A Second p53 Binding Site in the Central Domain of Mdm2 Is Essential for p53 Ubiquitination

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ABSTRACT: Mdm2 negatively regulates p53 by inhibiting its transcriptional activity and promoting its degradation by functioning as an E3 ubiquitin ligase. The primary p53 binding site on mdm2 is located in its *N*-terminal domain. Through binding to p53 at its *N*-terminal transactivation domain, mdm2 directly blocks the transcriptional activation function of p53. We discovered that truncated mdm2 protein constructs without the *N*-terminal p53 binding domain are at least as active as full-length mdm2 in catalyzing p53 ubiquitination. Furthermore, the deletion of the central acidic domain significantly reduces the E3 ligase activity of mdm2 toward p53. We have also performed GST pull-down experiments to probe the direct binding of various mdm2 domain constructs toward full length p53 and found that mdm2 constructs without the *N*-terminal p53 binding domain retain the ability to bind to p53. Our kinetic and binding data localize the second p53 binding site between amino acids 211 and 361, including the acidic domain and the zinc finger region. Our work, consistent with other reports, suggests that the p53 tetramer interacts with at least two sites on mdm2. Although the interaction between the *N*-termini of mdm2 and p53 blocks the transactivation activity of p53, the interaction between the central domain of mdm2 and the core domain of p53 is critical for the ubiquitination and degradation of p53. This second mdm2−p53 interaction site represents an alternative target for small molecule modulators of the mdm2−p53 pathway.

The tumor suppressor protein p53 functions as a transcription factor that binds DNA and induces the expression of a number of genes involved in cell growth arrest, DNA repair and apoptosis. Under normal conditions, p53 is maintained at a low steady-state level through proteasome-mediated degradation. Various genotoxic stresses initiate signaling pathways that stabilize p53 and activate its transcriptional activity, leading to either cell cycle arrest or apoptosis (*1*−*4*).

Mdm2 is a critical regulator of p53 activity and stability. By binding to the *N*-terminal transactivation domain of p53, mdm2 blocks p53's ability to activate transcription (*5*, *6*). Additionally, mdm2 serves as an E3 ubiquitin ligase to promote p53 degradation through the ubiquitin−proteosome pathway (*7*−*12*). The mdm2 gene is a direct target of p53 transcriptional activity, forming an auto feedback loop (*13*). Mdm2 has also been proposed to regulate p53 function by shuttling p53 from the nucleus to the cytoplasm, presumably through the monoubiquitination of p53 (*11*, *14*, *15*).

The ubiquitin−proteosome pathway plays an important role in controlling numerous biological events involving the cell cycle, differentiation, immune responses, DNA repair, and apoptosis (*16*, *17*). The ubiquitin (Ub[1]) cascade is initiated by the activation of Ub by the ubiquitin-activating enzyme (E1) to form a thioester bond between the active site Cys in E1 and the *C*-terminal Gly of Ub. The activated Ub is transferred to the active site Cys of one of several different ubiquitin-conjugating enzymes (Ubc or E2) in an ATP-independent manner. The Ub-conjugated E2 then functions in combination with an E3 ligase to transfer Ub to the target protein on Lys residues.

Mdm2 is a member of the RING finger domain family of E3 ubiquitin ligases. It contains at least four functionally independent domains (*13*), including (1) an *N*-terminal domain (a.a. 19−102) that recognizes the *N*-terminal Box-I domain of p53 (*5*, *18*, *19*); (2) a central acidic domain (a.a. 223−274) that binds to a number of proteins, including $p14^{ARF}$, L5, L11, L23, p300, and YY1 (*20*−*24*); (3) a putative zinc finger (a.a. 305−322) (*25*); and (4) a RING finger domain (a.a. 438−478) critical for its E3 ubiquitin ligase activity (*10*, *26*). The interaction between the *N*-terminal domains of mdm2 and p53 has been extensively studied (*5*, *18*, *19*, *27*−*29*), and several compounds have been reported to inhibit this interaction (*30*−*33*). A second mdm2 binding site has been proposed in the core domain of p53, and the mutation of this site increases the ubiquitination of p53 (*34*). Most recently, peptides derived from the acidic domain and part of the zinc finger domain of mdm2 were found to bind

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[1] Abbreviations: SDS−PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Ub, ubiquitin; E1, ubiquitin-activating enzyme; Ubc or E2, ubiquitin-conjugating enzyme; E3, ubiquitin ligase; GST, glutathione-S-transferase; a.a. amino acid; mdm2-FL, full length mdm2.

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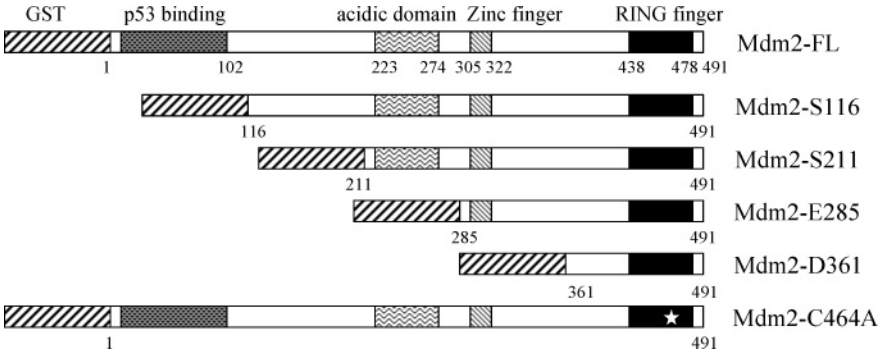

FIGURE 1: mdm2 constructs used for ubiquitin ligase assays and GST pull-down with p53. Regions of mdm2 corresponding to p53 binding, the acidic, the zinc finger and the RING finger domains, are labeled with different shading. The constructs were prepared as GST-tagged proteins. The numbers correspond to the amino acid residues of mdm2. The star indicates the Cys → Ala point mutation at a.a. 464 of mdm2.

to the specific DNA binding site of the core domain of p53, albeit with low affinity (*35*).

To understand how the binding interaction between mdm2 and p53 is related to the mdm2-catalyzed p53 ubiquitination, we compared the ubiquitin ligase activity and binding interaction of full length mdm2 (mdm2-FL) and various *N*-terminally truncated mdm2 constructs toward p53. Our results indicate that the interaction between the *N*-terminal domains of mdm2 and p53 is not required for the mdm2-catalyzed p53 ubiquitination. Our results further indicate a second p53 binding site on mdm2 that is critical for its ubiquitin ligase activity toward p53; this site is located between amino acids 211 and 361, a region that includes the acidic domain and the zinc finger region.

