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RESEARCH ARTICLE

Mutant p53 Sequestration of the MDM2 Acidic Domain Inhibits E3 Ligase Activity

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ABSTRACT Missense p53 mutants often accumulate in tumors and drive progression through gain of function. MDM2 efficiently degrades wild-type p53 but fails to degrade mutant p53 in tumor cells. Previous studies revealed that mutant p53 inhibits MDM2 autoubiquitination, suggesting that the interaction inhibits MDM2 E3 activity. Recent work showed that MDM2 E3 activity is stimulated by intramolecular interaction between the RING and acidic domains. Here, we show that in the mutant p53-MDM2 complex, the mutant p53 core domain binds to the MDM2 acidic domain with significantly higher avidity than wild-type p53. The mutant p53-MDM2 complex is deficient in catalyzing ubiquitin release from the activated E2 conjugating enzyme. An MDM2 construct with extra copies of the acidic domain is resistant to inhibition by mutant p53 and efficiently promotes mutant p53 ubiquitination and degradation. The results suggest that mutant p53 interferes with the intramolecular autoactivation mechanism of MDM2, contributing to reduced ubiquitination and increased accumulation in tumor cells.

KEYWORDS E3 ligase, MDM2, mutant p53, RING domain, acidic domain, conformation, ubiquitination

The p53 pathway is inactivated in the majority of human tumors. More than 50% of human tumors express p53 with missense mutations (1). Tumors retaining wild-type p53 often overexpress MDM2 and downregulate the expression of ARF, resulting in functional inactivation of p53. The majority of p53 mutations are missense substitutions in the DNA binding domain (core domain) that disrupt the p53-DNA interface (contact mutant) or cause misfolding (conformational mutant) (2). A unique feature of p53 mutants is their significant accumulation in tumor cells (3). Wild-type p53 is ubiquitinated by the E3 ligase MDM2 and rapidly degraded by the proteasome. DNA damage and various stress signals induce stabilization of wild-type p53, resulting in cell cycle arrest or apoptosis (4). In contrast, the turnover of mutant p53 in tumor cells is very slow, resembling that of wild-type p53 under stress. The accumulation of mutant p53 is thought to facilitate tumor development by promoting cell survival and invasion (5–7). This gain-of-function phenotype may involve interference in gene expression through binding to other transcription factors, such as p63, p73, NF-Y, and ETS (8, 9). Mice expressing mutant p53 have increased frequency of tumor metastasis compared to that in mice without p53, providing evidence of mutant p53 gain of function (6, 7).

Given the strong evidence of transforming activities of mutant p53, there is significant interest in targeting mutant p53 for cancer treatment. Knockdown of mutant p53 in tumor cell lines reduces their viability and invasive potential, suggesting that elimination of mutant p53 may have therapeutic benefits (10–12). Mutant p53s stably associate with heat shock proteins like hsp70 and hsp90 (13, 14). Misfolding of the mutated DNA binding core domain exposes hydrophobic regions, which may be recognized by molecular chaperones as denatured proteins. Previous studies showed that inhibitors of hsp90, such as geldanamycin, promoted mutant p53 degradation (15).

Citation Yang L, Song T, Cheng Q, Chen L, Chen J. 2019. Mutant p53 sequestration of the MDM2 acidic domain inhibits E3 ligase activity. Mol Cell Biol 39:e00375-18. https://doi.org/10.1128/MCB.00375-18.

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Received 25 July 2018
Returned for modification 30 August 2018
Accepted 13 November 2018
Accepted manuscript posted online 19 November 2018
Published 24 January 2019

Recent work showed that inhibition of hsp90 acetylation using histone deacetylase (HDAC) inhibitors promoted degradation of mutant p53 and conferred selective toxicity on tumor cells expressing mutant p53 (16, 17). Several other studies also described the identification of compounds that caused mutant p53 downregulation through different mechanisms (18, 19).

Numerous stress signals have been shown to cause wild-type p53 accumulation, mainly by blocking MDM2-mediated ubiquitination of p53. MDM2 promotes p53 degradation by forming a stable complex through N-terminal domains. The MDM2 C-terminal RING domain recruits ubiquitin-conjugating enzyme E2 that covalently modifies p53 lysine residues (20, 21). Phosphorylation of the p53 N terminus after DNA damage reduces MDM2 binding and contributes to p53 activation (22, 23). DNA damage also induces ATM-dependent phosphorylation of MDM2, which inhibits RING domain dimerization and p53 polyubiquitination (24–26). Oncogene activation induces the expression of ARF (alternative reading frame protein), which binds to MDM2 and inhibits p53 ubiquitination (27). Inhibition of nucleolar rDNA transcription promotes the release of ribosomal protein L11, which also binds to MDM2 and stabilizes p53 (28, 29). However, there is no evidence that these mechanisms are the main causes of mutant p53 stabilization in tumor cells.

We previously showed that mutant p53 overexpression caused MDM2 stabilization, suggesting that the MDM2 E3 function was inactivated after binding to mutant p53 (30). Constitutive binding between the misfolded mutant p53 and hsp90 appeared to be important for interference in MDM2 activity, since the hsp90 inhibitor geldanamycin promoted the degradation of mutant p53 and MDM2 (31). Recent studies showed that both MDM2 and another E3 ligase (CHIP) contributed to the degradation of mutant p53 upon hsp90 inhibition (32, 33). The mechanism of MDM2 inactivation by mutant p53 remained unresolved. Interestingly, mouse knock-in experiments revealed that mutant p53 was rapidly degraded by MDM2 in most (but not all) normal tissues and that it accumulated significantly only in tumor cells (34, 35). Therefore, unknown changes associated with malignant transformation facilitate the stabilization of mutant p53. Alterations in the molecular chaperones in tumor cells were suggested to promote mutant p53 accumulation (16).

Studies of wild-type p53 ubiquitination by MDM2 suggested that in addition to the p53-binding and RING domains, the central acidic domain (AD) of MDM2 (residues 220 to 300) was also critical for ubiquitination of p53 (36, 37). A small region of the MDM2 AD (residues 230 to 260) participates in an intramolecular interaction with the RING domain and stimulates the E3 ligase activity (38). The AD has features of a partially unstructured region that interacts with MDM2 regulators, such as ribosomal proteins and ARF (28, 39). The MDM2 AD has been shown to bind weakly to the p53 core domain (40–44). This interaction induces conformational change of wild-type p53 and exposes the Pab240 epitope, which is a feature of mutant p53. Therefore, it is possible that mutant p53 with a misfolded core domain has high affinity for binding MDM2 AD and interferes with its role in activating RING-mediated ubiquitination.

In this report, we investigate this hypothesis and present evidence that the mutant p53 core domain engaged in stable interaction with the MDM2 AD. The mutant p53-MDM2 complex was defective in promoting ubiquitin release from activated E2, consistent with loss of E3 ligase activity. MDM2 with extra copies of AD was resistant to inhibition by mutant p53 and efficiently promoted mutant p53 ubiquitination and degradation, suggesting that mutant p53 blocked MDM2 autoactivation. The results provide new insight on the mechanism of mutant p53 accumulation in tumors.

