Inhibition of p53 DNA binding function by the MDM2 acidic domain

Brittany Cross¹, Lihong Chen¹, Qian Cheng¹, Baozong Li¹, Zhi-Min Yuan², and Jiandong Chen¹* ¹Molecular Oncology Department, Moffitt Cancer Center, 12902 Magnolia Drive, Tampa, FL 33612, U.S.A. ²Department of Radiation Oncology, University of Texas Health Science Center, 7703 Floyd Curl Drive, San Antonio, TX 78229, U.S.A.

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*Address correspondence to: Jiandong Chen (jiandong.chen@moffitt.org, 813-745-6822)

MDM2 regulates p53 predominantly by promoting p53 ubiquitination. However, ubiquitination-independent mechanisms of MDM2 have also been implicated. Here we show that MDM2 inhibits p53 DNA binding activity in vitro and in vivo. MDM2 binding promotes p53 to adopt a mutant-like conformation, losing reactivity to antibody Pab1620, while exposing the Pab240 epitope. The acidic domain of MDM2 is required to induce p53 conformational change and inhibit p53 DNA binding. ARF binding to the MDM2 acidic domain restores p53 wild type conformation and rescues DNA binding activity. Furthermore, histone methyl transferase SUV39H1 binding to the MDM2 acidic domain also restores p53 wild type conformation and allows p53-MDM2-SUV39H1 complex to bind DNA. These results provide further evidence for an ubiquitination-independent mechanism of p53 regulation by MDM2, and reveal how MDM2-interacting repressors gain access to p53 target promoters and repress transcription. Furthermore, we show that the MDM2 inhibitor Nutlin cooperates with the proteasome inhibitor Bortezomib by stimulating p53 DNA binding and transcriptional activity, providing a rationale for combination therapy using proteasome and MDM2 inhibitors.

The tumor suppressor protein p53 encodes a transcription factor that plays a critical role in preventing malignant transformation by inducing cell-cycle arrest, DNA repair or apoptosis in response to various types of damage. Mutations or deletions in the p53 gene occur in over 50% of all human cancers, often in the centrally located sequence-specific DNA binding domain, resulting in the unfolding and accumulation of p53 in the nucleus (1-3). The p53 core domain has poor thermo stability at physiological temperatures and can undergo spontaneous denaturation (4-6). Changes in p53 conformation can inhibit the ability of p53 to induce expression of its downstream transcriptional targets (7,8).

P53 is maintained at low levels in unstressed cells with a short half-life. This is mainly achieved through the ability of MDM2 to bind p53 and act as an ubiquitin E3 ligase to promote its proteasomal degradation (9,10). MDM2 is also a transcriptional target of p53, forming an auto-regulatory feedback loop (11,12). The importance of MDM2 in the regulation of p53 is highlighted by the fact that mice deficient in MDM2 are embryonic lethal, while mice deficient for both MDM2 and p53 are viable (13,14). MDMX, a p53 binding partner with sequence homology to MDM2, is also an essential negative regulator of p53 (15). Unlike MDM2, MDMX lacks ubiquitin ligase activity and is unable to target p53 for proteasomal degradation (16,17). Recent studies suggest that MDMX is a bona fide p53 transcriptional target in certain cell types (17,18). MDMX can bind to p53 N-terminal transactivation domain and inhibit p53 transcription of target genes (19). MDMX-null mice are embryonic lethal despite expression of MDM2, suggesting a unique role for MDMX in the regulation of p53 (20). The prevailing view is that MDMX mainly functions by regulating p53 transcriptional activity, while MDM2 regulates p53 degradation (21,22).

MDM2 promotes p53 ubiquitination through a high affinity p53-binding domain at the N terminus, and a C terminal RING domain that recruits E2 ubiquitin conjugating enzymes. However, additional regions of MDM2 play critical roles in p53 ubiquitination and regulation. An intrinsically unstructured region near the RING domain contains multiple ATM phosphorylation sites important for DNA damage stabilization of p53 (23,24). The MDM2 acidic
domain has an important role in p53 ubiquitination (16,25). MDM2 degradation of p53 is regulated by the tumor suppressor ARF during mitogenic stress (26-28). ARF binds to the central acidic domain of MDM2, inhibiting its E3 ligase activity towards p53 and preventing MDM2-dependent nuclear export of p53 (29).

The MDM2 acidic domain also interacts with several transcription repressors, including YY1, KAP1 and SUV39H1 (30-32). These interactions suggest that MDM2 may, under some conditions, actively repress basal activity of p53 target genes by recruiting corepressors to promoters. Such a function would turn p53 from an activator to a repressor and expand its functional range, which is not achievable by regulating p53 degradation alone. An example of such an active mechanism is the regulation of E2F1 by pRb recruitment of HDAC and SUV39H1 to E2F1 target genes (33). In fact, previous studies showed that knockdown of KAP1 or SUV39H1 induced basal levels of p21 and MDM2 expression without affecting p53 level (31), indicating that MDM2 interactions with these repressors provide an additional level of control on p53 activity besides degradation.

Several reports suggest that MDM2 has additional non-degradation mechanisms for regulating p53 activity. A previous study showed that a temperature-sensitive p53 mutant does not bind DNA after forming a complex with MDM2 (34). EMSA experiments showed that full-length MDM2 does not interact with p53-DNA complex, suggesting that p53 interactions with DNA and MDM2 are mutually exclusive (35). However, a GST-MDM2-1-188 fragment was able to super-shift p53-DNA complex (36). More recent work shows that MDM2-hsp90 complex inhibits DNA binding by p53 in vitro and induces p53 unfolding (37). However, conflicting results suggest that MDM2 acts as a chaperone to promote p53 folding and stimulates p53 DNA binding in vitro (38). A recent study monitored p53 conformation under conditions in which MDM2-mediated degradation was inhibited and showed that MDM2 binding promotes conformational change, which preceded p53 ubiquitination and degradation (39). MDM2-mediated conformational change may expose lysine residues on p53 for ubiquitination, which can be opposed by overexpression of hsp90 (39,40).