MATERIALS AND METHODS

Materials. Ulp1 protease was prepared in-house. Glutathione Sepharose, Q-Sepharose, Mono-Q, Superdex 75, Superdex 200, G-25, Cy5 maleimide, and Oregon Green (OG) maleimide were obtained from GE Healthcare (Uppsala, Sweden). Streptactin Superflow and Ni-NTA agarose were from IBA GmbH (Göttingen, Germany) and Qiagen GMbH (Hilden, Germany), resepectively. TCEP (tris(2-carboxyehtyl)phosphine) and reduced glutathione were from Calbiochem (San Diego, CA). AEBSF (4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride) was from BP Biomedical (Irvine, CA).

Protein Expression and Purification of p53, mdm2, and E1. The human p53, mdm2, and E1 cDNA clones matched Genbank accession numbers M60950, Z12020, and M58028, respectively. Wild type p53 was modified by PCR to generate a Strep−Tev−p53 fusion, which was subcloned by Gateway recombination into the baculovirus expression vector, pDEST8. The Tev cleavage sequence was incorporated precisely to the 5′ end of E1, mdm2, and various mdm2 mutants (Figure 1) by PCR and subsequently cloned into pENTR/D. Mdm2 containing a single mutation of C464A was generated using the Quickchange site-directed mutagenesis kit (Stratagene #200518). The coding sequences were separately transferred into pDEST20 to generate an *N*-terminal GST fusion. Baculovirus expression was carried out using the bac-to-bac baculovirus expression system (Invitrogen, Carlsbad, CA). The baculovirus infection of exponentially growing Sf9 culture was carried out for 48 h at 28 °C. The infected Sf9 cell culture was harvested by centrifugation. The cell pellets were frozen and stored at −80 °C until they were processed for subsequent protein purification.

Sf9 cells expressing GST-E1, GST-mdm2, and GST-mdm2 mutants were lysed in buffer A (50 mM Tris-HCl at pH 8.0, 0.15 M NaCl, 5−10% Glycerol, 2−5 mM DTT) plus a protease inhibitor cocktail (1 μg/mL of leupeptin, 1 μg/mL of pepstatin A, 0.4 mM AEBSF) with 1% Triton X-100 and centrifuged to remove cellular debris and insoluble proteins. The soluble fraction was captured with glutathione sepharose, and the column was washed with buffer A. GST-E1 was eluted with 10 mM reduced glutathione in buffer A. GST-mdm2 and its mutants were eluted with 10 mM reduced glutathione in buffer A containing 0.1% Triton-X-100, and subsequently purified by Superdex200 or Superdex75 in buffer A with 0.1% octylglucoside.

Sf9 cells expressing His−Strep-tagged p53 were lysed by sonication in buffer A plus the protease inhibitor cocktail and 2 mM EDTA with 1% Triton X-100. After centrifugation, the soluble fraction was captured on Streptactin-superflow. The protein bound in the column was washed with buffer A and eluted with 3 mM desthiobiotin in buffer A. Affinity purified p53 was diluted 1:1 with water and purified on MonoQ IEX chromatography with a NaCl gradient in 25 mM Tris, 5% glycerol,and 0.2 mM TCEP. His−Strep−p53 was eluted at 250 mM NaCl.

Expression, Purification and Modification of UbcH5b and Ub. Smt3 DNA was cloned from *Saccharomyces cerevisiae* genomic DNA based on the genomic sequence of Chromosome IV (1469390−1469695) to generate a His-tagged Smt3 open reading frame (ending with Glycine 98, cleavable by Ulp1 protease), which was precisely fused in frame with the start of UbcH5b coding sequence in the *E.coli* expression vector pDEST14 (*36*). Cys-ubiquitin (Cys-Ub) cDNA was subcloned in pET22b. An amino terminal cysteine codon was precisely incorporated as a specific site for protein labeling with maleimide chemistry. Smt3-UbcH5b, Ulp1, and Cys-Ub were separately expressed in *E. coli* BL21 (DE3)* at 37 °C for 3 h in LB media containing 1 mM IPTG.

The *E. coli*-expressing His-Smt3-UbcH5b cell paste was lysed by pressure drop through a MicroFluidizer in a lysis buffer (buffer A plus inhibitor cocktail with 1% Triton X-100). After centrifugation, the soluble fraction was captured on Ni-NTA agarose. The column was washed with buffer A, and bound fractions were eluted with 300 mM imidazole in buffer A. After concentration, imidazole was removed by

desalting with a G-25 SEC column equilibrated with buffer A. The His−Smt3 tag was removed by enzymatic cleavage using His-tagged Ulp1 (made in-house) at a 25:1 substrate to enzyme molar ratio at 4 °C while progress of the enzymatic reaction was monitored by SDS−PAGE. Liberated His−Smt3, His-tagged Ulp1, and incompletely cleaved substrate were captured on Ni-NTA agarose. The resulting unbound fractions from Ni-NTA agarose were passed through Q-sepharose. The unbound proteins (untagged UbcH5b) were collected, concentrated, and desalted into a storage buffer of 50 mM Tris (pH 7.4), 150 mM NaCl, 10% Glycerol, and 1 mM DTT.

The *E. coli*-expressing Cys-Ub cell paste was lysed by pressure drop in lysis buffer A with 2 mM DTT. After centrifugation, the ice-bath-chilled soluble cell lysate was gradually acidified to pH 2 with acetic acid to precipitate most of the contaminating proteins. Soluble Cys-Ub was purified by C18 RP-HPLC with an acetonitrile gradient in 0.1% TFA. The fractions were analyzed by RP-HPLC and LC/MS. The Cys-Ub fractions were pooled, diluted with water, frozen, and lyophilized. Lyophilization was stopped prior to dryness and neutralized with 1 M Tris to pH 7. TCEP was added to a final concentration of 0.1 mM.

A 1.5 molar excess of Cy5-maleimide was added to specifically label the free sulfhydryl of Cys-Ub. The incorporation of Cy5 was monitored by LC/MS. The reaction was quenched by the addition of excess DTT. Cy5-maleimide Cys-Ub (Cy5-Ub) was separated from the crude reaction mixture by a G-25 sizing column and further purified by RP-HPLC. OG-Ub was prepared using a similar procedure.