RESULTS

Mutant p53 inhibits MDM2 E3 ligase activity. A previous study showed that coexpression of mutant p53 and MDM2 caused MDM2 stabilization and inhibited MDM2 self-ubiquitination (30). This observation was reproduced in a cotransfection experiment in which a panel of p53 mutants (conformation mutants with mutations R175H [a change of R to H at position 175] and G245S and contact mutants with mutations R248Q, R273H, and D281G) induced accumulation of MDM2 after coexpres-

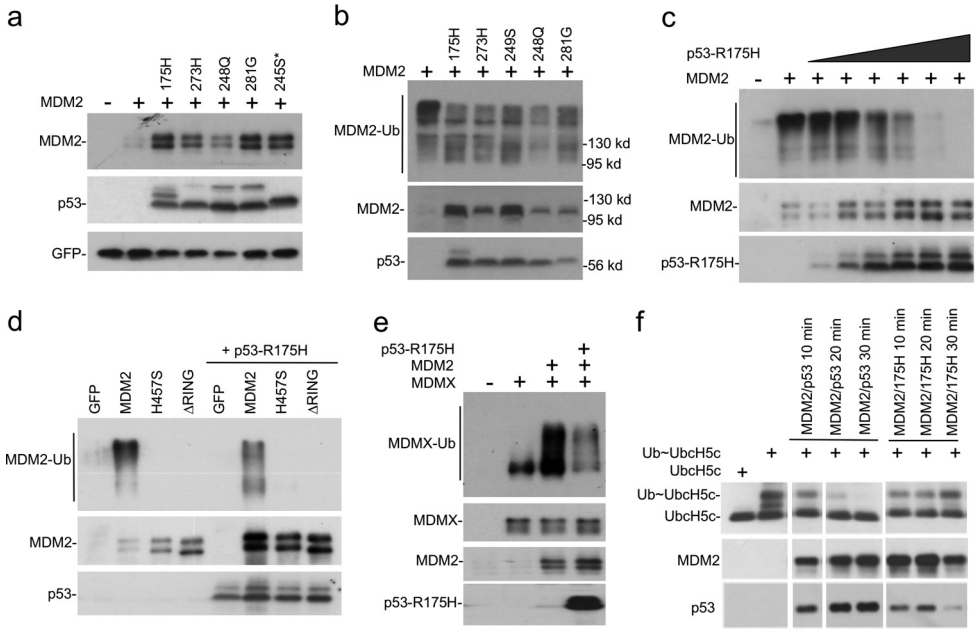

FIG 1 Mutant p53 inhibits MDM2 E3 ligase activity. (a) MDM2 was cotransfected with p53 mutants in H1299 cells for 48 h. Protein expression was detected by Western blotting. *, the G245S mutant contained N-terminal FLAG tag. (b) MDM2 was cotransfected with p53 mutants and His_6-ubiquitin in H1299 cells for 48 h. MDM2 self-ubiquitination was analyzed by Ni^{2+}-NTA pulldown and Western blotting. (c) Dose-dependent inhibition of MDM2 self-ubiquitination by mutant p53. (d) MDM2 self-ubiquitination was abrogated by point mutation (H457S) or deletion of the RING domain. (e) MDM2 ubiquitination of MDMX was inhibited by mutant p53. (f) Purified MDM2-p53 or MDM2-R175H mutant complex was incubated with charged E2. The release of ubiquitin from E2 was detected by Western blotting.

sion in p53-null H1299 cells (Fig. 1a). In an *in vivo* ubiquitination assay, coexpression of mutant p53 inhibited MDM2 self-ubiquitination in a dose-dependent manner (Fig. 1b and c), suggesting that MDM2 ubiquitin E3 ligase activity was inhibited. MDM2 ubiquitination *in vivo* was abrogated by point mutation or deletion of the RING, suggesting the assay detected self-ubiquitination rather than the activity of another E3 ligase (Fig. 1d). MDM2 is an E3 ligase for MDMX. The ability of MDM2 to promote MDMX ubiquitination was also inhibited by mutant p53 (Fig. 1e).

Ubiquitin E3 ligases recruit charged E2 to the proximity of substrates for ubiquitin transfer and also promote the release of activated ubiquitin from E2 to catalyze the transfer reaction (45). To test whether MDM2 in complex with mutant p53 has catalytic activity in promoting ubiquitin release, p53-MDM2 and R175H mutant-MDM2 complexes immunopurified from cotransfected cells were incubated with E2 charged with activated ubiquitin (Ub∼S-UbcH5c). Incubation of Ub∼S-UbcH5c with the p53-MDM2 complex resulted in time-dependent release of ubiquitin from the conjugate, whereas the R175H mutant-MDM2 complex was largely inactive for the ubiquitin release function (Fig. 1f), suggesting that MDM2 lost E3 activity when bound to mutant p53.

A protease-cleavable MDM2 protein for detecting domain interactions. The MDM2 acidic domain (AD) and RING domain engage in intramolecular interaction that stimulates the E3 activity (38). A minimal acidic domain (mAD) sequence of MDM2 (residues 230 to 260) is critical for this function. The MDM2 AD also interacts with the wild-type p53 core domain and induces a mutant-like conformational change in p53 (43). We hypothesized that the mutant p53 core domain in its default misfolded state may bind MDM2 AD with higher affinity and inhibit MDM2 E3 activity.

To analyze MDM2-mutant p53 domain interactions in a full-length MDM2-p53 complex, MDM2 was modified by inserting 3 PreScission protease cleavage sites and epitope tags into predicted disordered regions (Fig. 2a, MDM2GP; details in Materials and Methods). Cleavage of MDM2GP with PreScission produced fragments with epitope tags. A longer SQ-RING fragment (SQ designates the region with multiple ATM phosphorylation sites