MDM2 and p53 binding is mainly mediated by their N terminal domains. However it has been suggested that p53 has a second MDM2 interaction site (35,41). The central acidic region of MDM2 has also been shown to bind the p53 core domain and is sufficient to target p53 for ubiquitination in vitro (42,43). A biochemical study showed that purified ubiquitinated p53 does not bind DNA in vitro, and MDM2 can inhibit p53 DNA binding in vivo in an E3-dependent fashion (44). However, a MDM2 RING domain mutant still showed a measurable ability to inhibit p53 DNA binding in ChIP assay (44).

In this report we show that wild type p53-MDM2 complex does not bind DNA, and the MDM2 acidic region is responsible for promoting conformational change in p53 and inhibiting its DNA binding. Furthermore, these MDM2 functions are regulated by acidic domain binding partners such as ARF and SUV39H1. Our results suggest that ARF activates p53 in part by restoring its wild type conformation in the presence of MDM2. The histone methyltransferase SUV39H1 is targeted to p53 target promoters by binding MDM2 acidic domain and neutralizing its p53 conformational effect, forming a p53-MDM2-SUV39H1 complex capable of DNA binding and transcription repression.

**MATERIALS AND METHODS**

**Plasmids and cell lines.** MDM2, MDMX, p53, ARF and SUV39H1 constructs used in this study are of human origin. MDM2-MDMX hybrid constructs were described previously (25). Human pCIN4-HA-FLAG-p53 was kindly provided by Dr. Wei Gu (44). NARF6 (U2OS expressing IPTG inducible ARF) was provided by Dr. Dawn Quelle. MDM2 and MDMX deletion mutants were generated by PCR amplification and subcloning. H1299 (non-small cell lung carcinoma, p53-null), U2OS (osteosarcoma, wild-type p53), NARF6, SJSA, (osteosarcoma, wild-type p53, amplified MDM2), DLD1 (colon carcinoma, mutant p53), HCT116 (human colon carcinoma, wild-type p53), and H1299-V138 (H1299 stably transfected with temperature-sensitive mutant p53-V138) were maintained in DMEM with 10% fetal bovine serum.
Transfection of H1299 cells was performed using standard calcium phosphate precipitation protocol. MDM2 inhibitor Nutlin (Cayman) was used at 5–10 μM and the cells were incubated for 7 h. Proteasomal inhibitor Bortezomib (Selleck) was used at 50 nM (unless otherwise indicated) and the cells were incubated for 5 h.

**Western blot.** Cells were lysed in lysis buffer (50 mM Tris-HCl [pH 8.0], 5 mM EDTA, 150 mM NaCl, 0.5% NP-40, 1 mM phenylmethylsulfonyl fluoride), centrifuged for 10 minutes at 14,000 x g and the insoluble debris was discarded. Cell lysate (10 to 50 μg of protein) was fractionated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred to Immobilon P filters (Millipore). The filter was blocked for 1 h with phosphate-buffered saline (PBS) containing 5% nonfat dry milk, 0.1% Tween 20. The following antibodies were used: DO-1 for p53 (Pharmingen), 3G9 or 4B2 for MDM2, 10G11 or 8C6 for MDMX, 14P02 for ARF (NeoMarkers), mouse monoclonal antibody for FLAG tagged proteins and 9B11 for Myc tagged SUV (Cell Signaling). Bound primary antibody was detected by incubating for 1 h with horseradish peroxidase - goat anti -mouse Ig. The filter was developed using the ECL-plus reagent (Amersham Biosciences).

**Immunoprecipitation.** Cells were lysed in lysis buffer (50 mM Tris-HCl [pH 8.0], 5 mM EDTA, 150 mM NaCl, 0.5% NP-40, 1 mM phenylmethylsulfonyl fluoride), centrifuged for 10 minutes at 14,000 x g and the insoluble debris was discarded. Cell lysate (200-1000 μg of protein) was immunoprecipitated with 30 μl slurry of protein A agarose beads (Sigma) and MDM2 for 18 h at 4°C. For immunoprecipitation using conformation-specific p53 antibodies, the lysate was divided into equal halves and each immunoprecipitated for 18 h at 4°C with wild-type conformation specific antibody (Pab1620) or mutant conformation specific antibody (Pab240). For immunoprecipitation of FLAG tagged proteins the lysate was incubated with 50 μl slurry of M2-agarose beads overnight at 4°C (Sigma). The following antibodies were used to detect immunoprecipitated proteins by western blot: FL393 for p53 (Santa Cruz) and rabbit polyclonal antibodies for MDM2, MDMX and FLAG or Myc-tagged proteins.

**Chromatin Immunoprecipitation.** Chromatin immunoprecipitation was carried out by using a published procedure (64). Protein-DNA complexes from transiently transfected H1299 cells were immunoprecipitated with DO-1 antibody for p53 or 9B11 for Myc-tagged proteins and a 40 μl slurry of protein A agarose/salmon sperm DNA beads (Millipore). Coprecipitated chromatin was analyzed by quantitative PCR using primers (5’AGAAAGGGATGGTAGGAGA and 5’ACAAAGCACACATGCATC) to amplify the human p21 promoter and (5’CTGTGCGCTTTGCTGTGAGTAC and 5’CTAGCCCCAGGCAAGAGGAC) to amplify the human PUMA promoter, both containing p53 binding site. The readouts were normalized using 10% input chromatin for each sample.