Conjugation of UbcH5b with Labeled Ubiquitin. The preparation of Cy5 or OG-labeled Ub−UbcH5b (Cy5-Ub−UbcH5b or OG-Ub−UbcH5b) was similar to published procedures (*37*, *38*) except for the modification using 50 mM HEPES at pH 7.3, 50 mM NaCl, 0.1 mM TCEP for S-75 SEC column and storage.

All purified proteins were confirmed by LC-MS or peptide mapping. The purity of p53 and Cy5-Ub−UbcH5b was estimated to be >90% and 96%, respectively. The purity of mdm2, its mutants, and E1 was estimated to be at least >80%.

Analysis of mdm2-Catalyzed p53 and Autoubiquitination. Reactions were carried out in buffer B containing 15 mM HEPES (pH 7.5), 5 mM NaCl, and 10 mM octylglucoside in a 20 μL reaction volume at room temperature. The reaction mixtures contained Cy5-Ub−UbcH5b (70 nM or varied), p53 (700 nM or varied), and mdm2 at various concentrations. For the mdm2 autoubiquitination assay, p53 was not present in the reaction. The reactions were stopped with 4X SDS-reducing sample buffer, separated using 4−12% NuPAGE bis-Tris SDS−PAGE (Invitrogen), and the fluorescence intensity of the p53-Ub_n or mdm2-Ub_n bands were quantified on a Storm 860 PhosphoImager (Molecular Dynamics). Fluorescence intensity was converted to molar units of Cy-Ub−UbcH5b by referencing to a Cy5-Ub−UbcH5b calibration curve and assuming that the quantum yield for Cy5-Ub−UbcH5b fluorescence was not significantly affected by the formation of the Cy5-Ub−p53 or the Cy5-Ub−mdm2 complex.

Western blot analysis was carried out to confirm that the fluorescent bands assigned as Cy5-Ub−p53 products are indeed ubiquitinated p53. OG-Ub−UbcH5B was used instead of Cy5-Ub−UbcH5B to avoid fluorescent interference in the Odyssey Infrared Image system. After incubation at room temperature for 30 min, the ubiquitination reactions were terminated with 2X SDS-reducing sample buffer, separated on 4−20% TrisGlycine polyacrylamide gels, transferred onto a nitrocellulose membrane (Invitrogen), and analyzed by western blot using a monoclonal anti-p53 primary antibody (DO-1) (0.5 μg/mL) (BD PharMingen, Cat. 554293) and an Alexa Fluor 680 conjugated goat anti-mouse secondary antibody (0.8 μg/mL) (Molecular Probes, Cat. A-21057). Immunodetection was carried out using the Odyssey Infrared Image system (Li-Cor Bioscience).

Steady-State Analysis of mdm2-Mediated p53 Ubiquitination. The steady-state experiments were run at a single time point of 2 or 5 min in buffer B, using mdm2 (2 nM) or mdm2-S211 (0.4 nM) and various p53 and Cy5-Ub−UbcH5b concentrations. Prior to the steady-state analysis, we performed experiments to ensure that end-point analysis was within the linear range of the reaction (i.e., under initial velocity conditions). Data were globally fit to one of the following equations using the software program Grafit (Erithacus Software, Ltd.): eq 1, a Ping Pong Bi Bi steady-state mechanism; eq 2, a Rapid Equilibrium Random Bi Bi ternary complex steady-state mechanism (*11*, *39*).

$$v = \frac{V_{max}[AX][B]}{K_B[AX] + K_{AX}[B] + [AX][B]} \quad (1)$$

$$v = \frac{V_{max}[AX][B]}{\alpha K_{AX}K_B + \alpha K_B[AX] + \alpha K_{AX}[B] + [AX][B]} \quad (2)$$

In these equations, V_{max} is the maximal velocity obtained at infinite concentration of all substrates, [AX] and [B] are the concentrations of the group transfer donor and acceptor substrates (in our system [AX] = [Cy5-Ub−UbcH5b] and [B] = [p53]), K_{AX} and K_B are either Michaelis constants (in eq 1, Ping Pong mechanism) or equilibrium dissociation constants (in eq 2, Rapid Equilibrium Random Bi Bi mechanism) for substrates AX and B, respectively; α is the substrate cooperativity factor (e.g., the Michaelis constant is equal to α times the equilibrium dissociation constants when all other substrates are saturated).

In our current system, Cy5 or OG is linked to Ub though a Cys residue added to the *N*-terminus of Ub, rather than through the Lys 6 side chain. Using a competition assay, we have evidence that the Cy5 or OG labeling of Ub on the added *N*-terminal Cys residue does not alter the K_m for Ub−UbcH5b (data not shown), unlike our previous version of Og-Ub−UbcH5b with the Og modification on the side chain of Lys6 of Ub (*11*, *38*).

Binding Interaction of p53 to mdm2 by GST Pull-Down. The buffer conditions were based on ref 18 (*18*) with some modifications. Glutathione−Sepharose 4B beads (Pharmacia) were washed three times with the equilibrium buffer (20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 100 mM NaCl, and 0.5% Nonidet P-40) and incubated in the equilibrium buffer containing 0.5% nonfat milk for 30 min with shaking. The beads were washed three times again with the equilibrium buffer and stored in the same buffer at a concentration of 50% v/v. We used GST-E1 as a negative control for the pull-down.

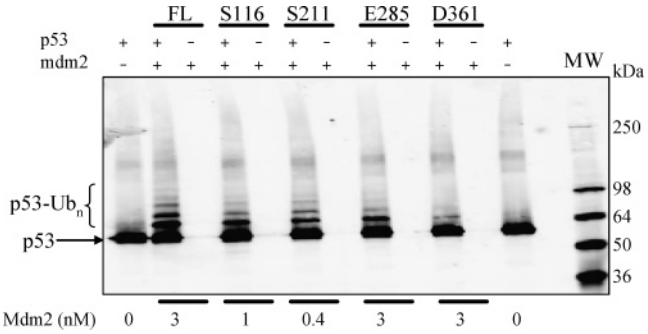

FIGURE 2: Western blot analysis of mdm2-mediated p53 ubiquitination. The ubiquitination reactions were carried out in the presence and absence of 100 nM p53 with 1 μM OG-Ub−UbcH5B and mdm2 at various concentrations. The ubiquitinated p53 products were analyzed by western blotting using an anti-p53 antibody (DO-1) as described in the Materials and Methods.

