Priority Report

Nutlin3 Blocks Vascular Endothelial Growth Factor Induction by Preventing the Interaction between Hypoxia Inducible Factor 1α and Hdm2

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Abstract

Hdm2 is elevated in numerous types of malignancies and is thought to impede the function of wild-type p53. Reactivation of p53 by disrupting the association with Hdm2 was the impetus for the development of Nutlin3. Although regulation of p53 has been the central focus of Hdm2 activity, it also binds other proteins through its p53-binding domain. Here, we show that hypoxia-inducible factor 1α (HIF1α) binds to Hdm2 in the domain designated to bind p53. HIF1α and p53 share a conserved motif that is required to bind Hdm2. Distinct complexes form between Hdm2-HIF1α and Hdm2-p53 as determined by immunoprecipitation of nuclear extracts and *in vitro*. The Hdm2 antagonist Nutlin3 prevents the association between Hdm2 and HIF1α. The vascular endothelial growth factor (VEGF) gene is a transcriptional target of HIF1α, and under normoxic or hypoxic conditions, Hdm2 increases HIF1α activity to induce VEGF production. Blocking the association of Hdm2 and HIF1α by Nutlin3, or ablating Hdm2 expression, diminished the level of VEGF under conditions of normoxia or hypoxia. Our findings establish a unique role for Nutlin3 in attenuating VEGF induction by preventing the association of Hdm2 with HIF1α. [Cancer Res 2007;67(2):450–4]

Introduction

The p53 tumor suppressor protein is a transcription factor that regulates numerous genes, the gene products of which govern DNA repair, cell cycle arrest, and apoptosis. Loss of p53 activity by point mutation or loss of heterozygosity is evident in a majority of human cancers supporting the notion that loss of function results in tumor progression. The tumors that retain wild-type p53 gene have circumvented normal mechanisms to activate p53. One mechanism of biochemically inactivating p53 involves p53 inducing the Mdm2 gene. Elevated Mdm2 protein binds to p53 to inactivate its transcriptional activity and facilitate the destabilization of p53. This interplay of p53 and Mdm2 has been characterized as the p53-Mdm2 autoregulatory feedback loop (1). Posttranslational modifications of Mdm2 play a dynamic role in regulating the ability of Mdm2 to destabilize p53 (2–4). Since the identification of the major role of Mdm2 in the regulation of the p53 function and considering that Mdm2 may be elevated by other factors in the tumor to inactivate p53 (5, 6), intense investigation has been undertaken to identify molecules that protect p53 from Mdm2. Several compounds have been identified that prevent the association between p53 and Mdm2 (Nutlin3 and Rita; refs. 7–9). In addition, compounds that target the ubiquitin ligase domain of Mdm2 can also protect or engage p53 activity (10).

Mdm2 will interact with numerous proteins, yet a select few interact within the p53-binding domain. Hypoxia-inducible factor 1α (HIF1α) has been shown to form a complex with Mdm2 or its human homologue Hdm2 (11, 12). In one report, increased association of HIF1α-Hdm2 protected p53 from Hdm2-mediated degradation (13). HIF1α is a transcription factor that regulates genes, the products of which are involved in metabolism and angiogenesis (11, 12). Hdm2-HIF1α association increases the induction of the vascular endothelial growth factor (VEGF).

We show that the p53-binding domain of Hdm2 associates in the NH_2 terminus of HIF1α,which shares a highly conserved motif with p53. HIF1α and Hdm2 form a distinct nuclear complex independent of p53. Furthermore, the Hdm2 antagonist Nutlin3 prevents the association of Hdm2 and HIF1α and inhibits the induction of VEGF. This work shows that Nutlin3 regulates the activities of Hdm2 involved in inducing the proangiogenic factor VEGF.

Materials and Methods

Cell culture. Cells were cultured in DMEM supplemented with 10% fetal bovine serum at 37°C under 5% CO_2. For growth under hypoxia, the media were changed to DMEM buffered with HEPES to maintain pH in the absence of CO_2, and oxygen was displaced with N_2 using a pro-oxy 100 regulator. Cells were cultured under 1% oxygen for 24 h.