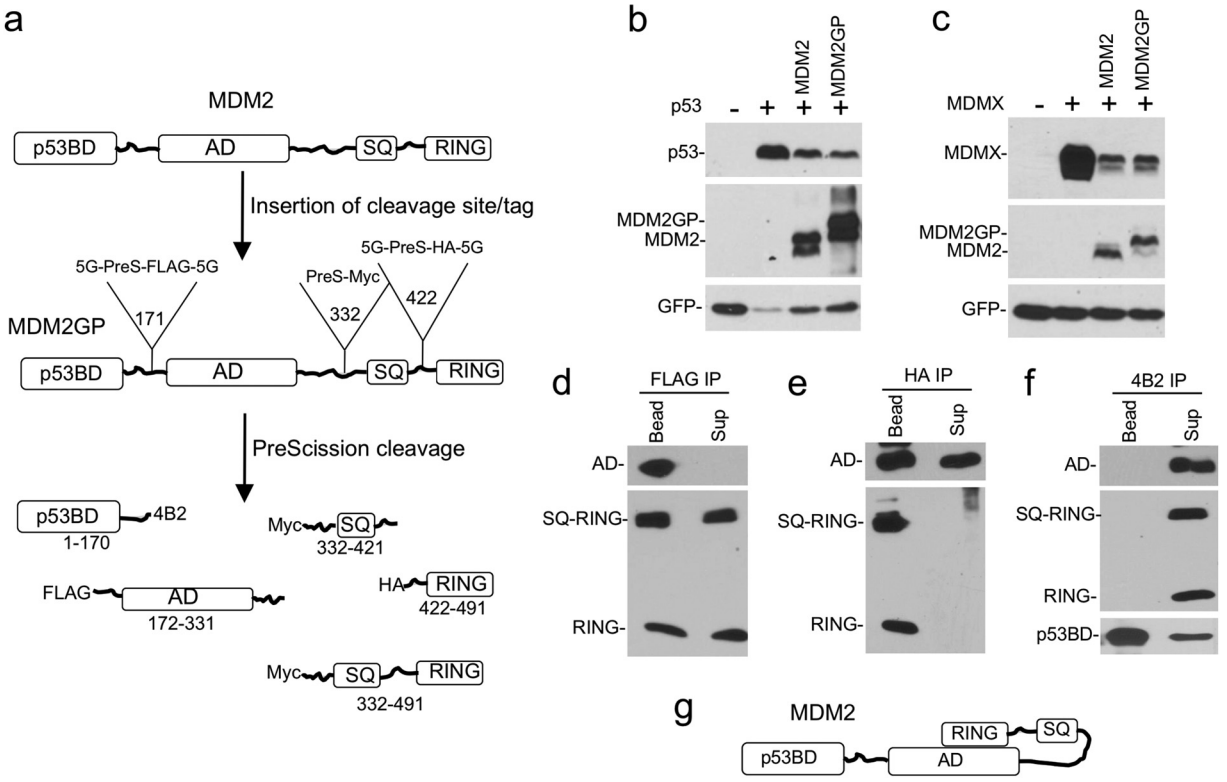

FIG 2 Construction of a cleavable MDM2 protein. (a) MDM2GP structure. PreScission cleavage site and epitope tags were inserted after residues 171, 332, and 422 of MDM2. (b, c) MDM2GP and MDM2 were cotransfected with p53 (b) or MDMX (c) in H1299 cells. The degradation of p53 and MDMX by MDM2 or MDM2GP was analyzed by Western blotting. (d) MDM2GP was immobilized on beads using anti-FLAG antibody and cleaved with PreScission for 1 h. SQ-RING and RING fragment dissociation from the immobilized AD was detected by HA Western blotting. IP, immunoprecipitation; Sup, supernatant. (e) MDM2GP was immobilized using HA antibody and cleaved with PreScission. AD fragment dissociation from the immobilized RING domain was detected by FLAG Western blotting. (f) MDM2GP was immobilized using anti-4B2 antibody and cleaved with PreScission. AD and RING fragment dissociation from the immobilized p53BD was detected by FLAG and HA Western blotting. (g) Model of intramolecular interaction between MDM2 AD and RING domain. SQ designates the region with multiple ATM phosphorylation sites (residues 386 to 429).

[residues 386 to 429]) was also produced due to incomplete cleavage between the SQ and RING regions. MDM2GP retained the ability to degrade p53 and MDMX in a cotransfection assay (Fig. 2b and c), suggesting that the modifications did not affect its function.

A previous study suggested that MDM2 AD and RING engage in intramolecular binding (Fig. 2g) (38). This interaction was confirmed using MDM2GP in a fragment release assay. Immobilization of MDM2GP by the FLAG epitope (located in the AD fragment) showed slow release of the RING and SQ-RING fragments after cleavage (Fig. 2d). Immobilization of the RING by the hemagglutinin (HA) epitope also resulted in slow release of AD (Fig. 2e). In contrast, immobilization using the N-terminal antibody 4B2 showed rapid release of AD and RING, suggesting that the MDM2 N terminus (p53 binding domain [p53BD]) did not form a stable intramolecular complex with the AD or RING (Fig. 2f).

Increased binding of mutant p53 to MDM2 AD and RING domains. To examine the domain interactions with p53, the p53/MDM2GP complex produced in H1299 cells was immobilized on beads using the anti-p53 antibody Pab421, followed by PreScission cleavage (Fig. 3a). The release of MDM2 fragments into the supernatant was analyzed. The MDM2 AD showed weak association with wild-type p53 after cleavage, since most of the AD fragment was released into the supernatant (Fig. 3b, top left). The RING and SQ-RING fragments showed moderate binding to wild-type p53 (Fig. 3b). The p53BD fragment was not detectable because it comigrated with a background band. Interestingly, the rates of release of AD, RING, and SQ-RING fragments from the R175H mutant complex were significantly slower than their rates of release from the wild-type p53

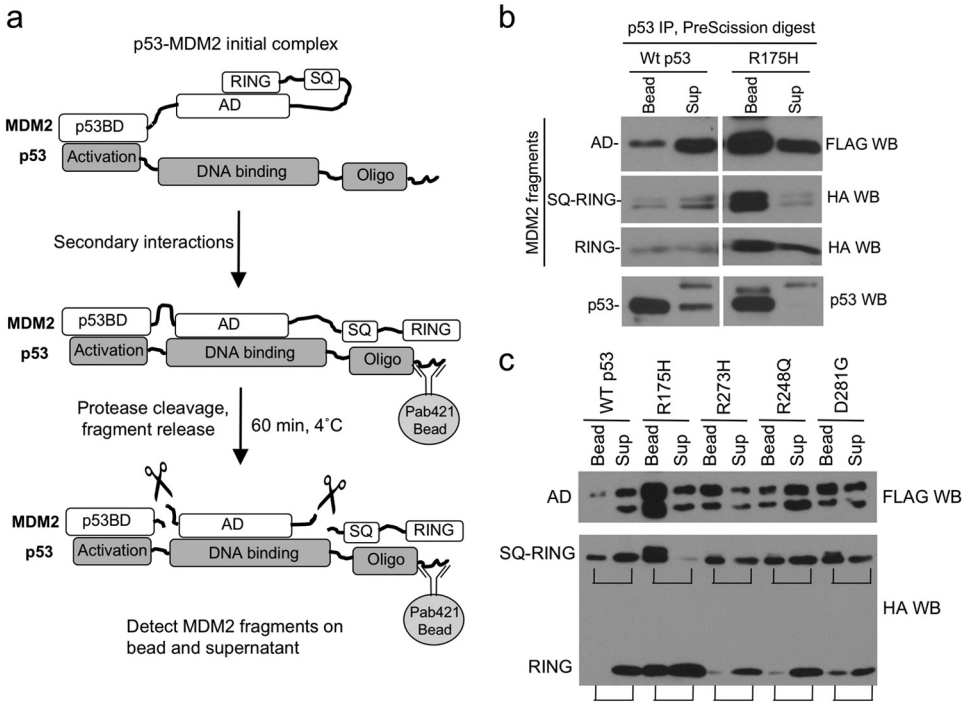

FIG 3 Increased binding of mutant p53 to MDM2 AD and RING domains. (a) Diagram of proteolytic fragment release (PFR) assay for detecting intermolecular interactions. MDM2GP-p53 complex from transfected H1299 cells was immobilized using anti-p53 antibody Pab421 conjugated to protein A beads. MDM2GP was cleaved by PreScission on the beads, and the release of MDM2 fragments was detected by Western blotting. (b) Comparison of MDM2 domain interactions with wild-type (Wt) and mutant p53. Wild-type p53 or R175H mutant was cotransfected with MDM2GP in H1299. MDM2GP-p53 complex was immobilized by Pab421 beads and cleaved by PreScission. MDM2 fragments that remained bound to the beads or dissociated into the supernatant were analyzed by Western blotting (WB). (c) Comparison of MDM2 domain interactions with wild-type and additional p53 mutants using the fragment release assay.