Ten microliters of p53 were incubated with 10 μL of GST-mdm2 (or GST-mdm2 mutants or GST-E1) (0.1 μM, respectively) in buffer B at room temperature for 20 min. The p53/mdm2 mixtures were diluted by 10-fold to 200 μL with the equilibrium buffer and then incubated with 20 μL of 50% pre-equilibrated beads for 1 h at 4 °C with shaking. The beads were washed three times with 1 mL of washing buffer (20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 250 mM NaCl, 1% Nonidet P-40), heated for 5 min at 95 °C in 40 μL of sample loading buffer and analyzed by western blot for bound p53 as described. The fluorescence intensity of the bound p53 was quantified using the Odyssey application software.

RESULTS

Deletion Mapping of mdm2 for p53 Ubiquitination Activity. To understand the domain requirements for the E3 ligase activity of mdm2 toward p53, we have expressed and purified a series of truncated mdm2 constructs as *N*-terminal GST-tagged fusion proteins (Figure 1), including: (1) mdm2-S116 (a.a. 116−491) with the *N*-terminal p53 binding domain deleted and all other domains intact; (2) mdm2-S211 (a.a. 211−491) retaining the central region (acidic domain and zinc finger) and the RING finger domain of mdm2; (3) mdm2-E285 containing the zinc finger and the RING finger domains; and (4) mdm2-D361 (a.a. 361−491) with only the RING finger domain. We have also prepared a full length mdm2 with a single Cys to Ala mutation C464A that has been shown to disrupt the RING finger and the ubiquitin ligase activity of mdm2 as a negative control (*10*).

The truncated mdm2 constructs were compared with full length mdm2 using an SDS−PAGE assay, monitoring the incorporation of Cy5-Ub onto p53. Instead of using the E1, E2, E3 cascade, we carried out the E1-mediated Ub transfer to the active site Cys of UbcH5b separately and used Cy5-Ub−UbcH5b to simplify our E3 reaction as described previously (*11*, *38*). Mdm2-mediated p53-Ub$_n$ formation was further confirmed by western blot using an anti-p53 antibody (Figure 2). Our results show that mdm2-S116 and mdm2-S211, both lacking the *N*-terminal p53 binding domain, are as active or slightly more active than mdm2-FL in catalyzing p53 ubiquitination (Table 1). Mdm2-E285, containing the zinc finger and RING finger domains, can still catalyze Ub transfer to p53 with only a 3-fold reduction in activity relative to that of mdm2-FL (Table 1). Mdm2-D361, containing only the RING finger domain, does not catalyze Ub transfer to p53. As expected, the Mdm2-C464A mutant with a disrupted RING finger has essentially no activity in catalyzing Ub transfer to p53, consistent with the requirement of the intact RING finger for the ubiquitination activity of mdm2.

Table 1: Comparison of Specific Activity of Full Length mdm2 and Its Mutants in Catalyzing P53 and Autoubiquitination Using the SDS−PAGE Assay[a]

enzyme	mdm2-catalyzed p53 ubiquitination specific activity[b]	mdm2 autoubiquitination specific activity
mdm2-FL	0.98 ± 0.05	0.49 ± 0.04
mdm2-S116	1.44 ± 0.09	0.73 ± 0.11
mdm2-S211	3.18 ± 0.12	0.66 ± 0.08
mdm2-E285	0.33 ± 0.08	1.59 ± 0.21
mdm2-D361	<0.01	2.79 ± 0.38
mdm2-C464A	<0.01	<0.01

[a] Each specific activity is the mean of two to three replicates ± SD. [b] Unit of specific activity: moles of ubiquitin transfer per min catalyzed by 1 mole of enzyme.

The truncated mdm2 constructs were also compared with mdm2-FL using the SDS−PAGE assay for mdm2 autoubiquitination (Table 1). Mdm2-S116, S211, E285, and D361 are at least as active as mdm2-FL in catalyzing autoubiquitination. The mdm2-C464A mutant is essentially inactive in autoubiquitination compared to mdm2-FL. In contrast with the requirement for the acidic domain and the zinc finger domain for catalyzing p53 ubiquitination, mdm2 containing the RING finger domain alone is sufficient to catalyze autoubiquitination.

Comparison of p53 Ubiquitination Kinetics for mdm2-FL and mdm2-S211. Mdm2-S211, a truncated form of mdm2 lacking the *N*-terminal p53 binding domain, is 2−3-fold more active than mdm2-FL in catalyzing p53 ubiquitination (Table 1). To further understand this result, we have compared the kinetic constants for this construct to that of mdm2-FL. The velocity of p53-Ub$_n$ formation was determined by varying Cy5-Ub−UbcH5b concentration with several fixed concentrations of p53. A series of parallel lines were obtained when the data are presented in a double-reciprocal format for both mdm2-FL and mdm2-S211 (Figure 3). The kinetic pattern of the substrate double titration is consistent with published reports (*11*), indicating either a hybrid Ping-Pong or a Rapid Equilibrium Random Bi Bi mechanism for both mdm2-FL and mdm2-S211. From the kinetic data alone, we were not able to uniquely assign a kinetic mechanism to mdm2-catalyzed p53 ubiquitination. Regardless of whether the kinetic mechanism is the hybrid Ping-Pong or the Rapid Equilibrium Random Bi Bi, the K_m values of Cy5-Ub−UbcH5b and p53 are similar for both full-length mdm2 and mdm2-S211 (40−100 and 700−800 nM, respectively; Table 2). The k_{cat} value of the reaction using mdm2-S211 is slightly higher than that of mdm2-FL.

These results suggest that the kinetic mechanism and constants are quite comparable between mdm2-FL and mdm2-S211, indicating that truncated mdm2, lacking the *N*-terminal p53 binding pocket, appears to be functionally equivalent to mdm2-FL in catalyzing p53 ubiquitination.

