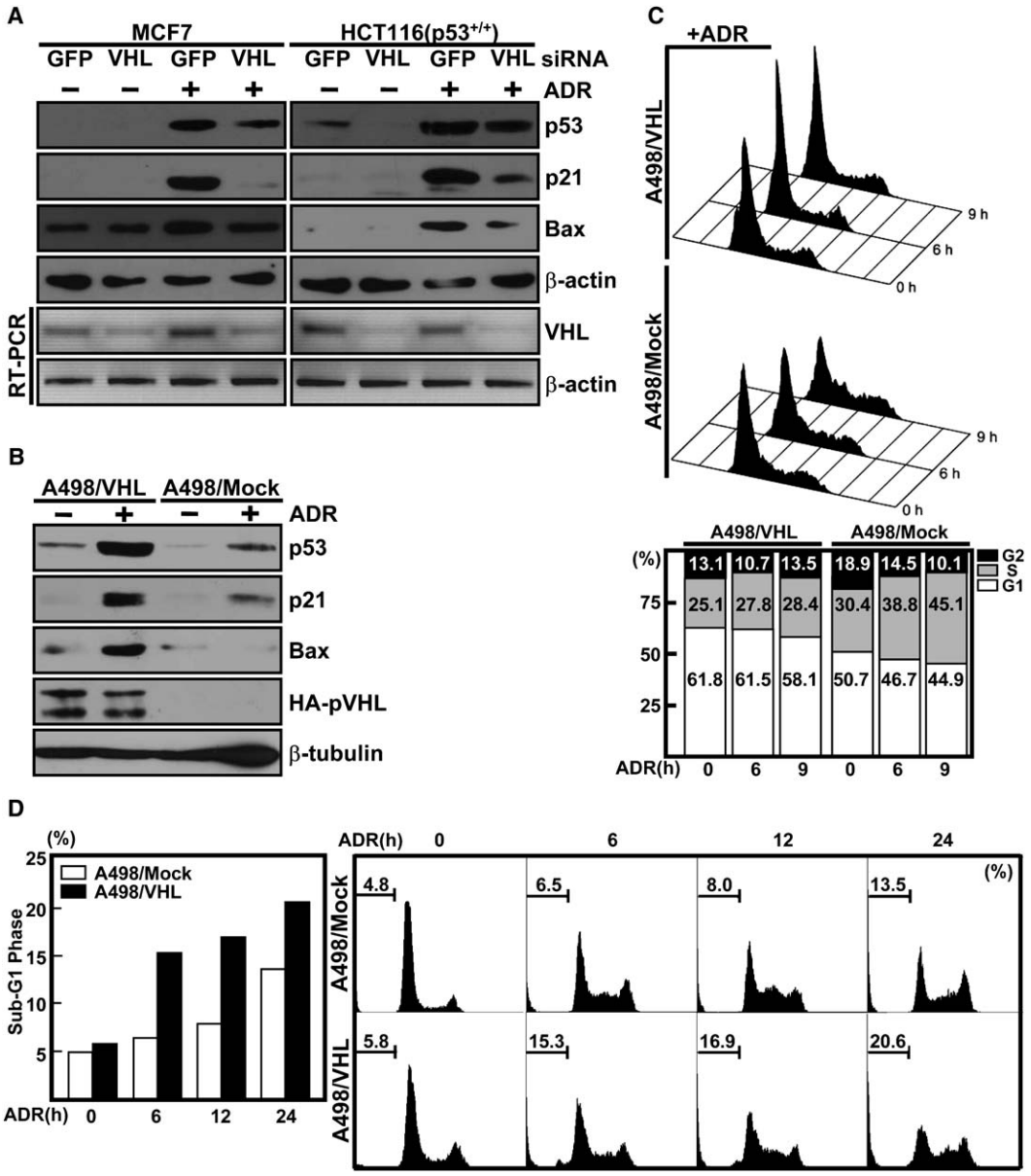

Figure 7. pVHL Affects p53-Mediated Cell Cycle Arrest and Apoptosis following DNA Damage

(A) The treatment of siVHL reduces the stabilization and transactivation of p53 under the DNA damage condition. Both MCF7 and HCT116 ($p53^{+/+}$) cells were treated with siVHL (100 pmol, 36 hr) and followed by the treatment of adriamycin (0.4 μg/ml, 16 hr). Harvested cells were separated into two parts. The one part was used for the detection of protein expressions. Cell lysates (50 μg) were loaded into 12% SDS-PAGE and probed with anti-p53 (DO-1), anti-p21, anti-Bax, and anti-β actin antibodies. The other part of cells was used for detection of mRNA level of VHL and β actin.