Immunoprecipitation and Western blotting. Antibodies to HIF1α (Novus Biologicals, Littleton, CO), Cdc2, p53 (Santa Cruz Biotechnology, Santa Cruz, CA), and Hdm2 (2A10 and IF2, Calbiochem, La Jolla, CA), were used for immunoprecipitations from 100 μg of nuclear extract isolated using the per-NUC/Cyto kit (Pierce, Rockford, IL). Briefly, 100 μg of nuclear extract was added to the antibody preadsorbed to protein A/G agarose, and the volume of the incubate was adjusted to 1 mL using the NP40 lysis buffer [50 mmol/L Tris (pH 7.6), 150 mmol/L NaCl, 0.5% NP40, 10 mmol/L sodium PPi, 10 mmol/L sodium fluoride, 1 mmol/L sodium orthovanadate, supplemented with 10 μg/mL aprotinin, leupeptin, and pepstatin A and 2 mmol/L phenylmethylsulfonyl fluoride]. After 1 h of incubation at 4°C, the incubates were centrifuged, the supernatant was siphoned, and 1 mL NP40 lysis buffer was added to the pellet, which was rotated at 4°C. This wash procedure was repeated thrice, and then Western blots were prepared. Blots were probed with a cocktail of 2A10, SMP14, and IF-2 for Hdm2, or with VEGF or glyceraldehyde-3-phosphate dehydrogenase antibodies, followed by secondary antibody coupled to horseradish peroxidase and detected by chemiluminescence reagent.

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Recombinant proteins. HIF1α and Hdm2 (V5 tagged) were produced using a rabbit reticulocyte lysate system (Promega, Madison, WI), except that the amino acid mixes were combined (cysteine and methonine) such that the reactions produced nonradiolabeled products. The proteins were mixed, and Hdm2 was purified by immunoprecipitation using a V5 antibody and washed with the buffers described above. HIF1α cDNA was digested with *Sma*1-*Sal*1 restriction enzymes. The *Sma*1-*Sal*1 fragment was ligated in frame into the pGEX4t-1 glutathione *S*-transferase (GST) expression vector. The Quik change site-directed mutagenesis kit (Stratagene, La Jolla, CA) mutated phenylalanine 37 to tyrosine in HIF1α. Mutagenesis was confirmed by sequencing. GST, GST-HIF1α, or GST-F37Y proteins were produced in bacteria and purified with glutathione beads and then incubated with recombinant human Hdm2, produced as previously described (14), and washed with PBS, and Western blot analysis was conducted.

Gene reporter assays. LipofectAMINE was used to transfect cells with Rous sarcoma virus β-galactosidase construct and *vegf* or *epo* promoters upstream of the luciferase gene, generous gifts from Amato Giaccia (Stanford University, Stanford, CA) and Gregg Semenza (Johns Hopkins University, Baltimore, MD), respectively. Thirty-six hours after transfection, the cells were harvested, and luciferase and β-galactosidase were assayed in a luminometer. The ratio of luciferase to β-galactosidase activity was calculated, and fold induction was determined relative to control. Each data point represents the mean of results from three independent transfections.

shRNA to Hdm2 in U87 cells. The virus was produced as previously described (15). U87 cells were infected with three rounds of virus encoding scrambled (shScr) or two sequences directed to Hdm2 (ShA/C; plasmids encoding the shRNA were a generous gift from Richard Iggo, Swiss Institute for Experimental Cancer Research, Epalinges, Switzerland) and selected with puromycin. The population of puromycin-resistant cells was used to conduct experiments.

Results and Discussion

Hdm2 forms distinct complexes with p53 and HIF1α. Several groups have reported the association of Hdm2 and HIF1α; however, the domains or motifs required for the association were not determined. To determine the domain required for the association of HIF1α and Hdm2, carboxyl-terminal truncations of Hdm2 (ΔRing finger, ΔZinc finger), and the 110 amino acid p53 interaction domain of Hdm2 were produced in a rabbit reticulocyte lysate system (Fig. 1*A*). The data in Fig. 1*A* show that HIF1α binds to the to p53-binding domain of Hdm2 (Fig. 1*A*).