**Purification of MDM2/MDMX associated p53 and DNA Affinity Immunoblotting.** H1299 cells were transiently transfected with FLAG tagged MDM2, MDMX and p53. Cells from a 10 cm plate were lysed in 1 ml lysis buffer [50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 150 mM NaCl, 0.5% NP-40, 1 mM phenylmethylsulfonyl fluoride], centrifuged for 10 minutes at 14,000 x g and the insoluble debris was discarded. The lysate was incubated with 40 μl slurry of M2-agarose beads (Sigma) for 18 h at 4°C. The beads were washed with lysis buffer and the FLAG-tagged proteins with their binding partners were eluted with 150 μl of lysis buffer containing 50 μg/ml FLAG epitope peptide (Sigma) for 2 h at 4°C. An aliquot of the eluted proteins was analyzed for expression levels by western blot. Lysate containing equal levels of p53 was added to a 200 μl DNA binding reaction mixture and incubated at 4°C for 30 min. The DNA binding reaction mixture contains 25 nM (0.01 nmole) double-stranded biotinylated oligonucleotide DNA representing the p53 binding site at the p21 promoter (Biotin-5’-TCGAGAGGCATGTCTAGGCATGTCTC annealed with 5’-GAGACATGCTAGACATGCCTTCGA), 2 μg poly(dI•dC), 5 mM DTT, 150 mM NaCl, 20
mM Tris-HCl (pH 7.2), 1 mM EDTA, 0.1% Triton X-100, and 4% glycerol. Mutant control oligonucleotide contains 5’-TCGAGAGGTCGCTCTAGGTCGCTCTC annealed with 5’-GAGAGCGACCTAGAGCGACCTCTCGA. The DNA/protein complexes were captured with 0.1 mg of magnetic Streptavidin beads (Promega) at 4°C for 30 min. The beads were collected using a magnet and washed 3 times with DNA binding buffer. The bound proteins were eluted by boiling in sample buffer [4% SDS, 20% glycerol, 200 mM DTT, 120 mM Tris (pH 6.8), 0.002% bromophenol blue]. The protein complexes were resolved by SDS-PAGE, and p53 was detected by western blot using DO-1 antibody.

RNA isolation and quantitative PCR. To determine the levels of p21, PUMA and GAPDH expression, total RNA was extracted using the Qiagen Rnease Mini Kit per the manufacturer instructions. cDNAs were prepared by reverse transcription of total RNA using the SuperScript III Invitrogen kit. The primers used for Sybrgreen quantitative PCR of human p21, PUMA and GAPDH mRNA are as follows. Human p21 forward: 5’ATGAAATTCACCCCCTTTCC; reverse: 5’AGGTGAGGGGACTCCAAAGT. PUMA forward: 5’TGTGGGTAGACCCAGTAAGG; reverse: 5’TGTGTTACTTCTGCCCTGCT. GAPDH forward: 5’GAGTCAACGGATTTGGTCGT; reverse: 5’GACAAGCTTCCCGTTCTCAG.

RESULTS

MDM2 inhibits p53 DNA binding

A previous study using the McKay assay suggests that MDM2-p53 complex does not bind DNA (34). However, several studies reported detection of MDM2 binding to p53 target gene promoters, indicating that the MDM2-p53 complex has DNA binding activity in vivo (45-49). To further investigate the effect of MDM2 on p53 DNA binding, we purified MDM2-p53 complex from H1299 cells cotransfected with p53 and FLAG-MDM2 using M2 beads for pull down and FLAG peptide for elution. The MDM2-p53 complex was incubated with biotinylated oligonucleotide DNA containing p53-binding sequence from the p21 promoter. P53-DNA complex was captured by streptavidin beads and analyzed by western blot. The analysis showed that p53 purified as FLAG-MDM2-p53 complex had very low DNA binding activity, whereas FLAG-p53 directly purified without MDM2 bound DNA efficiently (Figure 1a). In the same assay, purified FLAG-MDMX-p53 complex retained significant DNA binding, suggesting that only MDM2 has strong ability to inhibit p53 DNA binding. As a specificity control, FLAG-p53 did not bind to oligonucleotide with a mutated p53 consensus sequence.

To test whether MDM2 inhibits p53 DNA binding in vivo, H1299 cells were cotransfected with p53 and MDM2, treated with MG132 to block MDM2-mediated p53 degradation, and then analyzed by p53 ChIP and quantitative PCR (qPCR). Western blot of duplicate samples confirmed comparable expression of p53, MDM2 and MDMX levels (Figure 1b, lower panels). The p53 ChIP showed that coexpression of MDM2 significantly inhibited p53 binding to the p21 promoter and PUMA (not shown), whereas MDMX had a less dramatic effect. P21 protein expression was also significantly repressed by MDM2, compared to the modest repression by MDMX (Figure 1b, lower panels).

MDM2 acidic domain is critical for inhibiting p53 DNA binding

To identify the domain on MDM2 necessary for blocking p53 DNA binding, different C terminal mutants of FLAG-MDM2 were tested. The results showed that the MDM2 RING domain mutant (457S) was as active as wt MDM2. MDM2-1-300 (without the RING domain and zinc finger) still retained strong activity in blocking p53 DNA binding despite being expressed at lower levels. However, further deletion of the acidic domain (MDM2-1-200) completely abrogated the ability to inhibit p53 DNA binding (Figure 2a). In a control experiment, different C terminal truncation mutants of FLAG-MDMX were also tested. The results showed that full length or fragments of MDMX had no effect on p53 DNA binding despite forming complexes with p53 efficiently (Figure 2b). It is important to note that in this assay sample input was equalized for the amount
of co-precipitated p53. Some samples may have excess free MDM2, but is not expected to influence the result.