Binding of mdm2 Constructs to Full Length p53. To further test our hypothesis that mdm2 contains a second p53 binding

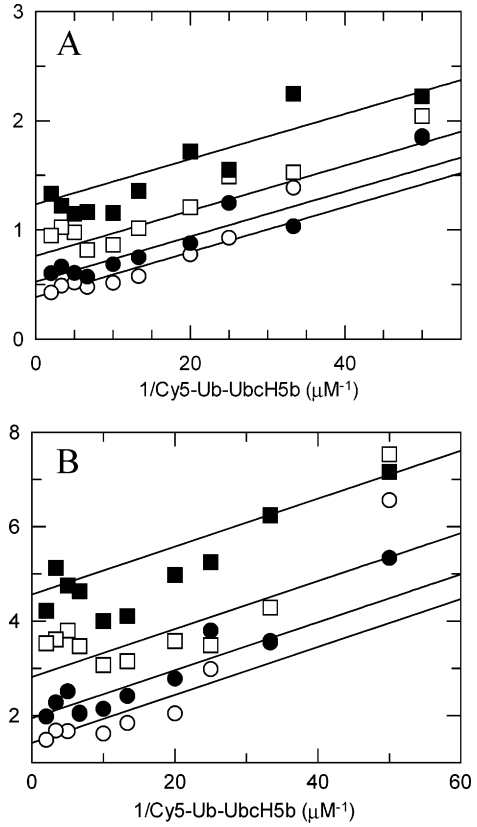

FIGURE 3: Kinetic analysis of Ub transfer to p53. The velocities of p53-Ub_n formation were determined as a function of Cy5-Ub−UbcH5b at several fixed concentrations of p53 catalyzed by mdm2-FL (A) or mdm2-S211 (B). The lines represent the global fitting of the entire data set to eq 1 using Grafit (p53 concentrations: ○, 2 μM; ●, 0.8 μM; □, 0.4 μM; ■, 0.2 μM).

site that is required for Ub transfer to p53, we carried out GST pull-down experiments to probe the direct interaction between mdm2-FL or deletion mutants and p53. Our results show that mdm2-S211 can bind p53 in a similar manner or in a manner only slightly weaker than that of mdm2-FL (Figure 4A and B). Similar to our kinetic result, mdm2-E285 can also bind p53 but with much reduced affinity. Mdm2-D361 does not bind to p53 because the amount of the p53 pull-down is no higher than the GST-E1 negative control used in these studies. Therefore, we conclude that the central region of mdm2 (acidic region and zinc finger; a.a. 211−361) appears to contain the second p53 binding site that is required for Ub transfer to p53. Although our GST pull-down experiments did not directly measure the binding affinity of the *N*-terminally truncated mdm2 constructs for p53, we estimate that the K_d value for this interaction is likely to be ≤ 1 μM, on the basis of the fact that it was maintained during a GST pull-down and multiple washing steps at sub-μM concentrations of both proteins.

Effect of Nutlin on mdm2-Mediated Ubiquitination of p53. On the basis of the crystal structure of mdm2 bound to a peptide from the transactivation domain of p53 (*5*), small molecule antagonists called Nutlins have been reported to inhibit the mdm2−p53 interaction leading to p53 stabilization (*32*). We tested Nutlin-3 for its effects on mdm2-mediated p53 ubiquitination and found it to be a weak inhibitor of the ubiquitin ligase activity of mdm2 (IC_{50} ~6−10 μM) in contrast to its effect on blocking the *N*-terminal mdm2−p53 interaction (IC_{50} reported to be 0.09 μM (*32*)). To further investigate the effects of Nutlin-3 on mdm2-catalyzed p53 and autoubiquitination, we compared the IC_{50} values of this inhibitor using various truncated mdm2 mutants as the enzyme source. Our results show that Nutlin-3 inhibits mdm2-catalyzed p53 ubiquitination and autoubiquitination with IC_{50} values around 10 μM regardless of whether full length or various truncated mdm2 constructs were used as the enzyme source. Therefore, it is likely that the inhibitory activity of Nutlin-3 in the ubiquitination assay is nonspecific. As we have demonstrated that the *N*-terminal p53 binding domain of mdm2 is not critical for mdm2-catalyzed p53 ubiquitination, it is not surprising that Nutlin-3 is not very active as an inhibitor of mdm2-catalyzed p53 ubiquitination.

DISCUSSION

We have shown that the *N*-terminal domain of mdm2 is not required for its ubiquitination activity toward p53. Using GST pull-down, we have further demonstrated that there is a second p53 binding site on mdm2, which includes the acidic domain and the zinc finger domain. This second interaction is critical for the mdm2-mediated ubiquitination of p53. We have also confirmed that the intact RING finger domain alone of mdm2 is sufficient for its autoubiquitination activity.

The region of mdm2 critical for complex formation with p53 was originally mapped to the *N*-terminal domain by genetic and biochemical methods (*18*, *19*). The interaction between peptides derived from the p53 *N*-terminus and the *N*-terminal domain of mdm2 has been extensively studied (*5*, *27*−*29*). The K_d values for this interaction were reported to be 60−700 nM depending on the length of the p53 peptides (*5*, *27*−*29*). However, there have also been reports of secondary binding sites on mdm2 for the p53 core domain that affect p53 ubiquitination (*34*, *40*). By using domain swapping between mdm2 and mdmX, Meulmeester et al. described that the central domain of mdm2 (202−302) is essential for p53 ubiquitination (*41*). Similar findings on the contribution of mdm2's central acidic domain to p53 ubiquitination were also reported by Kawai and co-workers (*42*). Recently, Yu et al. reported that the p53 core domain binds to peptides derived from mdm2's acidic domain and a part of its zinc finger domain, albeit with relatively weak affinity (100−300 μM) (*35*). Our results are consistent with the presence of a second p53 binding site on mdm2, located within a region spanning its acidic and zinc finger domains. However, the K_d value estimated from our GST pull-down experiment as well as the K_m value of p53 measured from the ubiquitination kinetic analysis are consistent with relatively high affinity (<1 μM) for this second binding site. It is possible that although the individual peptides derived from the acidic domain and zinc finger of mdm2 have weak affinity toward p53 (*35*), there may be multiple contacts to form a specific site with higher affinity in binding to p53 within the central domain of mdm2. We have further shown that this second interaction is critical for the p53 ubiquitination activity of mdm2, whereas the interaction between the *N*-terminal domains of mdm2 and p53 is dispensable. Taken together, the data suggest that the p53 tetramer interacts with at least two sites on mdm2—the *N*-terminal domain of p53 binds to the *N*-terminal domain of mdm2, whereas the core domain of p53 binds to the central acidic domain of mdm2. Each of these interactions has a different