complex (Fig. 3b, right). This result suggests that the R175H mutant has stronger binding to the MDM2 AD fragment (residues 171 to 332) and RING domain (residues 422 to 491) than does wild-type p53. Two contact mutants (the R273H and D281G mutants) also showed increased binding to the MDM2 AD and RING, although this was less pronounced than for the R175H conformational mutant (Fig. 3c). The R248Q contact mutant had the weakest effect. The detection of strong RING binding by the R175H mutant in the fragment release assay was consistent with a previous coimmunoprecipitation (co-IP) analysis showing that MDM2 bound the R175H mutant differently than wild-type p53, with the RING contributing significantly to the R175H mutant's binding (33).

A minimal region of MDM2 AD (mAD [residues 230 to 260]) is important for stimulating the RING domain E3 ligase activity. To further test whether mutant p53 binds to the MDM2 mAD, a green fluorescent protein (GFP)-mAD fusion protein was coexpressed with p53 in H1299 cells to compare binding efficiencies by co-IP. The result showed that most contact and conformational mutants coprecipitated GFP-mAD with higher efficiency than wild-type p53 (Fig. 4a). In contrast, the SQ-RING fragment (361 to 491) showed no increase or even weaker binding to mutant p53 in the co-IP (Fig. 4b), suggesting that other MDM2 domains in the full-length complex were needed to initiate the strong RING binding to mutant p53. Therefore, the strong AD-mutant p53 interaction was detectable using two different assays. The GFP-mAD8GS mutant, in which 8 hydrophobic residues (comprising W, L, V, and F) in the mAD were replaced by hydrophilic G or S, showed reduced binding to mutant p53 (Fig. 4c), suggesting that the mAD interacts with exposed hydrophobic regions of the mutant p53 core domain (see below) (38).

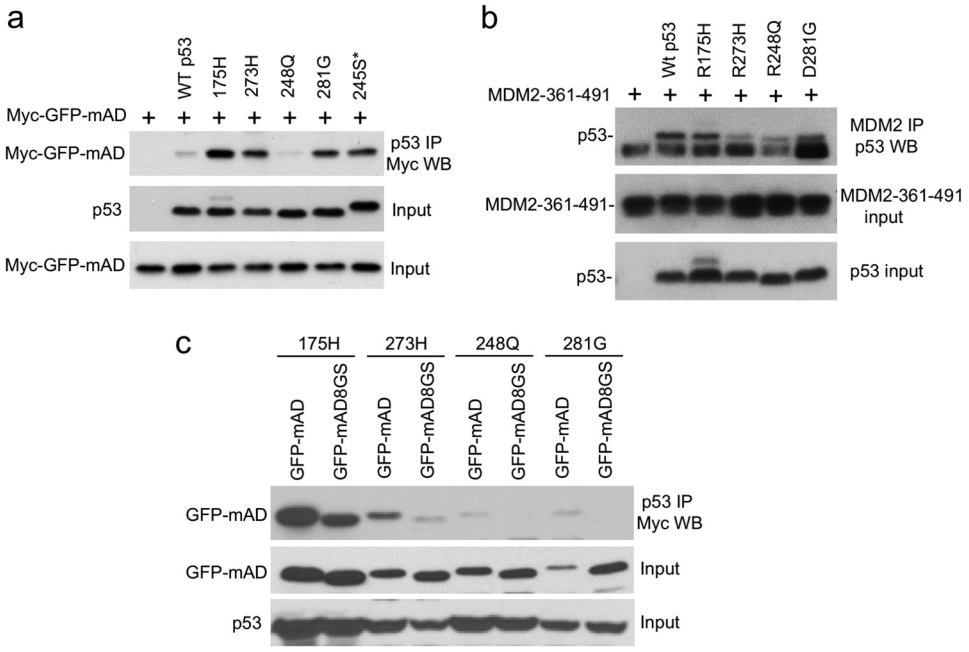

FIG 4 Mutant p53 binding to MDM2 AD in coexpression and pulldown assay. (a) The minimal AD (mAD, residues 230 to 260) was expressed as a fusion to Myc-tagged GFP. Myc-GFP-mAD was coexpressed with p53 in H1299 cells. The interaction of mAD with p53 was analyzed by IP-Western blotting. (b) H1299 cells were transfected with the MDM2 RING domain fragment (residues 361 to 491) and p53. The interaction of RING and p53 was analyzed by IP-Western blotting. (c) H1299 cells were transfected with GFP-mAD8GS (containing hydrophobic-to-hydrophilic substitution of 8 residues) and p53 mutants. The binding between mAD and p53 mutants was analyzed by IP-Western blotting.

Overall, the results showed that mutant p53 had significantly stronger binding to the MDM2 AD, and certain mutants, such as the R175H mutant, also bound strongly to the RING in the full-length complex, suggesting potential mechanisms for inhibiting MDM2 E3 ligase activity.

MDM2 AD and RING domains bind to the p53 core domain. To map the region of p53 involved in binding to MDM2 AD and RING, immobilized glutathione *S*-transferase (GST)–p53 deletion constructs were used to capture MDM2GP expressed in H1299 cells. The GST-p53/MDM2GP complex formed *in vitro* was cleaved with PreScission and analyzed for the release of MDM2 fragments. As expected, the MDM2 N terminus (p53BD) bound to all constructs containing the p53 N terminus (Fig. 5a). The AD bound to the p53 construct comprising residues 1 to 300 (p53-1-300) but not to p53-1-82 (Fig. 5a), suggesting that residues 82 to 300 contained the main binding site for the MDM2 AD. The SQ-RING and RING fragments also bound to all constructs containing the p53 core but lost binding to p53-1-82, suggesting that the RING also interacts mainly with the p53 core (Fig. 5a). The results suggest that the p53 core domain is the major binding site for both MDM2 AD and RING (Fig. 5c).