When p53 DNA binding in vivo was analyzed by ChIP in transfected cells, the effects of MDM2 mutants were consistent with the in vitro DNA pull down assay. Full-length MDM2 and MDM2-457S were both efficient in blocking p53 DNA binding. However, deletion of both acidic domain and RING domains (MDM2-1-200) completely restored p53 DNA binding (Figure 2c, Supplemental Figure S1a & S1b). Treatment with MG132 for 5 hours before ChIP analysis produced similar results (data not shown), suggesting that MDM2 can inhibit p53 DNA binding in vivo without targeting it for degradation. As expected, MDMX wild type or mutant showed no difference in regulating p53 DNA binding in ChIP assay, consistent with the in vitro DNA binding result (Figure 2d, Supplemental Figure S1c & S1d). These results showed that the MDM2 acidic domain has an important role in regulating p53 DNA binding.

The functional difference between MDM2 and MDMX acidic domains are consistent with their low sequence identity (28%) (Figure 1d). Despite their names, the net charges of the acidic domains also differ significantly (MDM2: -29 at pH7.0. MDMX: -14 at pH7.0). These features may be the basis for their different protein binding specificity.

**MDM2 but not MDMX induces p53 conformational change**

DNA binding by p53 requires proper folding of a conformation-labile core domain. To determine how MDM2 inhibits p53 DNA binding, we tested the ability of MDM2 and MDMX in inducing p53 conformational change. A recent study showed that MDM2 induces p53 conformational change as measured by reactivity to wild type-specific Pab1620 and mutant-specific Pab240, which recognize epitopes located in the p53 core domain (39). Using this assay, p53 was cotransfected with MDM2 or MDMX into H1299 cells, incubated without or with MG132 to prevent p53 degradation, and analyzed by Pab1620/Pab240 IP. The ratios of p53 with wild type and mutant conformation were determined by western blot with pan-specific p53 antibody FL393.

The results showed that p53 was predominantly in a wild type conformation when expressed alone (Figure 3a). Coexpression with MDM2 induced a significant switch from wild type to mutant conformation as reported previously (39). In addition, MDM2-457S RING mutant retained full effect, indicating that E3 ligase function was not required for inducing p53 conformational change. Furthermore, MDMX expression did not alter p53 conformation (Figure 3a). In the absence of MG132, p53 with mutant conformation did not accumulate, presumably because of subsequent ubiquitination and degradation by the proteasome (Figure 3a). In contrast, MDM2-457S-induced p53 conformational change was readily detectable in the absence of MG132 because it did not promote p53 ubiquitination and degradation (Figure 3a). These results showed that MDM2 inhibition of p53 DNA binding correlates with induction of conformational change before ubiquitination.

To test whether MDM2-induced p53 conformational change occurs with endogenous proteins, SJSA cells (MDM2 amplification) was analyzed in comparison to HCT116 cells (no MDM2 amplification). The result showed that whereas p53 was present mostly in wild type conformation in HCT116, >50% of endogenous p53 in SJSA was present in the mutant conformation (Figure 3b). In comparison, endogenous mutant p53 in DLD1 cells was nearly 100% in the mutant conformation. As another control, the temperature-sensitive p53-V138 mutant stably expressed in H1299 cells showed conformational switch between permissive and non-permissive temperatures as expected (Figure 3b).

To further test whether mutant conformation p53 in SJSA cells was due to interaction with overexpressed MDM2, cells were treated with Nutlin to disrupt p53-DMM2 binding. The result showed that Nutlin treatment not only increased total p53 level, but also increased the ratio of Pab1620-positive p53 over Pab240-positive p53 (Figure 3c). As expected, MDM2 coprecipitation with p53 was reduced, but not eliminated after Nutlin treatment, presumably due to the fact that Nutlin induces very high level MDM2 expression (Figure 3c, right panels).
Taken together, the data suggests that a direct interaction between endogenous MDM2 and p53 is needed to change p53 conformation.

**MDM2 acidic domain is critical for inducing p53 conformational change**

To map the MDM2 domain that mediates p53 conformational change, a panel of MDM2 deletion mutants were co-expressed with p53 in H1299 cells (Figure 4a). Conformational analysis of p53 showed that deletion of the MDM2 acidic domain (1-230, 1-200, Δ220-325) abrogated the conformational effect (Figure 4b). As expected, deleting the N terminal p53-binding domain (50-491, 230-491) also abrogated the activity, whereas mutations affecting the RING domain (1-440, 1-290, 457S) had no effect. The expression and binding between p53 and MDM2 mutants were confirmed by IP-western blot analysis (Figure 4c, 4d). Therefore, the MDM2 acidic domain has a critical role in inducing the p53 switch to mutant conformation, but requires targeting by the high-affinity N terminal p53-binding domain.

To further test the functional significance of the MDM2 acidic domain, we took advantage of the fact that MDMX has negligible activity in p53 conformational change. A panel of MDM2-MDMX hybrid constructs was analyzed for induction of p53 mutant conformation (Figure 5a & 5c). The results showed that although MDMX did not alter p53 conformation, replacing its acidic domain and C terminal half with the MDM2 counterpart conferred partial to full activity (Figure 5a & 5c, #4, #6). In contrast, transplanting the MDM2 RING domain alone to MDMX had no effect (Figure 5a & 5c, #8, #10). Additionally, the MDMX acidic domain had no activity if transplanted to MDM2 (Figure 5a & 5c, #3, #5). We should note that the phenotypes of #5 and #6 were somewhat ambiguous in multiple experiments, possibly because placement of the junction was not optimal for protein function. Overall, these results corroborate the MDM2 deletion analysis and reveal a critical role of the MDM2 acidic domain in p53 conformational change. Furthermore, the p53-binding domain of MDM2 simply provides a targeting function, which can be replaced by the MDMX N terminus.