Table 2: Summary of Kinetic Constants (average from $n = 2$ determinations) for mdm2-Catalyzed Ubiquitin Transfer to P53[a]

	Ping Pong mechanism		RERBB[b] mechanism	
kinetic constant	mdm2-FL	mdm2-S211	mdm2-FL	mdm2-S211
Cy5-Ub−UbcH5b, K_m	70 ± 1.7 nM	40 ± 11 nM	100 ± 14 nM	51 ± 14 nM
p53, K_m	731 ± 109 nM	759 ± 150 nM	764 ± 101 nM	813 ± 115 nM
k_{cat}	1.8 ± 0.1 min^{-1}	3.2 ± 1.3 min^{-1}	1.8 ± 0.1 min^{-1}	3.2 ± 1.3 min^{-1}
α[d]	NA[c]	NA	332 ± 144	254 ± 72

[a] Each specific activity is the mean of two to three replicates ± SD. [b] RERBB, Rapid Equilibrium Random Bi Bi. [c] NA, not applicable to this mechanism. [d] α is the substrate cooperativity factor (e.g., the Michaelis constant is equal to α times the equilibrium dissociation constants when all other substrates are saturated).

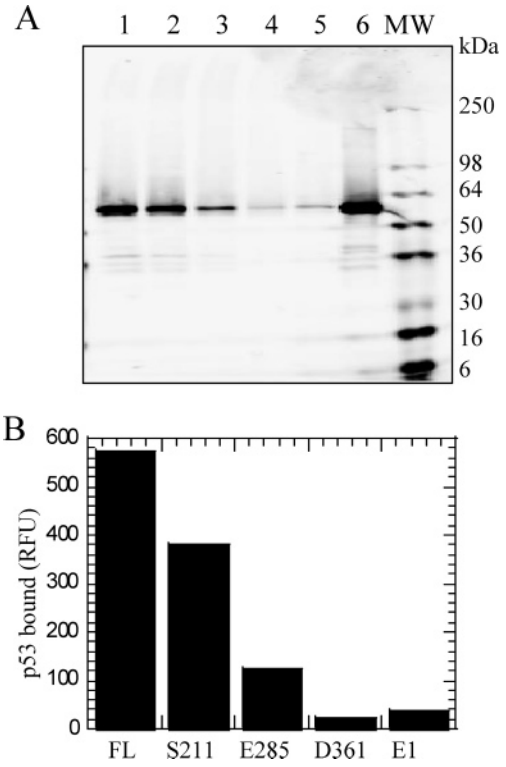

FIGURE 4: Binding of p53 to GST-mdm2 fusion proteins using GST pull-down. The preincubated mixture containing p53 and GST-mdm2, mutants, or GST-E1 were incubated with glutathione−sepharose 4B beads as described in Materials and Methods. (A) Binding of p53 to GST-mdm2 detected by western blot and quantified using the Odyssey Infrared Image system. Lane 1, mdm2-FL; lane 2, mdm2-S211; lane 3, mdm2-E285; lane 4, mdm2-D361; lane 5, GST-E1 control; lane 6, 1 pmol p53 protein as a control for antibody detection. MW, molecular mass markers. (B) Fluorescence intensity of bound p53 in western blot quantified using Odyssey application software and expressed as Relative Fluorescence Units (RFU).

functional consequence. Although the interaction between the *N*-termini blocks the transactivation of p53, the interaction between the central domain of mdm2 and the core domain of p53 is critical for the ubiquitination and degradation of p53.

The central domain of mdm2, including the acidic domain and the zinc finger region, provides the binding site for many interaction partners of mdm2, including p14Arf (*20*, *43*), L5 (*22*, *44*), L11 (*24*, *45*), L23 (*21*, *46*), TBP (*47*), and Rb (*48*). These interactions have been reported to inhibit mdm2-mediated p53 ubiquitination and degradation. One possible mechanism for the inhibitory action of these molecules is the direct blocking of the interaction of p53 with the central domain of mdm2 and, thus, interfering with mdm2-mediated p53 degradation. As Fersht and co-workers have pointed out (*35*), the central domain of mdm2 can also be phosphorylated at multiple sites, which may also affect the interaction between mdm2 and p53 and mdm2-mediated p53 ubiquintation and degradation.

Although the solution structure of the C4 zinc finger of mdm2 has recently been solved (*25*), the structure of the acidic domain remains unknown. Structural information on how the second p53 binding site on mdm2 interacts with the core domain of p53 will be extremely helpful toward an increased understanding of the molecular interactions between mdm2 and p53 as well as toward the design of inhibitors to regulate this interaction. It will be even more useful to gain structural insights on how full length p53 interacts with full length mdm2, how the primary and secondary binding sites are spatially arranged, and how they contribute to the binding energy of the mdm2−p53 interaction.

In the past few years, there have been intense efforts toward identifying small molecule modulators of the mdm2−p53 pathway by targeting the E3 ligase activity of mdm2 (*49*, *50*) or by inhibiting the interaction between the *N*-terminal domain of mdm2 and the *N*-terminal domain of p53 (*30*−*33*, *40*). Vassilev et al. reported the discovery of small molecule inhibitors, referred to as Nutlins, which bind to the *N*-terminal domain of mdm2 in a small hydrophobic cleft and block the interaction with p53 (*32*). In tumor cells with wild type p53, Nutlins activate p53, leading to cell cycle arrest, apoptosis, and growth inhibition of human tumor xenografts in nude mice. We have tested Nutlin-3 for its ability to inhibit mdm2-mediated p53 ubiquitination and found that the compound is a relatively poor inhibitor of this activity. This result is consistent with our demonstration that the interaction between the *N*-terminal domains of mdm2 and p53 is not required for the E3 ligase activity of mdm2. We thus postulate that Nutlins are likely to only disrupt the interaction between the *N*-terminal domain of mdm2 and p53 and may not dissociate fully the mdm2−p53 complex. However, because Nutlins can activate p53 in cells, it suggests that the interaction between mdm2 and p53 may be dynamic and that blocking the *N*-terminal domain of mdm2 suffices to allow the transactivation domain of p53 to interact with the transcriptional machinery and activate downstream genes.

In light of our determination that the second p53 binding site located in the central domain of mdm2 is critical for the E3 ubiquitin ligase activity of mdm2 toward p53, we propose that targeting this second binding site could be an alternative means of inhibiting the E3 ligase activity of mdm2, leading to p53 activation.