Because Hdm2-p53 interaction requires a defined amino acid sequence in p53, we compared the amino acid sequences of HIF1α and p53. p53 and other proteins (p73 and E2F) that bind to the NH_2 terminus of Hdm2 share a conserved motif (Fig. 1*B*; ref. 16). Upon examination of the HIF1α amino acid sequence, we identified the conserved motif in the NH_2 terminus (Fig. 1*B*). For the p53-Hdm2 interaction, phenylalanine 19 of p53 inserts into the hydrophobic pocket of Hdm2 and makes van der Waals contacts with isoleucine 61 and glycine 58 (16). Because phenylalanine is important for the p53-Hdm2 association, we generated a HIF1α mutant whereby phenylalanine 37 was changed to a tyrosine (F37Y). The first 245 amino acids of HIF1α and F37Y were produced as GST fusion proteins and incubated with recombinant Hdm2. Western blot analysis showed that Hdm2 bound GST-HIF-1α, but not GST or GST-F37Y (Fig. 2*B*), demonstrating the dependence of the conserved motif in HIF1α.

To show that p53 and HIF1α compete for the same binding site in Hdm2, we did *in vitro* binding assay. Recombinant Hdm2 was preincubated with 10 pg, 100 pg, or 1 ng of recombinant p53. The Hdm2-p53 reactions were then added to GST-HIF1α-glutathione beads. The data in Fig. 2*A* show by Western blot analyses that p53 binding to Hdm2 prevents the association with HIF1α, further demonstrating the competition of both proteins for binding to Hdm2 in the p53 interaction domain.

To examine the complex formation of HIF1α bound to Hdm2 *in vivo*, mouse embryo fibroblasts *p53*$^{-/-}$ (MEF), HEPG2, and MCF7 cells were grown under normoxia, and nuclear extracts were isolated and used to purify the HIF1α and Mdm2 by immunoprecipitation. Western blots show that Mdm2 forms a complex with HIF1α in the absence of p53 (Fig. 2*B*) in MEFs. Immunoprecipitations of nuclear extracts from HEPG2 and MCF7 cells show that both p53 and HIF1α complex with Hdm2. However, HIF1α did not copurify with p53, and p53 did not copurify with HIF1α. Having shown a p53-independent association of Hdm2 and HIF1α under normoxia, we tested for the interaction of the proteins isolated from MEFs, *p53*$^{-/-}$ MEFs, H1299 (p53 null), and T47D cells (mutant p53) grown under hypoxia. The Hdm2/HIF1α complex was isolated from nuclear fractions of each cell type and Western blotted for HIF1α and Hdm2/Mdm2 (Fig. 2*C*). The results in Figs. 1

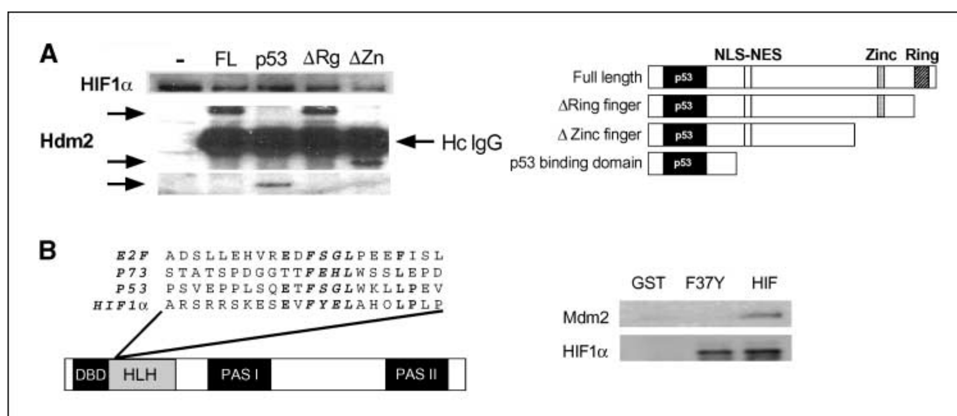

Figure 1. Mapping the HIF1α-Hdm2 interaction. *A,* rabbit reticulocyte system produced full-length and deletion mutants of Hdm2 and HIF1α. HIF1α was mixed with either Hdm2 or the deletion mutants, and an Hdm2 antibody was used to immunopurify Hdm2. *Row 1,* input HIF1α; *rows 2–5,* the immunoprecipitated Hdm2 (V5 epitope). Western blot analysis detected HIF1α(*top*) Hdm2 or the deletion mutants of Hdm2 (*bottom*). The schematic of Hdm2 showing the ring finger domain, zinc finger domain, the nuclear import and export sequences (*NLS-NES*), and the p53-binding domain and carboxyl-terminal deletions of Hdm2. *B,* sequence alignment of E2F, p73, p53, and HIF1α. *Bold letters,* conserved amino acids. The schematic of the first 245 amino acids of HIF1α shows the DNA binding domain (*DBD*), the helix-loop-helix domain (*HLH*), and Per-ARNT-Sim (*PAS*) domains I and II. GST, GST-HIF1α, and F37Y HIF1α bound to glutathione beads were incubated with recombinant human Hdm2, washed, and processed for Western blot analysis.