Similar analysis using GST-R175H mutant truncation constructs showed that mutant p53 also used the core domain to interact with MDM2 AD and RING (Fig. 5b). The GST-R175H mutant construct expressed in *Escherichia coli* showed stronger binding to the MDM2 AD than the GST-p53 construct (Fig. 5b), suggesting that the conformational defect of the R175H mutant was sufficient to increase binding to MDM2 AD. The AD and RING of MDMX also interact with wild-type p53 and inhibit DNA binding (46). Using a protease-cleavable MDMXc3 construct similar to MDM2GP in design (PreScission site inserted after MDMX residues 140, 350, and 429) (47), the interactions of MDMX AD and RING with wild-type and mutant p53 were compared. The MDMX AD binding to the GST-R175H mutant construct was only modestly stronger than its binding to GST-p53. Furthermore, MDMX RING binding was not enhanced by mutation in p53 (data not

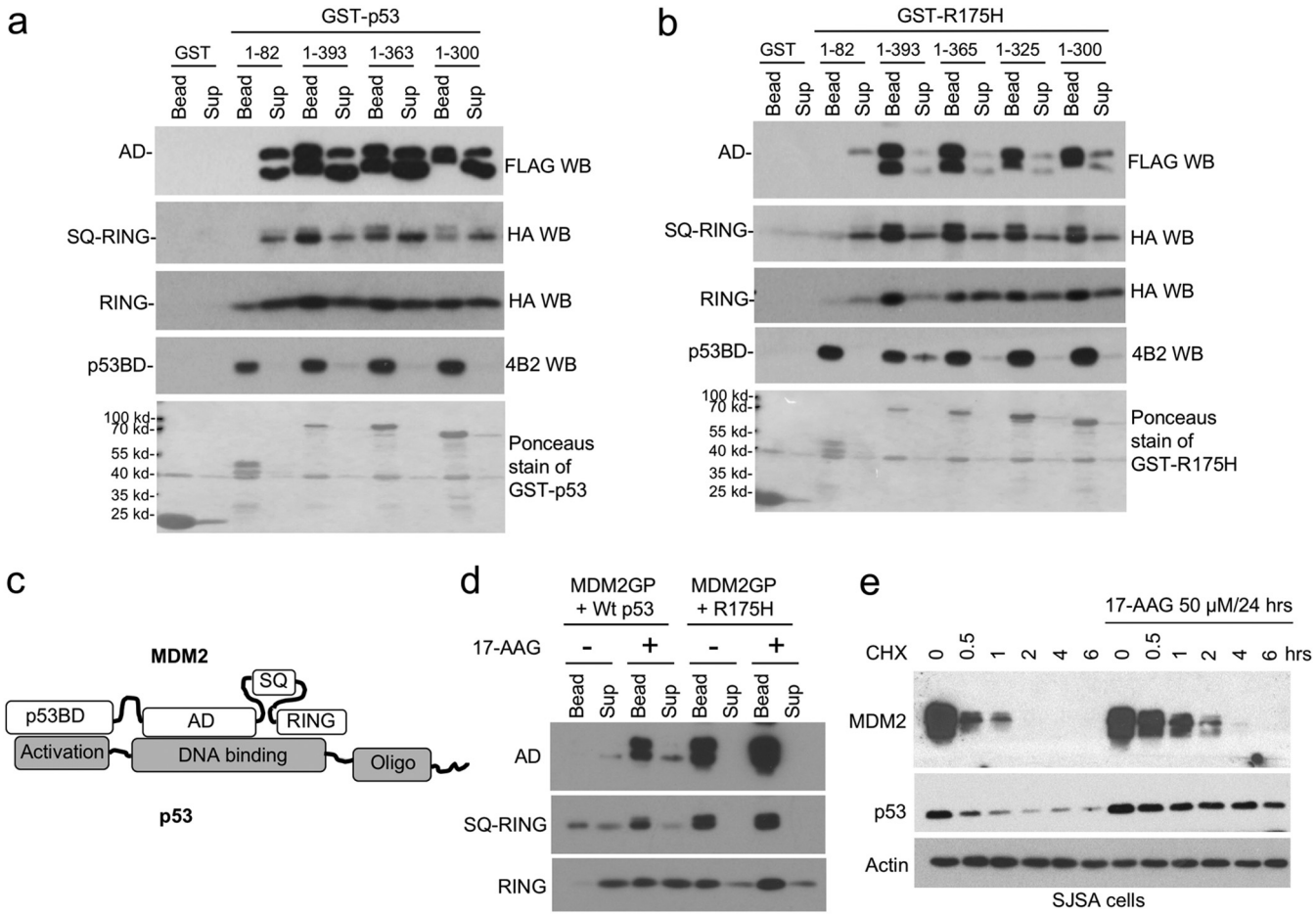

FIG 5 MDM2 AD and RING domains bind to p53 core domain. (a, b) Beads loaded with GST-p53 and GST-R175H deletion mutants were incubated with H1299 lysate containing MDM2GP to form p53/MDM2GP complexes that were then cleaved with PreScission. The release of MDM2 fragments from the p53 complex was detected by Western blotting. Numbers above lanes show p53 residues present in each construct. (c) Diagram of MDM2 AD and RING binding to p53 core domain. (d) H1299 cells cotransfected with MDM2GP and p53 were treated with 20 μM 17-AAG for 5 h. MDM2GP-p53 complex was captured on Pab421 beads and cleaved by PreScission. The release of MDM2 fragments from p53 complex was detected by Western blotting. (e) SJSA cells were cultured in medium containing 17-AAG (50 μM) for 24 h. The turnover of MDM2 and p53 was analyzed by Western blotting after treatment with cycloheximide (CHX; 100 μg/ml).

shown). Therefore, MDM2 AD and RING binding was significantly enhanced by mutation of the p53 core domain, whereas MDMX AD and RING binding was less affected.

Inhibition of hsp90 stimulates wild-type p53 binding to MDM2 AD. Inhibitors of hsp90, such as geldanamycin and 17-AAG (tanespimycin [17-*N*-allylamino-17-demethoxygeldanamycin]), promote mutant p53 degradation partly by restoring MDM2-mediated ubiquitination (31, 32). Therefore, it was of interest to determine whether hsp90 inhibitors act by disrupting the binding between mutant p53 and MDM2 AD. H1299 cells cotransfected with p53 and MDM2GP were treated with 17-AAG. The p53-MDM2 complex was immobilized and analyzed for MDM2 fragment release after protease cleavage. Inhibition of hsp90 did not block the strong binding between the R175H mutant and the MDM2 AD and RING fragments (Fig. 5d). The result suggests that binding of mutant p53 to MDM2 AD and RING does not require hsp90. Therefore, hsp90 inhibitor-mediated mutant p53 degradation does not involve the release of MDM2 AD from p53.