We also attempted to determine whether sub regions of the MDM2 acidic domain are critical for p53 conformational change using a series of acidic domain small internal deletion mutants (Supplemental Figure S2a). The results showed that all small internal deletions partially abrogated the conformational effect (Supplemental Figure S2b & 2c). This suggests that different parts of the MDM2 acidic domain contribute to p53 conformational change in an additive fashion. Consistent with this notion, a study showed that the binding affinity of MDM2 acidic domain to p53 core is proportional to the length of the acidic domain tested (43).

Previous studies suggested that phosphorylation of MDM2 acidic domain on S256 is needed for efficient degradation of p53 (50). Our mass spectrometric analysis of MDM2 identified two additional phosphorylation sites S232 and S290 in the acidic domain (unpublished results). While S232A and S290A mutants had no obvious effect on p53 degradation, S256A mutation caused a moderate defect in p53 degradation as reported (data not shown). To test whether phosphorylation of these sites play a role in p53 conformational change, alanine substitution mutants were analyzed. The result showed no significant difference compared to wild-type MDM2 (Supplemental Figure S3), suggesting that acidic domain phosphorylation is not necessary for induction of p53 conformational change.

**Acidic domain-p53 binding correlates with p53 conformational change**

MDM2 acidic domain has been shown to interact weakly with p53 core domain (42,43,51). Whether MDMX acidic domain has such activity has not been reported. To further test whether the ability to induce p53 conformational switch correlates with p53 core domain binding, MDM2-100-361 and MDMX-100-361 fragments were tested for p53 binding after cotransfection into H1299 cells. The result showed that MDM2-100-361 coprecipitated with p53 at a clearly detectable level, although much weaker than full-length MDM2 as expected (Figure 5d). In contrast, MDMX-100-361 showed no interaction with p53. This result adds further evidence that the MDM2 acidic domain is unique compared to MDMX in its ability to bind p53, which may be why MDM2, not MDMX, promotes p53 misfolding and inhibits p53 DNA binding.
ARF prevents p53 conformational change and restores DNA binding

The ARF protein is critical for p53 activation in response to hyper proliferative stress through interacting with MDM2 acidic domain (52). Although the best-established effect of ARF is to cause p53 stabilization, we asked whether ARF also stimulate p53 DNA binding by interfering with the acidic domain of MDM2. When tested in the oligonucleotide DNA pull down assay, coexpression of ARF significantly rescued the DNA binding activity of p53-MDM2 complex (Figure 6a). Furthermore, ARF expression also stimulated p53 binding to p21 and PUMA promoters in vivo (Figure 6b, Supplemental Figure S4a & S4b). Western blots of duplicate samples were used to verify equal p53 expression and confirm expression of ARF and MDM2 (Supplemental Figure S4a, bottom panels).

Next, the effect of ARF on MDM2-mediated p53 conformational change was analyzed by co-transfection in H1299. The result showed that ARF blocked p53 conformational change in a dose-dependent fashion (Figure 6c). This occurred without affecting p53-MDM2 complex formation. In fact, ARF coprecipitated with p53 in the presence of MDM2, indicating formation of p53-MDM2-ARF trimeric complex (Figure 6c). Furthermore, restoring ARF expression in SJSA cells (ARF-negative, MDM2 amplification) by infection with ARF adenovirus partially reverted endogenous p53 to wild type conformation (Figure 6d). In additional experiments, expression of ARF in U2OS cells with IPTG-inducible ARF (NARF6 cells) also showed that endogenous p53 reverted back to wild-type conformation (Figure 6d). These results showed that ARF has a novel function in preventing p53 conformational change mediated by MDM2.

Since p53-MDM2-ARF trimeric complex was detectable after coexpression (Figure 6d), ARF may be recruited to DNA by binding to MDM2 and keeping p53 in a wild type conformation. As expected, ChIP analysis using Myc-tagged ARF showed that it was detected at the p21 and PUMA promoters in an MDM2 and p53-dependent manner (Supplemental Figure S4c, S4d). Besides restoring p53 DNA binding, it remains to be determined whether chromatin recruitment of ARF has other functional consequence.

SUV39H1 blocks p53 conformational change to access p53 target promoter

The MDM2 acidic domain interacts with several transcription repressors including YY1, KAP1 and SUV39H1. These interactions suggest that MDM2 may actively repress p53 targets by recruiting corepressors to promoters. We hypothesized that similar to ARF, co-repressors may also prevent MDM2 acidic domain from blocking p53 DNA binding, thus allowing recruitment of the p53-MDM2-repressor complex to promoters and repress transcription.

We tested this hypothesis using SUV39H1 as an example due to its efficient MDM2 binding (32). In vitro DNA binding assay showed that co-expression of SUV39H1 with MDM2 partially restored p53 DNA binding function (Figure 7a). Co-expression of SUV39H1 with MDM2 and p53 also led to an increase in p53-DNA binding in vivo (Figure 7b, Supplemental Figure S6a & S6b). In a p53 conformational analysis, overexpression of SUV39H1 partially inhibited the ability of MDM2 to induce p53 mutant conformation (Figure 7c). Co-precipitation of p53, MDM2 and SUV39H1 indicated that these proteins also formed a trimeric complex (Figure 7c). SUV39H1 ChIP analysis showed that coexpression of p53 and MDM2 promoted SUV39H1 recruitment to p21 and PUMA promoters (Figure 7d, Supplemental Figure S6c). Additional attempts to detect recruitment of endogenous SUV39H1 to the p21 and PUMA promoters by p53 and MDM2 were not informative, possibly limited by the SUV39H1 antibody. These results showed that similar to ARF, SUV39H1 binding to MDM2 acidic domain prevents p53 misfolding by MDM2, thus allowing the trimeric repressive complex to bind p53 target promoters.