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REFERENCES

1. Harris, S. L., and Levine, A. J. (2005) The p53 pathway: positive and negative feedback loops, *Oncogene 24*, 2899−2908.
2. Lakin, N. D., and Jackson, S. P. (1999) Regulation of p53 in response to DNA damage, *Oncogene 18*, 7644−7655.
3. Levine, A. J. (1997) p53, the cellular gatekeeper for growth and division, *Cell 88*, 323−331.
4. Prives, C., and Hall, P. A. (1999) The p53 pathway, *J. Pathol. 187*, 112−126.
5. Kussie, P. H., Gorina, S., Marechal, V., Elenbaas, B., Moreau, J., Levine, A. J., and Pavletich, N. P. (1996) Structure of the MDM2 oncoprotein bound to the p53 tumor suppressor transactivation domain, *Science 274*, 948−953.
6. Momand, J., Zambetti, G. P., Olson, D. C., George, D., and Levine, A. J. (1992) The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation, *Cell 69*, 1237−1245.
7. Ashcroft, M., and Vousden, K. H. (1999) Regulation of p53 stability, *Oncogene 18*, 7637−7643.
8. Haupt, Y., Maya, R., Kazaz, A., and Oren, M. (1997) Mdm2 promotes the rapid degradation of p53, *Nature 387*, 296−299.
9. Kubbutat, M. H., Jones, S. N., and Vousden, K. H. (1997) Regulation of p53 stability by Mdm2, *Nature 387*, 299−303.
10. Fang, S., Jensen, J. P., Ludwig, R. L., Vousden, K. H., and Weissman, A. M. (2000) Mdm2 is a RING finger-dependent ubiquitin protein ligase for itself and p53, *J. Biol. Chem. 275*, 8945−8951.
11. Lai, Z., Ferry, K. V., Diamond, M. A., Wee, K. E., Kim, Y. B., Ma, J., Yang, T., Benfield, P. A., Copeland, R. A., and Auger, K. R. (2001) Human mdm2 mediates multiple mono-ubiquitination of p53 by a mechanism requiring enzyme isomerization, *J. Biol. Chem. 276*, 31357−31367.
12. Honda, R., Tanaka, H., and Yasuda, H. (1997) Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53, *FEBS Lett. 420*, 25−27.
13. Freedman, D. A., Wu, L., and Levine, A. J. (1999) Functions of the MDM2 oncoprotein, *Cell Mol. Life Sci. 55*, 96−107.
14. Roth, J., Dobbelstein, M., Freedman, D. A., Shenk, T., and Levine, A. J. (1998) Nucleo-cytoplasmic shuttling of the hdm2 oncoprotein regulates the levels of the p53 protein via a pathway used by the human immunodeficiency virus rev protein, *EMBO J. 17*, 554−564.
15. Brooks, C. L., Li, M., and Gu, W. (2004) Monoubiquitination: the signal for p53 nuclear export? *Cell Cycle 3*, 436−438.
16. Hershko, A., and Ciechanover, A. (1998) The ubiquitin system, *Annu. Rev. Biochem. 67*, 425−479.
17. Pickart, C. M., and Eddins, M. J. (2004) Ubiquitin: structures, functions, mechanisms, *Biochim. Biophys. Acta 1695*, 55−72.
18. Chen, J., Marechal, V., and Levine, A. J. (1993) Mapping of the p53 and mdm-2 interaction domains, *Mol. Cell. Biol. 13*, 4107−4114.
19. Freedman, D. A., Epstein, C. B., Roth, J. C., and Levine, A. J. (1997) A genetic approach to mapping the p53 binding site in the MDM2 protein, *Mol. Med. 3*, 248−259.
20. Bothner, B., Lewis, W. S., DiGiammarino, E. L., Weber, J. D., Bothner, S. J., and Kriwacki, R. W. (2001) Defining the molecular basis of Arf and Hdm2 interactions, *J. Mol. Biol. 314*, 263−277.
21. Dai, M. S., Zeng, S. X., Jin, Y., Sun, X. X., David, L., and Lu, H. (2004) Ribosomal protein L23 activates p53 by inhibiting MDM2 function in response to ribosomal perturbation but not to translation inhibition, *Mol. Cell. Biol. 24*, 7654−7668.
22. Marechal, V., Elenbaas, B., Piette, J., Nicolas, J. C., and Levine, A. J. (1994) The ribosomal L5 protein is associated with mdm-2 and mdm-2-p53 complexes, *Mol. Cell. Biol. 14*, 7414−7420.
23. Sui, G., Affar, e. B., Shi, Y., Brignone, C., Wall, N. R., Yin, P., Donohoe, M., Luke, M. P., Calvo, D., Grossman, S. R., and Shi, Y. (2004) Yin Yang 1 is a negative regulator of p53, *Cell 117*, 859−872.
24. Zhang, Y., Wolf, G. W., Bhat, K., Jin, A., Allio, T., Burkhart, W. A., and Xiong, Y. (2003) Ribosomal protein L11 negatively regulates oncoprotein MDM2 and mediates a p53-dependent ribosomal-stress checkpoint pathway, *Mol. Cell. Biol. 23*, 8902−8912.
25. Yu, G. W., Allen, M. D., Andreeva, A., Fersht, A. R., and Bycroft, M. (2006) Solution structure of the C4 zinc finger domain of HDM2, *Protein Sci. 15*, 384−389.
26. Honda, R., and Yasuda, H. (2000) Activity of MDM2, a ubiquitin ligase, toward p53 or itself is dependent on the RING finger domain of the ligase, *Oncogene 19*, 1473−1476.
27. Lai, Z., Auger, K. R., Manubay, C. M., and Copeland, R. A. (2000) Thermodynamics of p53 binding to hdm2(1-126): effects of phosphorylation and p53 peptide length, *Arch. Biochem. Biophys. 381*, 278−284.
28. Sakaguchi, K., Saito, S., Higashimoto, Y., Roy, S., Anderson, C. W., and Appella, E. (2000) Damage-mediated phosphorylation of human p53 threonine 18 through a cascade mediated by a casein 1-like kinase. Effect on Mdm2 binding, *J. Biol. Chem. 275*, 9278−9283.
29. Schon, O., Friedler, A., Bycroft, M., Freund, S. M., and Fersht, A. R. (2002) Molecular mechanism of the interaction between MDM2 and p53, *J. Mol. Biol. 323*, 491−501.
30. Duncan, S. J., Gruschow, S., Williams, D. H., McNicholas, C., Purewal, R., Hajek, M., Gerlitz, M., Martin, S., Wrigley, S. K., and Moore, M. (2001) Isolation and structure elucidation of Chlorofusin, a novel p53-MDM2 antagonist from a Fusarium sp, *J. Am. Chem. Soc. 123*, 554−560.
31. Stoll, R., Renner, C., Hansen, S., Palme, S., Klein, C., Belling, A., Zeslawski, W., Kamionka, M., Rehm, T., Muhlhahn, P., Schumacher, R., Hesse, F., Kaluza, B., Voelter, W., Engh, R. A., and Holak, T. A. (2001) Chalcone derivatives antagonize interactions between the human oncoprotein MDM2 and p53, *Biochemistry 40*, 336−344.
32. Vassilev, L. T., Vu, B. T., Graves, B., Carvajal, D., Podlaski, F., Filipovic, Z., Kong, N., Kammlott, U., Lukacs, C., Klein, C., Fotouhi, N., and Liu, E. A. (2004) In vivo activation of the p53 pathway by small-molecule antagonists of MDM2, *Science 303*, 844−848.
33. Zhao, J., Wang, M., Chen, J., Luo, A., Wang, X., Wu, M., Yin, D., and Liu, Z. (2002) The initial evaluation of nonpeptidic small-molecule HDM2 inhibitors based on p53-HDM2 complex structure, *Cancer Lett. 183*, 69−77.
34. Shimizu, H., Burch, L. R., Smith, A. J., Dornan, D., Wallace, M., Ball, K. L., and Hupp, T. R. (2002) The conformationally flexible S9-S10 linker region in the core domain of p53 contains a novel MDM2 binding site whose mutation increases ubiquitination of p53 in vivo, *J. Biol. Chem. 277*, 28446−28458.
35. Yu, G. W., Rudiger, S., Veprintsev, D., Freund, S., Fernandez-Fernandez, M. R., and Fersht, A. R. (2006) The central region of HDM2 provides a second binding site for p53, *Proc. Natl. Acad. Sci. U.S.A. 103*, 1227−1232.
36. Li, S. J., and Hochstrasser, M. (1999) A new protease required for cell-cycle progression in yeast, *Nature 398*, 246−251.
37. Wee, K. E., Lai, Z., Auger, K. R., Ma, J., Horiuchi, K. Y., Dowling, R. L., Dougherty, C. S., Corman, J. I., Wynn, R., and Copeland, R. A. (2000) Steady-state kinetic analysis of human ubiquitin-activating enzyme (E1) using a fluorescently labeled ubiquitin substrate, *J. Protein Chem. 19*, 489−498.
38. Auger, K. R., Copeland, R. A., and Lai, Z. (2005) Quantitative assays of mdm2 ubiquitin ligase activity and other ubiquitin-utilizing enzymes for inhibitor discovery, *Methods Enzymol. 399*, 701−717.
39. Copeland, R. A. (2005) *Evaluation of Enzyme Inhibitors in Drug Discovery. A Guide for Medicinal Chemists and Pharmacologists*, John Wiley & Sons, Hoboken, NJ.
40. Argentini, M., Barboule, N., and Wasylyk, B. (2001) The contribution of the acidic domain of MDM2 to p53 and MDM2 stability, *Oncogene 20*, 1267−1275.
41. Meulmeester, E., Frenk, R., Stad, R., de Graaf, P., Marine, J. C., Vousden, K. H., and Jochemsen, A. G. (2003) Critical role for a central part of Mdm2 in the ubiquitylation of p53, *Mol. Cell. Biol. 23*, 4929−4938.
42. Kawai, H., Wiederschain, D., and Yuan, Z. M. (2003) Critical contribution of the MDM2 acidic domain to p53 ubiquitination, *Mol. Cell. Biol. 23*, 4939−4947.
43. Lohrum, M. A., Ashcroft, M., Kubbutat, M. H., and Vousden, K. H. (2000) Contribution of two independent MDM2-binding domains in p14(ARF) to p53 stabilization, *Curr. Biol. 10*, 539−542.
44. Dai, M. S., and Lu, H. (2004) Inhibition of MDM2-mediated p53 ubiquitination and degradation by ribosomal protein L5, *J. Biol. Chem. 279*, 44475−44482.