Interestingly, the interactions of wild-type p53 with MDM2 AD and RING were significantly stimulated by 17-AAG (Fig. 5d). hsp90 participates in the folding and activation of wild-type p53 (48, 49). The result suggests that inhibition of hsp90 produces misfolded wild-type p53 that binds tightly to MDM2 AD. We found that treatment of cells expressing wild-type p53 (SJSA cell line) with 17-AAG caused stabilization of both p53 and MDM2, consistent with the interpretation that a fraction

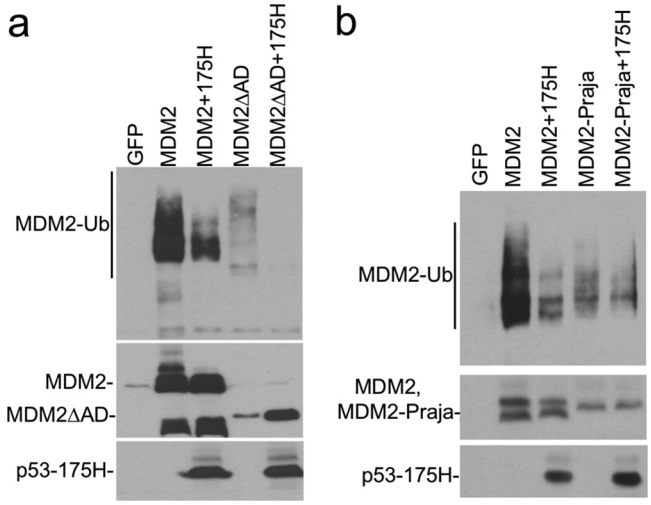

FIG 6 Mutant p53 interferes with MDM2 RING function. (a) MDM2 or MDM2ΔAD (with deletion of residues 210 to 290) was coexpressed with mutant p53 and His_6-ubiquitin in H1299 cells. MDM2 self-ubiquitination was analyzed by Ni^{2+}-NTA pulldown and MDM2 Western blotting. (b) MDM2 or MDM2-Praja (MDM2 RING replaced with Praja RING) was cotransfected with p53 and His_6-ubiquitin in H1299 cells. MDM2 self-ubiquitination was analyzed by Ni^{2+}-NTA pulldown and MDM2 Western blotting.

of wild-type p53 adopts a mutant-like conformation and becomes stabilized by tightly binding and inhibiting the MDM2 AD (Fig. 5e).

Mutant p53 interferes with both AD and RING functions. To test whether mutant p53 inhibits MDM2 RING activity, the MDM2ΔAD mutant (without the AD) was coexpressed with mutant p53. Similar to full-length MDM2, MDM2ΔAD self-ubiquitination was strongly inhibited by the R175H mutant (Fig. 6a), suggesting that RING was inhibited by mutant p53. When the MDM2 RING domain was replaced with the RING domain from the E3 ligase Praja (MDM2-Praja), the MDM2-Praja self-ubiquitination was not sensitive to mutant p53 (Fig. 6b), suggesting that mutant p53 specifically inhibits MDM2 RING activity (20).

The MDM2 AD stimulates RING activity through intramolecular interaction. The strong binding of mutant p53 core domain to the AD is likely to inhibit its RING-activating function. We reasoned that MDM2 with extra copies of the AD should be resistant to inhibition by mutant p53, since one p53 core domain would not be able to bind and inactivate multiple ADs simultaneously in a complex. To test this hypothesis, the MDM2-3AD construct, containing 2 extra tandem copies of the AD (residues 221 to 280), was analyzed (Fig. 7a) (38).

In a cotransfection assay, the self-ubiquitination of MDM2-3AD was less sensitive to inhibition by p53 mutants (Fig. 7b). MDM2-3AD was significantly more efficient than MDM2 in ubiquitinating mutant p53 (Fig. 7c). MDM2-3AD also degraded mutant p53 more efficiently than MDM2 in a cotransfection assay (Fig. 7d). When tumor cells (MDA-231 cell line) expressing endogenous mutant p53 were transfected with MDM2 and MDM2-3AD, only MDM2-3AD was able to significantly degrade endogenous mutant p53 despite having a lower expression level than MDM2 (Fig. 7e). The results suggest that restoring the AD function of MDM2 may be a potential strategy to degrade mutant p53 in tumor cells and eliminate gain of function.

DISCUSSION

Previous studies showed that mutant p53 expressed in transformed cells was resistant to ubiquitination by MDM2 (32). The binding of mutant p53 to MDM2 also inhibited MDM2 self-ubiquitination and caused MDM2 stabilization, suggesting that mutant p53 inhibits MDM2 E3 activity (30, 31). Our current analysis of domain interactions between MDM2 and mutant p53 showed that the mutant p53 core domain bound to the MDM2 AD and RING domains with higher avidity than wild-type p53. The