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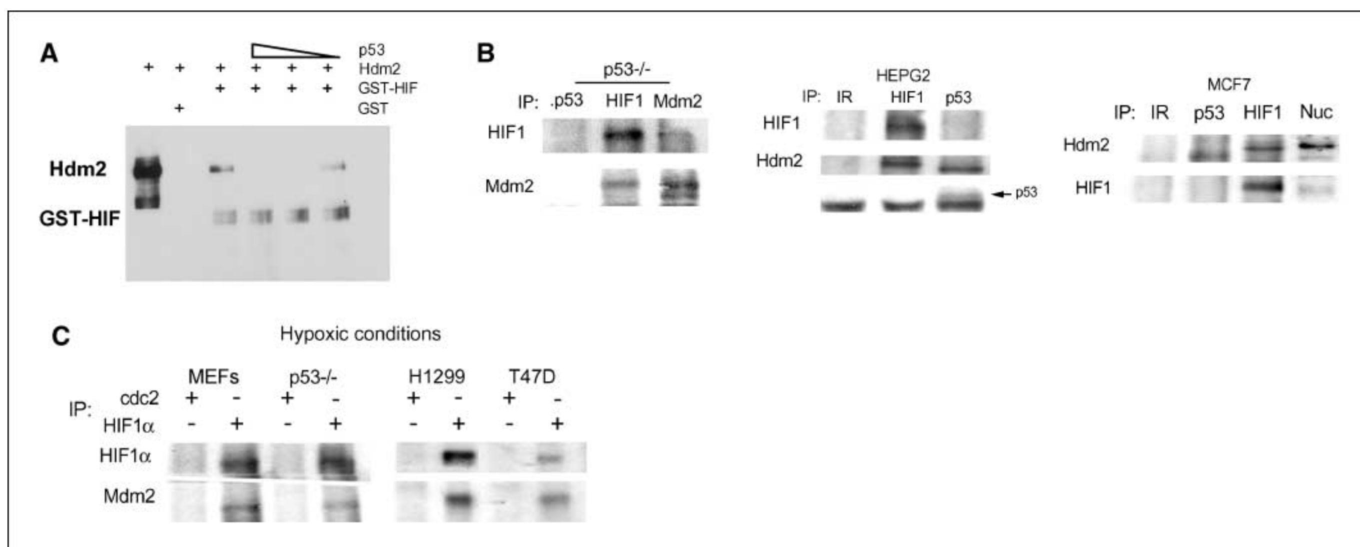

Figure 2. Characterization of p53, HIF1α, and Hdm2 complexes *in vitro* and *in vivo*. *A,* recombinant p53 (1 μg, 100 pg, and 10 pg) was preincubated with Hdm2 and then added to GST-HIF1α. Complexes were purified by glutathione beads and analyzed by Western blot. *B,* Cdc2, HIF1α, and Mdm2 were immunoprecipitated from nuclear extracts from $p53^{-/-}$ MEFs. Western blot analysis was done to detect HIF1α and Mdm2 (*left*). HEPG2 (*middle*) nuclear extracts were used to immunoprecipitate and Western blot for Hdm2, HIF1α, and p53. Nuclear extracts were immunoprecipitated for insulin receptor, HIF1α, or p53. *Nuc* is input of 50 μg of nuclear extracts from MCF7 (*right*). Western blot analysis was used to detect HIF1α, p53, and Hdm2 immunocomplexes. *C,* immunopurified HIF1α isolated from nuclear extracts in $p53^{-/-}$ or wild-type MEFs, H1299 cells, or T47D cells under hypoxic conditions (1% oxygen for 24 h). Western blots were prepared and probed for Hdm2 and HIF1α.

and 2 show that Mdm2 or Hdm2 (human homologue) complex with HIF1α, and this complex does not copurify p53, and immunopurified p53 does not copurify HIF1α. These observations are incongruent with a report that shows a trimeric complex of p53/HIF1α/Mdm2 isolated from whole cell lysates (11). There could be a simple explanation for the different observation. The authors only used an Mdm2 antibody to show a complex of p53 and HIF1α, which would pull down both p53 and HIF1α and would not show the distinct complexes.