Inhibition of MDM2 cooperates with Bortezomib to activate p53

Given the observation that p53 accumulated after proteasome inhibition is
partially misfolded due to MDM2 binding, we asked whether this phenomenon has clinical relevance. The proteasome inhibitor Bortezomib (Velcade) induces cell death independent of p53 (53). Our results suggest that proteasome inhibition stabilizes both p53 and MDM2, forming complexes that are partially deficient for DNA binding or transcription activation. Thus the p53-mediated anti-tumor activity is not fully exploited by Bortezomib. If this is the case, Nutlin should cooperate with Bortezomib to induce p53 targets by inhibiting MDM2.

When SJSA and U2OS cells were treated with Bortezomib, there was negligible or weak increase in protein levels of PUMA and p21. Combination of Bortezomib and Nutlin resulted in strong induction of PUMA and p21, without further increasing p53 level (Figure 8a). RT-PCR analysis showed that in MDM2-overexpressing SJSA cells, Bortezomib stabilized p53 but did not induce PUMA and p21 mRNA. Bortezomib and Nutlin combination induced PUMA and p21 mRNA to levels similar to Nutlin alone (Figure 8b). ChIP analysis showed that p53 DNA binding was moderately induced by Bortezomib in SJSA cells, and was further enhanced by Nutlin (Figure 8c). Similar results were observed in U2OS cells, although the phenotypes were less dramatic (Supplemental Figure S7), as expected from the lower level of MDM2 in this cell line. These results showed that p53 stabilized by Bortezomib has poor DNA binding and poor transcriptional activity due to interaction with MDM2. Inhibition of MDM2 restores the DNA binding and transcription functions of p53 in Bortezomib-treated cells.

Discussion

The experiments described above showed that MDM2 binding to p53 leads to its conformational change to a state similar to misfolded mutant p53. This activity does not require the ubiquitin ligase activity of MDM2, consistent with recent reports from the Maki lab (39,40). Furthermore, our study produced several new observations: (1) MDM2 inhibits p53 binding to DNA through an E3-independent mechanism. (2) The acidic domain of MDM2 is critical for inducing p53 misfolding and inhibiting p53 DNA binding. (3) The MDMX acidic domain does not bind p53 or induce p53 misfolding. (4) ARF prevents p53 misfolding by MDM2. (5) SUV39H1 binding to MDM2 acidic domain prevents p53 misfolding and allows its recruitment to p53 target promoters.

The detailed mechanism by which MDM2 binding induces p53 conformational change remains to be determined. The core domain of p53 is known to have poor thermo stability and spontaneously denatures at physiological temperature in vitro (4). Presumably, p53 in cells also exists in a dynamic equilibrium as wild type and mis-folded forms. A simple scenario is that the MDM2 acidic domain preferentially interacts with p53 at a mutant-like conformation with exposed internal residues. The presence of MDM2 will alter the p53 conformation equilibrium and trap it in a mutant conformation. It is also possible that MDM2 binding to p53 N terminus causes allosteric changes in its core domain, which is then trapped by subsequent interaction with the MDM2 acidic domain. Molecular chaperones that normally promote protein folding, such as hsp90, have been shown to antagonize the effect of MDM2 (39), possibly by competing with MDM2 acidic domain for p53 binding.

It is noteworthy that the use of conformation-sensitive p53 antibodies result in the arbitrary definition of wild type (Pab1620-positive) and mutant (Pab240-positive) conformational states. In reality, p53 is likely to also exist in many intermediate states between Pab1620 and Pab240-reactive conformations. After binding to MDM2, p53 may rapidly lose DNA binding activity before fully adopting a Pab240-positive conformation. Consistent with this notion, MDM2 inhibits p53 DNA binding in ChIP assay in the absence of MG132, when most p53 complexed with MDM2 are still Pab1620-positive. The MDM2-457SE3 mutant also efficiently inhibits p53 DNA binding in vivo while only switching a fraction of p53 to Pab240-positive conformation. Therefore, MDM2-induced p53 misfolding may be more efficient than the Pab240 reactivity suggests. This distinct function may enable MDM2 to act rapidly without relying on ubiquitination and degradation of p53, or serve as a backup against p53 deubiquitinating enzymes.

Our results underscore the importance of the MDM2 acidic domain in p53 regulation. The
The central region between residues 200-300 of MDM2 is a busy hub for binding multiple transcription corepressors and coactivators, and contains several phosphorylation sites (50,54). Important p53 activators such as ARF and many ribosomal proteins also bind to the acidic domain. The region is predicted to be structurally disordered, which is a pre-requisite for interaction with multiple partners by adopting different conformations (55). The acidic domain has also been shown to bind to p53 core domain, and our results add a new function to this interaction, which is blocking DNA binding by p53. Surprisingly, the MDMX central domain does not have this activity, probably because of low sequence homology to MDM2 acidic domain. In fact, most MDM2 acidic domain-binding partners do not bind MDMX (including ARF, L5, L11, L23, SUV39H1, EHMT1). Although it is not clear which is the primitive member of the MDM2/X family, we speculate that MDM2 may be an evolved version of MDMX that gained a multitude of new regulatory capabilities to control p53. Alternatively, MDMX may have evolved in a different direction to perform distinct but essential functions in p53 regulation.

The specific ability of MDM2 to switch p53 to a mutant conformation should enable more effective p53 inhibition than MDMX. This is consistent with findings in animal models that MDM2 is a more critical regulator of p53 than MDMX in adult organs (56,57). By simultaneously promoting p53 ubiquitination and blocking DNA binding, MDM2 efficiently neutralizes p53 function without entirely relying on its degradation. This function is further enhanced by recruitment of corepressors to p53 target genes that directly repress transcription. It is possible that p53 misfolding by MDM2 also serves to increase ubiquitination efficiency by increasing access to lysine residues in the core or C terminal domains. Although the novel activities of MDM2 acidic domain are most evident in overexpression assays, they are likely to be physiologically relevant, as shown using endogenous proteins in SJSAs cells.