(B) pVHL is involved in the stabilization of p53 and in the upregulation of its downstream genes. Both A498/Mock and A498/VHL were treated with adriamycin (0.4 μg/ml) for 12 hr. The expression of proteins was immunoblotted with anti-p53, anti-p21, and anti-Bax antibodies. pVHL expression was detected with anti-HA antibody. β-tubulin expression was used as a control.

(C) pVHL exerts its effect on p53-mediated G1 arrest following DNA damage. The detailed information is described in Experimental Procedures.

(D) Reintroduction of pVHL in RCC cells accelerates the adriamycin-induced apoptosis. Both unsynchronized A498/Mock and A498/VHL were treated with adriamycin (0.4 μg/ml) and harvested at different time intervals. Cells were stained with PI, and apoptotic rate was detected by measuring the population of sub-G1. In (C) and (D), each figure is a representative of three independent experiments.

component for the formation of ATM-p53 complex. Furthermore, we also found that pVHL enhances the lysine acetylation of p53. In addition to pVHL-ATM interaction, pVHL also associates with p300 in a p53-independent manner. Taken together, we suggest that pVHL serves as a cofactor for strengthening the formation of ATM-p53-p300 complex under DNA-damaged conditions.

A number of proteins have been reported to affect the stability and transactivation of p53. However, each protein has a different mechanism for activating p53 function. HAUSP deubiquitinizes p53 and competes with Mdm2-mediated ubiquitination (Li et al., 2002). ATF3 directly binds to the COOH-terminal region of p53 and physically blocks the ubiquitination of lysine residues

(Yan et al., 2005). Pin1 binds to the p(S/T)-P domain and induces the conformational change of p53 (Zacchi et al., 2002; Zheng et al., 2002). A family of ING tumor suppressor induces the acetylation of lysine residues at the COOH terminal of p53 (Garkavtsev et al., 1998; Nagashima et al., 2001). The promyelocytic leukemia (PML) potenitates the p53 function by CBP-dependent acetylation and Chk2-dependent Ser-20 phosphorylation (Louria-Hayon et al., 2003; Bernardi et al., 2004). Like PML, pVHL stabilizes p53 by simultaneously affecting both NH_2-terminal phosphorylation and COOH-terminal acetylation of p53. However, PML mediates the Chk2-dependent Ser-20 phosphorylation, whereas pVHL is involved in ATM-mediated Ser-15-phosphorylation of p53. To date, little has been known about whether pVHL may be involved in the Chk2- or ATR-mediated activation of p53. It will be interesting to determine the relationship of pVHL with these kinases involved in p53 phosphorylation after DNA damage.

Galban et al. (2003) have shown that reintroduction of pVHL in RCC cells markedly accumulated p53. This was due to the pVHL-enhanced binding of the RNA binding protein HuR to 3′ untranslated region of p53 mRNA. In this study, we also found that pVHL reintroduction alone, to a lesser extent, appears to stabilize p53. However, the drastic effect of pVHL on p53 stabilization was found under DNA damage. We showed that pVHL has an ability to associate with both p53-ATM and p53-p300 (or pCAF) complex and to increase the phosphorylation of Ser-15 and acetylation of lysine residues of p53. In addition, we showed that p53 strongly associates with p300 (Figure 5D) and is more stabilized in A498/VHL cells than in A498/Mock cells upon treatment with adriamycin (Figure 7B). These results indicate that pVHL serves as an important component for p53 stabilization and activation under DNA damage.

In contrast to our findings reported here, Stickle et al. (2005) reported that the expression of p53 in RCC cells is independent of pVHL. They showed that pVHL elevated p53 expression only in p53-mutated RCC cells, while pVHL has no influence on the elevation of p53 in RCC cells containing wild-type p53. However, we observed that transient transfection of pVHL increased the phosphorylation and acetylation of p53 (Figures 3A and 5A), thus elevating p53 expression in both RCC and non-RCC cells (Figures 4A, 4B, and 7B). The overexpression of pVHL in A498/VHL stabilizes and transactivates wild-type p53 and subsequently induces its downstream genes when adriamycin is treated (Figure 7B). Even though they used the similar types of cells and methods, it is unclear why pVHL-mediated elevation of p53 was not observed in RCC and non-RCC cells.