VEGF is regulated by loss of Hdm2 or Nutlin3 treatment, which prevents Hdm2-HIF1α complex formation. Experiments were conducted with *mdm2*-deficient MEFs to assess the functional aspect of the HIF1α-Hdm2 complex. As knock-out of *mdm2* is embryonically lethal, experiments were conducted with $p53^{-/-}$ and $p53^{-/-}mdm2^{-/-}$ MEF. By comparison with MEFs, HIF1α activity was greater in $p53^{-/-}$ MEFs grown under normoxia or hypoxia as measured by *vegf* reporter assay (Fig. 3*A*). Transactivation of the *vegf* reporter was diminished in $p53^{-/-}mdm2^{-/-}$ MEFs (Fig. 3*A*), suggesting a molecular role for Mdm2 in the production of VEGF (Fig. 2). To show that this observation was not limited to murine cells, U87 cells were generated with a scrambled shRNA (scrRNA) or shRNA directed to Hdm2 (A/C). U87 cells under hypoxic conditions did not induce VEGF when ShRNA decreased Hdm2. U87 cells are wild type for p53, and to eliminate the possibility of p53 regulating HIF1α, we used H1299 cells that lack p53. Transient overexpression of Hdm2 under condition of normoxia or hypoxia in H1299 cells augmented *vegf* promoter activity, whereas the expression of Hdm2 in the reverse orientation (2mdH) diminished reporter activity (Fig. 3*C* and *D*). Although the Hdm2-HIF1α complex led to an increase in VEGF production, it is not clear how the complex works (17). Both Hdm2 and HIF1α bind with p300. Hdm2 may be involved in the formation of a p300-Hdm2-HIF1α complex that would be responsible for inducing HIF1α target genes. Further investigation is required to resolve the functional aspect of this complex.

To test if Nutlin3 would have an effect on *vegf* production, the *vegf* luciferase construct was transiently transfected into H1299 cells. Transfected cells were then treated with 10 μmol/L Nutlin3 and incubated under normoxic or hypoxic conditions for 24 h. Nutlin3 effectively prevented the induction of *vegf* in transient assays comparable to transient transfection of antisense Hdm2 (Fig. 3*C*). Additionally, we examined another HIF1α-responsive promoter (erythropoietin) linked to luciferase (*epo*). Nutlin3 decreased the induction of the *epo* promoter (Fig. 3*D*) similar to that of the *vegf* promoter (Fig. 3*C*) under conditions of normoxia or hypoxia.

Given that Nutlin3 diminished *vegf* and *epo* promoter activity in transient assays, we next examined *in vitro* how Nutlin3 would affect the formation of the HIF1α-Hdm2 complex. Nutlin3 (1 nmol/L, 10 nmol/L, and 1 μmol/L) was preincubated with recombinant Hdm2 and incubated with GST-HIF1α or p53. Complexes were purified with glutathione beads (HIF1α) or DO-1 agarose beads (p53) and analyzed by Western blot. The data in Fig. 4*A* show that Nutlin3 prevented the association of p53 or HIF1α with Hdm2. Next, we examined the ability of Nutlin3 to influence the Hdm2-HIF1α association by immunoprecipitation of Hdm2 from nuclear extracts isolated from cells under hypoxic conditions in the absence or presence of 10 μmol/L Nutlin3. The data in Fig. 4*B* show that the nuclear HIF1α-Hdm2 complex was disrupted by the presence of Nutlin3 in H1299 cells. Similar results were observed in U87 cells (wild-type p53), in that Nutlin3 prevented the complex formation of Hdm2 with HIF1α when Hdm2 was immunoprecipitated (Fig. 4*D*). An examination of endogenous VEGF levels in U87 cells shows diminished levels when cells are incubated under normoxic or hypoxic conditions in the presence of Nutlin3 (Fig. 4*D*). Thus, Nutlin3 is able to block the formation of the complex of Hdm2 and HIF1α, and this disruption has a dramatic effect on the levels of VEGF in the absence or presence of p53.