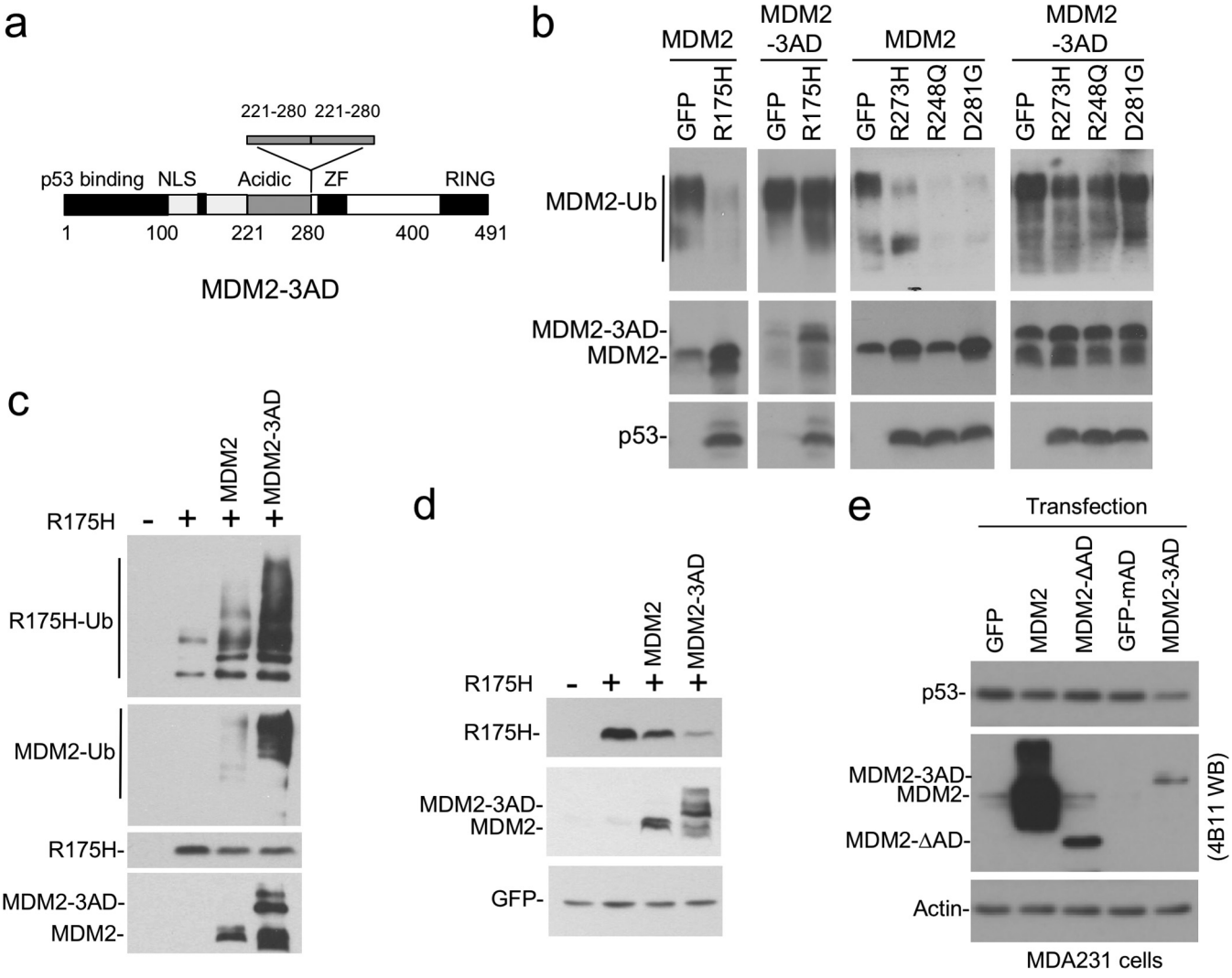

FIG 7 MDM2-3AD is resistant to inhibition by mutant p53. (a) The structure of MDM2-3AD. Two extra copies of AD (residues 221 to 280) were inserted. (b) The effect of mutant p53 on MDM2-3AD self-ubiquitination was detected by coexpressing MDM2-3AD and mutant p53 in H1299 cells. MDM2 ubiquitination was analyzed by Ni^{2+}-NTA pulldown and MDM2 Western blotting. (c) H1299 cells were cotransfected with MDM2-3AD, p53, and His_6-ubiquitin. MDM2 and p53 ubiquitination was determined by Ni^{2+}-NTA pulldown and Western blotting for MDM2 and p53. (d) H1299 cells were cotransfected with MDM2-3AD and p53-R175H. P53 degradation was determined by Western blotting. (e) MDA-MB-231 (R280K) cells were transiently transfected with the indicated plasmids. The level of endogenous p53 was determined by Western blotting.

results suggest that mutant p53 may interfere with MDM2 E3 ligase function using two different mechanisms, (i) binding and abrogating the autoactivating effect of the AD and (ii) binding and inhibiting the RING domain.

At present, the structural details of AD and RING interactions with mutant p53 and the mechanism of MDM2 E3 ligase inhibition remain unknown. Previous work showed that the intramolecular interaction between the MDM2 mAD region (amino acids [aa] 230 to 260) and RING significantly enhanced E3 ligase activity (38). Therefore, the strong binding of the mutant p53 core domain to AD may interfere with the autoactivation mechanism and suppress RING activity. The mAD sequence is also the ARF binding site on MDM2, suggesting that mutant p53 and ARF use similar mechanisms to inhibit MDM2 E3 activity. The presence of multiple domain interactions between MDM2 and p53 prevented us from determining whether mutant p53 disrupts the intramolecular AD-RING binding. It should be noted that ARF binding to the MDM2 AD inhibits p53 ubiquitination without disrupting AD-RING interaction (38). Our analysis using MDM2 mutants without the AD showed that mutant p53 also directly inhibited RING domain

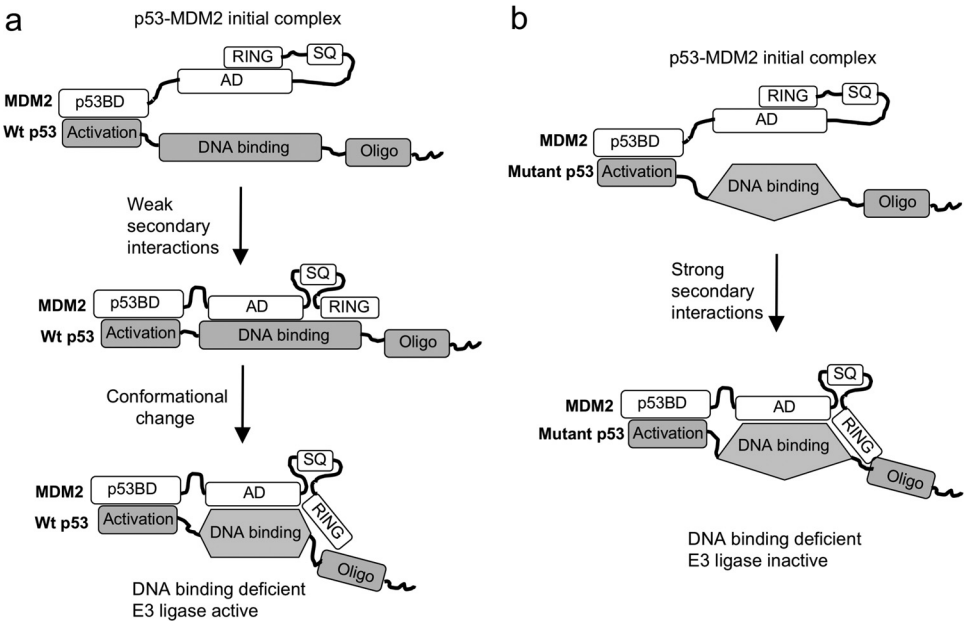

FIG 8 A model of MDM2 inhibition by mutant p53. (a) Wild-type p53 binding to MDM2 is initiated through N-terminal-specific interaction, followed by weak secondary interactions between the AD and RING with the core domain. The AD induces mutant-like conformational change in the core and inhibits p53 DNA binding. The weak AD and RING interactions with the core do not interfere with MDM2 E3 activity and p53 degradation. (b) Mutant p53 interaction with MDM2 is also initiated by specific N-terminal binding. Subsequently, the exposed hydrophobic residues of the mutant p53 core domain bind AD and RING with high avidity, blocking the MDM2 E3 activity. These interactions cooperate with other mechanisms, such as chaperone binding, to inhibit mutant p53 ubiquitination and degradation.

activity. The significance of this interaction in mutant p53 stabilization is unclear at present.

What is the structural basis for the strong binding between mutant p53 core and MDM2 AD? Previous work indicated that the MDM2 AD bound to the wild-type p53 core domain with low affinity and induced the misfolding of p53 to a mutant-like conformation, suggesting that the AD favored binding to p53 with a mutant conformation (43, 44). The wild-type p53 conformation is thermolabile and is thought to exist in a dynamic equilibrium between wild-type and mutant conformations (50). MDM2 AD binding will shift the conformation equilibrium of wild-type p53 toward the mutant, as reported previously (Fig. 8a). Naturally misfolded mutant p53 core domain is expected to have high avidity for the MDM2 AD (Fig. 8b). The MDM2 AD has a high degree of intrinsic disorder and is capable of binding to different partners. The mutant p53 core domain with exposed hydrophobic sequences may be a favored binding target for the AD. Such strong binding is a nonspecific by-product of p53 mutations rather than an evolutionarily fine-tuned function. However, because mutant p53 retains specific binding to MDM2 through the N terminus, strong AD-core binding and inhibition of MDM2 E3 function occur by default, resulting in the stabilization of most p53 mutants (Fig. 8b).