We demonstrated that pVHL enhances p53 transcriptional activity and upregulates p53-downstream genes p21 and Bax, which ultimately lead to RCC cell cycle arrest and apoptosis after DNA damage. Furthermore, we demonstrated that the loss of VHL impairs the p53-mediated cell cycle arrest and apoptosis (Figures 7C and 7D). It is worth noting that several reports showed that pVHL could affect cell cycle in a p53-independent manner: the loss of VHL enhances Cyclin D1 expression (Bindra et al., 2002) and downregulates the expression of p27 in renal tumors (Osipov et al., 2002). In addition, the reintroduction of pVHL was reported to increase the expression of p27 (Kim et al., 1998). We also observed that pVHL-mediated enhancement of p27 is independent of p53 (Figure S6). Since HuR was reported to enhance the translation of p27 (Millard et al., 2000), it is predicted that ectopic expression of pVHL probably increases the translation of p27 mRNA. Conversely, Mack et al. (2005) reported that the growth of $VHL^{-/-}$-transformed MEF is retarded due to the elevation of p21 and p27 level. It has been speculated that this discrepancy probably occurs in the different genetic backgrounds between $VHL^{-/-}$ RCC cells and $VHL^{-/-}$-transformed MEF.

In conclusion, we provide a mechanism by which the tumor suppressor pVHL crosstalks with another tumor suppressor p53 under conditions of DNA damage. Thus, VHL-deletion is likely to attenuate p53 activation in the presence of DNA damage and to trigger the aberrant upregulation of HIF-α, the combination of which exerts synergistic effects on tumorigenesis in RCC. It has important implications in both the diagnosis and treatment of renal cancer.

Experimental Procedures

Cells

HEK293, H1299, MCF7, and A498 cells were obtained from ATCC. HCT116 ($p53^{+/+}$) and HCT116 ($p53^{-/-}$) were obtained from B. Vogelstein (The Johns Hopkins University Medical Institutions). A498/VHL stable cells were selected using G418 (1 mg/ml) from A498 cells transiently transfected with pCR3-HA-VHL plasmid. Cells were cultured in DMEM containing 10% (v/v) fetal bovine serum and 50 U/ml of streptomycin and penicillin.

DNA and Transfection

Mammalian expression vectors of full-length or deletion mutants of p53 were obtained by inserting PCR fragments of p53 into pcDNA3-HA, pEGFP-C2, or pCS2+MT vectors. The mammalian expression vector for mGST-p53 (pEBG-p53) was obtained from Dr. J.H. Choe (KAIST, Korea). The mammalian expression vector for pVHL was as described previously (Choi et al., 2004). The expression vector for myc-tagged elongin C was cloned by inserting cDNA of elongin C into pcDNA3.1-myc vector (Invitrogen). The ATM expression vector (pcDNA3-flag-ATM) was described previously (Kim et al., 1999). Various point mutants used in this study were made using QuickChange Site-Directed Mutagenesis Kit (Stratagene). Reporter genes used in this study were as follows: a p21-luciferase plasmid from Dr. B. Vogelstein (The Johns Hopkins University Medical Institutions), a Bax-luciferase plasmid from Dr. C.W. Lee (National Cancer Center, Korea), a Noxa-luciferase plasmid from Dr. T. Taniguchi (University of Tokyo, Japan), and a pG13-luciferase plasmid from Dr. G. Lozano (The University of Texas M.D. Anderson Cancer Center). More detailed information about the DNAs used in this study is available on request. DNA transfections were carried out using lipofectamine reagent according to the manufacturer's instructions (Invitrogen).

Endogenous Immunoprecipitation

pVHL antibody was chemically crosslinked to protein G bead using ImmunoPure Protein G IgG Orientation Kit (Pierce). MCF7 cells were treated with adriamycin (0.4 μg/ml) for 12 hr. Cell lysates were immunoprecipitated with immobilized pVHL-protein G beads. Immunoprecipitates were subjected to 12% SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with anti-p53 (#9282) polyclonal antibody.

Purification of Recombinant Proteins

For recombinant GST-fused VHL mutants, cDNAs covering the full-length, α domain, or β domain of pVHL were inserted into pGEX-4T1 vector (Amersham). *E. coli* DH5α harboring each of the GST-fusion vectors was grown at 37ºC for 5 hr after adding 1 mM IPTG. GST-fusion proteins were purified through a conventional procedure using glutathione-Sepharose (Amersham). For recombinant $(His)_6$-p53, full-length cDNA of p53 was inserted into pRSET-A vector

(Invitrogen). *E. coli* BL21 (DE3pLysS) harboring pRSET-p53 was treated with 1 mM IPTG and further grown at 25ºC overnight. $(His)_6$-p53 proteins were purified using TALON metal affinity resin (Clontech).