Great interest has been generated recently in developing compounds that can reestablish p53 activity. Numerous approaches

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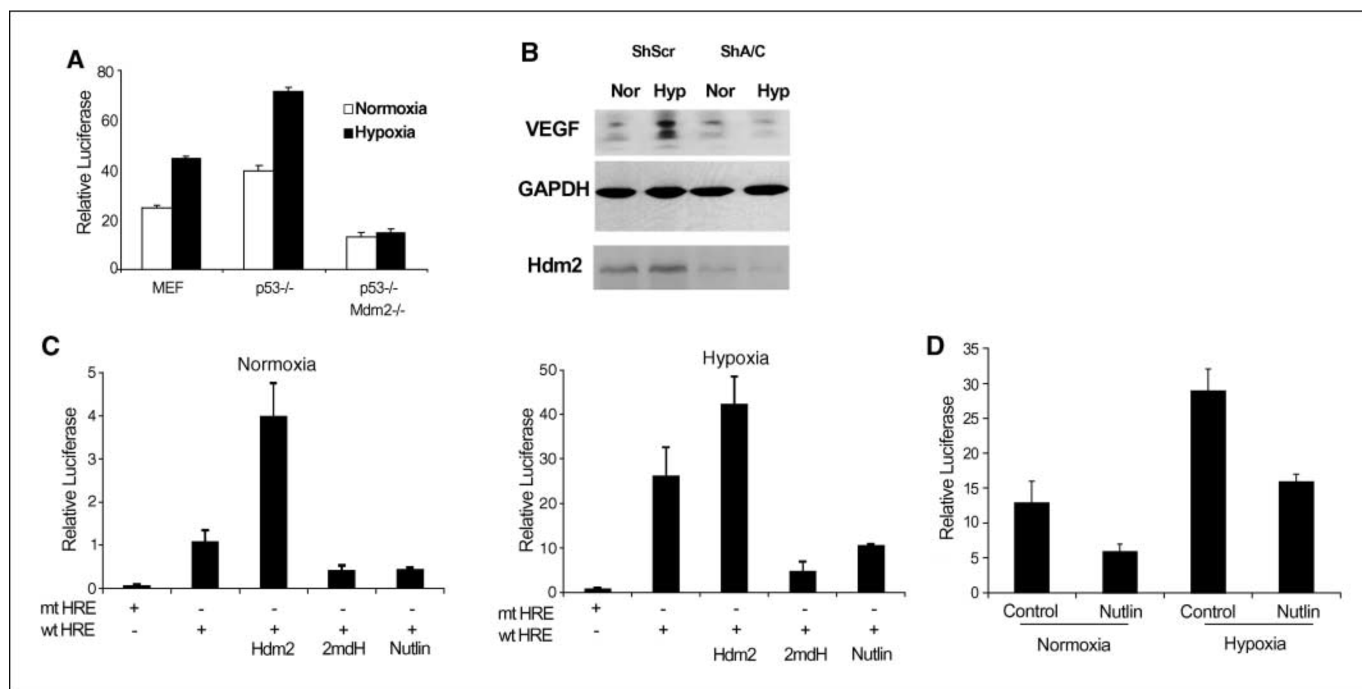

Figure 3. *Vegf* reporter activity. *A,* parental, *p53*$^{-/-}$, and *p53*$^{-/-}$*mdm2*$^{-/-}$ MEFs were transfected with the *vegf* promoter and β-gal and then cultured under normoxia or hypoxia. *B,* U87 scrambled (ShScr) or Hdm2 knockdown (ShA/C) cells were treated under normoxic or hypoxic conditions, and cellular extracts were harvested for Western blot analysis of Hdm2, VEGF, and glyceraldehyde-3-phosphate dehydrogenase. *C,* normoxia (*left*), hypoxia (*right*). H1299 cells were transiently cotransfected with wild-type or mutant (mutant HRE elements) *vegf* promoter, β-gal expression vector, and Hdm2 or Hdm2 expressed in the reverse orientation (*2mdH*). H1299 cells transiently transfected with the *vegf* reporter and β-gal were pretreated with 10 μmol/L Nutlin3 for 10 min, then cells were incubated under hypoxia or normoxia for 24 h and harvested for analysis thereafter. *D,* H1299 cells were transiently transfected with the *epo* promoter driving luciferase, and β-gal then 24 h thereafter were treated with 10 μmol/L Nultin3 for 24 h under normoxia or hypoxia and then harvested for analysis. *Columns,* mean reporter assays from three independent transfections; *bars,* SD.