Our results reveal novel mechanisms by which p53 can be regulated (Figure 9). The ARF tumor suppressor is best known for its ability to block p53 ubiquitination by MDM2. Here we showed that ARF binding to the MDM2 acidic domain also prevents p53 misfolding, and stimulates p53 DNA binding. ARF binding to MDM2 has been shown to promote stable beta sheet formation (58,59), which may trap MDM2 acidic domain in a conformation that cannot bind p53 core domain or induce p53 misfolding. It is still not clear how p53-MDM2-ARF complex can activate transcription after binding DNA. Possibly because p53 binds DNA as a tetramer, a fraction of the p53 molecules have N terminal domain that are not concealed by MDM2. Furthermore, p53-DNA binding may be more stable than p53-MDM2 interaction, eventually leaving free p53 on the DNA.

If MDM2-p53 complex does not bind DNA, why is endogenous MDM2 detected on p53 target promoters in tumor cells devoid of ARF (45-49)? Our results suggest that binding of MDM2 acidic domain by SUV39H1 has effects similar to ARF, preventing p53 misfolding and allowing p53-MDM2 complex to bind DNA. It is possible that other proteins that interact with the acidic domain of MDM2 also protect p53 from misfolding. Therefore, when MDM2 is detected on p53 target promoters, it is likely to be in complexes with acidic domain binding partners. In the case of p53 activators such as ARF and ribosomal proteins, these interactions promote p53 DNA binding and transcription activation. In the case of corepressors such as KAP1 and SUV39H1, the interactions allow their recruitment to p53 target promoters to repress transcription. Furthermore, ARF has been shown to displace KAP1 and SUV39H1 from MDM2 (31,32), suggesting that ARF binding to p53-MDM2 complex on chromatin may block the recruitment of corepressors by MDM2. These mechanisms enable the MDM2 acidic domain to function as an important signal conduit for both activators and inhibitors of p53.

Our findings also have obvious translational implications. The proteasome inhibitor Bortezomib was initially shown to induce cell death independent of p53 status (53). However, there is evidence that Bortezomib induces pro-apoptotic protein NOXA more efficiently in the presence of Wt p53 (60). Recent studies showed that Nutlin synergizes with Bortezomib to induce cell death in multiple myelomas. The molecular mechanism of this cooperation was either not investigated (61,62), or
was shown to be due to induction of p53 target genes (63). Our results suggest that proteasome inhibition stabilizes both p53 and MDM2, forming complexes that are partially deficient for DNA binding due to conformational switch by MDM2, or bind DNA but inactive for transcription due to MDM2 recruitment of repressors. As such, Nutlin cooperates with Bortezomib by relieving the repressive effects of MDM2, resulting in high-level expression of p53 target genes. This finding provides a molecular rationale for combination therapy using proteasome and MDM2 inhibitors against tumors that express wild type p53.

Acknowledgement

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Reference

Figure 1. Inhibition of p53 DNA binding by MDM2. (a) H1299 cells were transfected with FLAG-p53, or un-tagged p53 with FLAG-MDM2 or FLAG-MDMX plasmids. The protein complexes were purified using M2 Beads and eluted using a FLAG peptide. Elutes containing equal amounts of p53 were captured with biotinylated oligonucleotide DNA containing p53 binding site (wt) or a mutated sequence (mut). An aliquot of eluate was analyzed to confirm similar levels of p53 expression. (b) H1299 cells were transfected with p53, MDM2 or MDMX and analyzed by ChIP, followed by quantitative PCR (qPCR) to detect p53 DNA binding to the p21 promoter. WCE (whole cell extracts) of parallel samples were analyzed to confirm levels of protein expression. (c) Diagrams of MDM2 and MDMX domains. NLS: nuclear localization signal. Acidic: acidic domain. Zn: zinc finger. RING: RING domain. (d) Alignment of MDM2 and MDMX central acidic region showing the low degree of sequence identity (shaded boxes).

Figure 2. MDM2 acidic domain inhibits p53 DNA binding. (a) & (b) H1299 cells were transiently transfected and the FLAG-tagged protein complexes were immunopurified. Eluates containing equal amounts of p53 were captured with biotinylated oligonucleotide DNA containing p53 binding site. An aliquot of eluate was analyzed to confirm similar levels of p53 expression. (c) & (d) H1299 cells were transiently transfected with the indicated plasmids and analyzed by ChIP qPCR to detect p53 DNA binding to the p21 promoter.

Figure 3. MDM2 but not MDMX induces p53 conformational change. (a) H1299 cells were transfected with the indicated plasmids for 24 h and left untreated or incubated with 30 µM MG132 for 5 h. Cell lysates were immunoprecipitated with wild-type conformation specific (Pab1620) or mutant conformation specific (Pab240) antibodies. The precipitated p53 was detected by western blot. WCE were analyzed for protein levels. The amount of sample used for the IP was empirically adjusted to compensate for large changes in expression levels. P53-249S is a conformational mutant as positive control. (b) Cell lines expressing different levels of endogenous MDM2 and wt or mutant p53 were treated with 30 µM MG132 for 5 h and immunoprecipitated with Pab1620 or Pab240. Protein expression was confirmed by western blot of WCE. (c) SJSA cells were treated with 30 µM MG132 for 5 h with or without 5 µM Nutlin, and analyzed for p53 conformation by Pab1620 and Pab240 IP. MDM2 coprecipitated with p53 was detected by western blot.