45. Lohrum, M. A., Ludwig, R. L., Kubbutat, M. H., Hanlon, M., and Vousden, K. H. (2003) Regulation of HDM2 activity by the ribosomal protein L11, *Cancer Cell 3*, 577−587.
46. Jin, A., Itahana, K., O'Keefe, K., and Zhang, Y. (2004) Inhibition of HDM2 and activation of p53 by ribosomal protein L23, *Mol. Cell. Biol. 24*, 7669−7680.
47. Leveillard, T., and Wasylyk, B. (1997) The MDM2 C-terminal region binds to TAFII250 and is required for MDM2 regulation of the cyclin A promoter, *J. Biol. Chem. 272*, 30651−30661.
48. Sdek, P., Ying, H., Chang, D. L., Qiu, W., Zheng, H., Touitou, R., Allday, M. J., and Xiao, Z. X. (2005) MDM2 promotes proteasome-dependent ubiquitin-independent degradation of retinoblastoma protein, *Mol. Cell 20*, 699−708.
49. Lai, Z., Yang, T., Kim, Y. B., Sielecki, T. M., Diamond, M. A., Strack, P., Rolfe, M., Caligiuri, M., Benfield, P. A., Auger, K. R., and Copeland, R. A. (2002) Differentiation of Hdm2-mediated p53 ubiquitination and Hdm2 autoubiquitination activity by small molecular weight inhibitors, *Proc. Natl. Acad. Sci. U.S.A. 99*, 14734−14739.
50. Yang, Y., Ludwig, R. L., Jensen, J. P., Pierre, S. A., Medaglia, M. V., Davydov, I. V., Safiran, Y. J., Oberoi, P., Kenten, J. H., Phillips, A. C., Weissman, A. M., and Vousden, K. H. (2005) Small molecule inhibitors of HDM2 ubiquitin ligase activity stabilize and activate p53 in cells, *Cancer Cell 7*, 547−559.

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