have been used to free Hdm2 from p53, such as altering the levels of Hdm2 by antisense oligonucleotides and using small disruptive peptides or small molecules that target the p53-binding domain of Hdm2. Several lead compounds have proven to be successful in preventing such interaction. Nutlin3 was successful in preventing the association of p53 and Hdm2 and, in xenograph models, decreased tumor volume similar to doxorubicin treatment. We previously showed that the Nutlin3 compound was specific to Hdm2 and not to its family member Hdmx (Mdm4) in preventing the association with p53 (18). This shows that Nutlin3 effects are

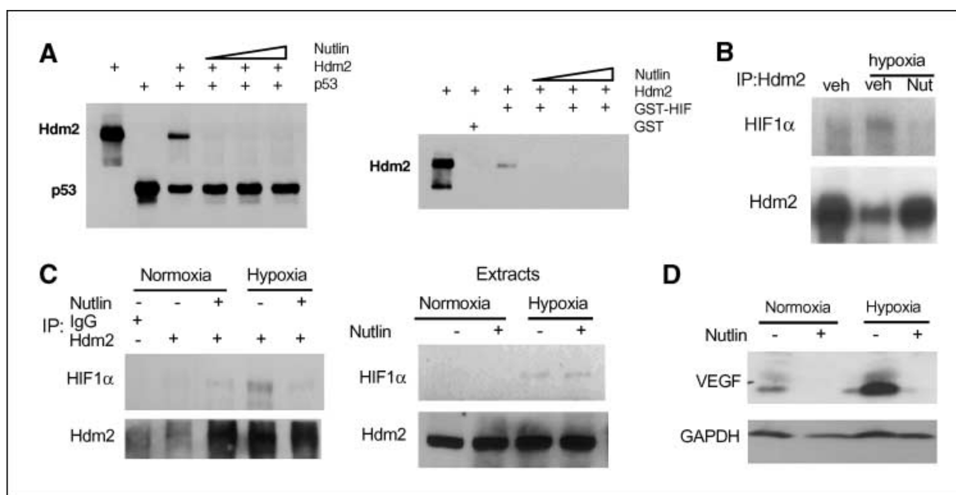

Figure 4. Nutlin3 prevents Hdm2-HIF1α association. *A,* recombinant Hdm2 was preincubated with Nutlin3 (1 nmol/L, 10 nmol/L, or 1 μmol/L), and GST or GST-HIF1α was added. Western blot analysis was done for Hdm2 using 2A10 antibody (*left*). Experiments were done as with HIF1α, except that recombinant p53 was added and DO-1 (p53) agarose beads were used to purify complexes. 2A10 detected Hdm2, and rabbit polyclonal antibody detected p53 (*right*). *B,* H1299 cells were treated with either vehicle or 10 μmol/L Nutlin3 and incubated under hypoxia or normoxia for 24 h. Hdm2 or control immunoglobulin G was used to immunopurify Hdm2 from protein extracts. Western blot detected Hdm2 and HIF1α. *C,* Hdm2 was immunopurified from cellular extracts of U87 cells pretreated with Nutlin3 (10 μmol/L) under conditions of normoxia or hypoxia, and HIF1α or Hdm2 was detected by Western blot (*left*) or cellular extracts (*right*). *D,* whole cell lysates were prepared for Western blot analysis for the detection of VEGF and glyceraldehyde-3-phosphate dehydrogenase.

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specific to Hdm2 and the utility of this small molecule inhibitor not only to protect p53 from Hdm2 but also to prevent Hdm2 from activating HIF1αand mediating VEGF induction.

Hdm2 plays a role in tumorigenesis that can be independent of p53. Indeed, hemangiosarcoma, a highly vascularized tumor, was observed with high frequency in Mdm2 transgenic mice. Moreover, tumor development in these mice was independent of p53 (19). About 70% of patients with hemangiosarcoma have high levels of VEGF (20). These two studies and our work presented herein support a role of Hdm2 in promoting the angiogenic factor VEGF. The oncogenic activity of Hdm2 may manifest itself by playing a dual role in blocking p53 activity while also enhancing HIF1α activity. However, targeting Hdm2 using Nutlin3 or Nutlin3-like molecules in the treatment of patients may target the tumor by reactivating p53 to bring about a decrease in tumor volume, which has been shown in xenograph models, and by inhibiting HIF1α-mediated angiogenesis.

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