Figure 4. Mapping the MDM2 domain that mediates p53 conformational change. (a) Diagram of MDM2 deletion and point mutants. (b) H1299 cells were transfected with the indicated plasmids, treated with MG132 (30 µM, 5 h) and analyzed by Pab1620 and Pab240 IP. (c) MDM2 mutants were co-transfected with p53 into H1299 cells. MDM2 was immunoprecipitated with a mixture of 4B2, 5B10 and 3G9 antibodies and co-precipitation of p53 was detected by western blot. Immunoprecipitation of MDM2 mutants were confirmed by western blot (bottom panel). (d) Expression of p53 and MDM2 mutations were confirmed by western blot of WCE.

Figure 5. Induction of p53 conformational change by MDM2-MDMX hybrid constructs. (a) H1299 cells were transfected with p53 and FLAG tagged MDM2-MDMX hybrid constructs. Cells were treated with MG132 (30 µM, 5 h) and immunoprecipitated with Pab1620 or Pab240 followed by western blot for p53. Co-immunoprecipitation of the hybrid proteins was detected by western blot. (b) Expression of p53 and FLAG-tagged MDM2-MDMX hybrid proteins were confirmed by western blot of WCE. (c) Diagram of MDM2-MDMX hybrid constructs and summary of results in (a). (d) The indicated FLAG tagged proteins were transiently co-transfected with p53 into H1299 cells. FLAG-DM2 or MDMX was immunoprecipitated using M2 Beads. Co-precipitated p53 was detected by western blot.
Figure 6. ARF inhibits p53 conformational change and restores p53-DNA binding. (a) H1299 cells were transiently transfected and the FLAG-tagged protein complexes were immunopurified. Eluates containing equal amounts of p53 were captured with biotinylated oligonucleotide DNA containing p53 binding site. (b) H1299 cells were transfected with the indicated plasmids and analyzed by ChIP-qPCR to detect p53 DNA binding to the p21 promoter. (c) H1299 cells were transfected with the indicated plasmids and increasing amounts of ARF. Cells were treated with MG132 (30 µM, 5 h) and analyzed by Pab1620 and Pab240 IP. Co-immunoprecipitation of ARF and MDM2 were detected by western blot. (d) SJSA cells were infected with adenovirus expressing ARF or GFP (MOI=50) for 24 h. Cells were treated with MG132 (30 µM, 5 h) and analyzed by Pab1620 and Pab240 IP. Co-immunoprecipitation of ARF and MDM2 were detected by western blot. Expression levels were confirmed by western blot of WCE (right panels).

Figure 7. SUV39H1 inhibits p53 conformational change and restores p53-DNA binding. (a) H1299 cells were transfected and the FLAG-tagged protein complexes were immunopurified. Eluates containing equal amounts of p53 were captured with biotinylated oligonucleotide DNA containing p53 binding site. (b) H1299 cells were transfected with the indicated plasmids and analyzed by ChIP-qPCR to detect p53 DNA binding to the p21 promoter. (c) H1299 cells were transfected with the indicated plasmids and increasing amounts of SUV39H1. Cells were treated with MG132 (30 µM, 5 h) and analyzed by Pab1620 and Pab240 IP. Co-immunoprecipitation of SUV39H1 and MDM2 were detected by western blot. (d) H1299 cells were transfected with Myc-SUV39H1, MDM2 and p53. Cells were analyzed by ChIP using anti-Myc antibody. SUV39H1 recruitment to p21 promoter by p53 and MDM2 was detected by qPCR of ChIP samples.

Figure 8. Nutlin activates p53 in Bortezomib-treated cells. (a) SJSA & (b) U2OS cells were treated with the indicated drugs and analyzed by western blot at indicated time points for expression of p53 targets. (b) SJSA cells were treated with 8 µM Nutlin and 50 nM Bortezomib for 5 h. Total RNA isolated from the cells was analyzed by quantitative RT-PCR. PUMA and p21 mRNA levels were normalized to GAPDH. (d) SJSA cells were treated with 8 µM Nutlin and 50 nM Bortezomib for 5 h. Cells were analyzed by p53 ChIP and qPCR of PUMA and p21 promoters.

Figure 9. A model for ubiquitin-independent regulation of p53 by MDM2. MDM2 binding to p53 leads to subsequent conformational change of p53 core domain, resulting in loss of DNA binding. ARF binding to MDM2 acidic domain inhibits ubiquitination of p53, but also prevents p53 conformational change and loss of DNA binding activity, resulting in stabilized p53 competent in transcription activation. SUV39H1 binding to MDM2 acidic domain prevents p53 conformational change, which allows p53-DM2-SUV39H1 trimeric complex to bind DNA and repress p53 target genes.
Figure 1

a) Oligonucleotide

- p53 bound to DNA
- Input FLAG-p53
- Input MDM2

b) p53 ChIP, p21 promoter PCR

- % Occupancy
- Comparison of p53, p53 + MDM2, p53 + MDMX

WCE p53
WCE p21

WCE FLAG (MDM2, MDMX)

c) p53 binding

- NLS, Acidic, Zn, RING

MDM2
1 100 200 300 400 491

MDMX
1 100 200 300 400 491

d) Sequence alignment

- Comparison of sequences from different proteins

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Figure 4

a) Schematic representation of the p53 conf. switch, p53 binding sites, and their corresponding acidic, Zn, and RING domains.


c) Immunoblot analysis of MDM2 and p53 IPs and MDM2 WB, with + and ++ indicating positive and strong positive signals.

d) Western blot analysis of MDM2 and p53 constructs with WCE and MDM2 WB.
Figure 5

[Diagram of experimental results showing bands for different conditions, with labels for IP, p53, M2/MX, GFP, and WCE, along with a table with columns for p53-binding, Acidic, Zinc, and RING, and a column for p53 conf. change with entries such as +, -, +/-, and +/-.]
Inhibition of p53 DNA binding function by the MDM2 acidic domain
Brittany Cross, Lihong Chen, Qian Cheng, Baozong Li, Zhi-Min Yuan and Jiandong Chen
J. Biol. Chem. published online March 17, 2011

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