RNA Interference and RT-PCR

The siRNA for VHL and GFP were chemically synthesized by Dharmacon. siVHL corresponds to positions 609–629 of the coding region (Berra et al., 2003). As a control, siGFP was used (5′-GGCUAC GUCCAGGAGCGCACCUU-3′, 5′-GGUGCGCUCCUGGACGUAGCC UU-3′). Both MCF7 and HCT116 ($p53^{+/+}$) cells were treated with 100 pmol of siVHL using oligofectamine reagent (Invitrogen). Total RNAs were extracted with Trizol reagent (Life Technologies, Inc.). cDNAs were synthesized using reverse transcriptase cDNA synthesis kit (Takara, Japan) with total RNAs as template. PCR primers used in this study were as follows: pVHL, 5′-GCGTCGTGCTGC CCGTATG'3′, 5′-TTCTGCACATTTGGGTGGTCTTC-3′; and β actin, 5′-GGCATCCACGAAACTACCTT-3′, 5′-CTGTGTGGACTTGGGAGA GG-3′.

Cell Cycle Analysis

For G0 synchronization, both A498/Mock and A498/VHL (about 20% confluency) were cultured in DMEM containing 0.5% (v/v) FBS for 30 hr. Cells were released from G0 arrest by replacement of media with DMEM containing 10% (v/v) FBS for 12 hr, followed by treatment with adriamycin (0.4 μg/ml) for various times. Cells were fixed in 50% (v/v) ethanol and stained with 1 ml of a solution containing RNase A (50 μg/ml) and propidium iodide (PI) (50 μg/ml). Cells were analyzed using a Coulter Epics XL flow cytometer (Beckman-Coulter).

Detection of Apoptosis

Apoptosis was judged by the result of FACS analysis using PI. A498 and A498/VHL cells were treated with adriamycin (0.4 μg/ml) for various times. For analysis, cells were collected and fixed overnight with 50% ethanol in PBS at 4ºC. After washed twice with PBS, cells were incubated for 30 min with a solution containing 50 μg/ml RNaseA and 50 μg/ml PI. At least 10,000 cells were collected and analyzed with Coulter Epics XL flow cytometer (Beckman-Coulter). Percentages of Sub-G1 were calculated with Muticycle for Windows software (Coulter).

Immunoprecipitation and Immunoblotting

Cells were lysed with lysis buffer (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.5% [v/v] NP-40, 1× protease inhibitor cocktail [Roche]), and cell lysates were incubated with a suitable antibody in the presence of protein A/G beads (Santa Cruz Biotechnology) for 2 hr. Immunoprecipitates were boiled with SDS sample buffer, loaded into SDS-PAGE, and probed with suitable antibodies. The antibodies used in this study were as follows: anti-Flag (M2) and anti-β actin monoclonal antibodies were purchased from Sigma; anti-HA and anti-c-*myc* monoclonal antibodies from Covance; anti-p53 (DO-1), anti-ubiquitin (P401), anti-Bax (SC-493), anti-p300 (N-15), anti-pCAF (H-369), anti-GST (B-14), and anti-β-tubulin (D-10) from Santa Cruz Biotechnology; anti-pVHL and anti-HIF-1α from Pharmingen; anti-Mdm2 (Ab-1) from Oncogene; anti-p21 from Transduction; anti-acetyl-p53 (K373/382) from Upstate; and anti-His (27E8), anti-p53 (#9282), and anti-phospho-p53 (Ser-15) antibodies from Cell Signaling.

Immunofluorescent Staining

H1299 cells were transfected with different combinations of mammalian expression vectors harboring GFP-p53, Mdm2, or pVHL and grown on coverslides. After 24 hr of transfection, cells were fixed with 4% (w/v) paraformaldehyde and blocked with 2% (w/v) bovine serum albumin in phosphate-buffered saline. The cells were then stained with anti-HA (Covance) monoclonal antibody and Rhodamine Red-X-conjugated anti-mouse antibody (Jackson Immunoresearch). Immunofluoresence was examined using a Zeiss LSM 510 laser scanning microscope.

In Vivo Ubiquitination Assay

H1299 cells were transfected with different combinations of HA-ubiquitin, Mdm2, and/or wild-type of pVHL (wt) or a mutant of pVHL (Y98N). Transfected cells were treated with 10 μM MG132 (Calbiochem) for 16 hr and lysed with lysis buffer. Supernatants were immunoprecipitated with anti-p53 (DO-1) antibody, and the washed immunoprecipitates were separated by SDS-PAGE and probed with anti-ubiquitin antibody. For detecting the endogenous ubiquitination of p53, HCT116 ($p53^{+/+}$) cells were treated siVHL (100 pmol) for 48 hr and further incubated with 20 μM MG132 for 6 hr, and then cell lysates were immunoprecipitated with anti-p53 (#9282) polyclonal antibody and probed with either anti-p53 (DO-1) antibody or anti-ubiquitin (P401) antibody.

Supplemental Data

Supplemental Data include six figures and can be found with this article online at http://www.molecule.org/cgi/content/full/22/3/395/DC1/.

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