Our findings provide a new clue to the mechanism of mutant p53 stabilization. However, its connections to mutant p53 stabilization in transformed cells and degradation by hsp90 inhibitors remain unclear. hsp90 inhibitors disrupt hsp90 binding to p53 and promote mutant p53 degradation, leading to the conclusion that stable hsp90 binding to mutant p53 inhibits MDM2 E3 activity (31, 32). However, hsp90 inhibitors may also directly affect MDM2 activity to promote mutant p53 degradation (51). Our results showed that hsp90 inhibition did not affect the binding of AD to mutant p53, ruling out the AD-core interaction as a target of hsp90 inhibitors. MDM2 has been reported to ubiquitinate other proteins in addition to p53 and MDMX. Our results suggest that mutant p53 may affect the ubiquitination of other MDM2 substrates, which may contribute to gain-of-function effects.

Mouse models showed that mutant p53 accumulated only in tumors and was degraded as rapidly as wild-type p53 in normal tissues (7, 34). However, recent analysis using more sensitive antibodies detected mutant p53 accumulation in the proliferating cells of intestinal crypts, suggesting that stabilization can occur to some degree in nontransformed cells (35). The abnormal mutant p53 interaction with MDM2 AD and RING may be responsible for the stabilization in normal cells. The mechanisms of mutant p53 stabilization in tumor cells remain undefined but may include multiple pathways, such as oncogene activation, increased ARF expression, chronic DNA damage signaling, and changed chaperone activities (16, 52). For technical reasons, our analysis of mutant p53 interaction with MDM2 AD and RING used tumor cells in order to obtain sufficient protein levels. Whether the interactions are different in transformed and normal cells remains to be determined, and the answer may provide further insight into the phenomenon of tumor cell-specific accumulation of mutant p53.

hsp90 inhibition achieves only partial degradation of mutant p53 and does not affect binding to MDM2 AD, indicating that targeting hsp90 alone does not fully restore MDM2 function. Our current findings suggest potential new targets for stimulating mutant p53 degradation in tumor cells. Proof-of-concept experiments using the MDM2-3AD construct showed that extra copies of the AD increased resistance to inhibition by mutant p53 and enhanced the ubiquitination/degradation of mutant p53. The results suggest that small molecule drugs that disrupt mutant p53-AD interaction may stimulate MDM2 E3 activity. Alternatively, small molecules may be developed that interact with and activate the RING similarly to the mAD. Such compounds may have therapeutic potential against tumors that accumulate gain-of-function mutant p53. Therefore, it will be informative to determine the structures of the AD-mutant p53 core domain complex and the AD-RING complex to understand the mechanisms of autoactivation and mutant p53-mediated inhibition.

MATERIALS AND METHODS

Plasmids, cell lines, and reagents. All MDM2 and p53 constructs used in this study were human cDNA clones. MDM2 constructs for expression in mammalian cell culture were generated by PCR cloning and inserted into the pCMV-neo-Bam vector. The MDM2GP sequence contains the sequences GGGGGL EVLFQGPDYKDDDDKGGGGG, LEVLFQGPEEQKLISEEDL, and GGGGGLEVLFQGPYPYDVPDYAGGGGG inserted after MDM2 residues 171, 332, and 422, respectively. MDM2 and p53 constructs for expression in *E. coli* were generated by cloning cDNA sequences into the pGEX-2T vector (GE Healthcare Life Sciences). The human NSCLC cell line H1299 (p53 null), osteosarcoma cell line SJSA (p53 wild type), and human breast adenocarcinoma cell line MDA-MB-231 (p53 R280K) were purchased from ATCC (Manassas, VA) and maintained in Dulbecco modified Eagle medium (HyClone) with 10% fetal bovine serum (Sigma). Cells were cultured at 37°C in an incubator with a humidified atmosphere containing 5% CO_2. The HSP90 inhibitor 17-AAG was obtained from Selleck Chemicals (Houston, TX). Cycloheximide was purchased from Sigma-Aldrich (St. Louis, MO). Anti-MDM2 monoclonal antibodies 4B2, 4B11, and 3G9 and anti-MDMX monoclonal antibody 8C6 were generated previously (53, 54). Anti-FLAG polyclonal antibody and anti-Myc polyclonal antibody were purchased from Sigma-Aldrich (St. Louis, MO). Anti-HA polyclonal antibody and mouse anti-β-actin antibody were obtained from Cell Signaling Technology (Danvers, MA). Mouse anti-p53 antibody DO1 was purchase from BD Biosciences (San Jose, CA). Anti-GFP polyclonal antibody was obtained from Santa Cruz Biotechnology (Dallas, TX). Polyethylenimine (PEI) was purchased from Polysciences (Warrington, PA).

Proteolytic fragment release assay. H1299 cells were transfected with MDM2GP and p53 using the PEI method. Cells were lysed in immunoprecipitation buffer (150 mM NaCl, 50 mM Tris-HCl [pH 8.0], 0.5% Nonidet P-40, 2.0 mM NaF, 1.0 mM dithiothreitol [DTT], 10% [vol/vol] glycerol). Cell lysate (~1 mg protein) was incubated with Pab421 conjugated to protein A beads (30 μg packed volume) for 18 h at 4°C. The beads were washed twice with PreScission buffer (150 mM NaCl, 10 mM HEPES pH 7.5, 0.05% Nonidet P-40, 1.0 mM DTT, 10% glycerol) and suspended in 100 μl of PreScission buffer with PreScission protease (0.2 μg/μl). After incubation at 4°C for 1 h with shaking, the mixture was centrifuged to separate the beads (bound material) and supernatant (released material). The beads were washed once briefly with PreScission buffer to remove the residual supernatant. The beads and supernatant were boiled in Laemmli sample buffer and subjected to SDS-PAGE for Western blotting to analyze the bound/released ratio of each fragment using anti-FLAG, anti-Myc, anti-HA, and anti-4B2 antibodies.

Western blotting. To detect proteins by Western blotting, cells were lysed in lysis buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.5% NP-40, 1 mM phenylmethylsulfonyl fluoride [PMSF], 1× protease inhibitor cocktail) and centrifuged at 4°C for 10 min at 14,000 × g. The supernatant was boiled in Laemmli sample buffer for 5 min and subjected to SDS-PAGE and Western blotting to detect the expression of proteins indicated in the figures using the corresponding antibodies. To determine the half-life of MDM2 and p53 in SJSA cells after 17-AAG (50 μM for 24 h) treatment, cells were incubated

with 100 μg/ml of cycloheximide. The samples collected at different time points were analyzed by Western blotting.

GST pulldown and proteolytic fragment release assay. GST-p53 or GST-R175H full-length and deletion mutants were induced by 0.1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) and 150 μM $ZnCl_2$ at 18°C for 20 h. The bacterial pellet from 250 ml of culture was suspended in 10 ml 1× PBS and sonicated. Triton X-100 (final concentration, 0.5%) was added to the lysate, followed by 30 min of incubation on ice and 10 min of centrifugation at 10,000 × g. The GST-p53 fusion protein from 0.2 ml of lysate was captured by glutathione-agarose beads (20-μl packed volume). The loaded beads were washed with PreScission buffer and incubated with H1299 lysate containing MDM2GP or MDMXc3 at 4°C for 18 h. The beads were washed twice with PreScission buffer and suspended in 100 μl PreScission buffer, and PreScission protease was added (0.2 μg/μl). After incubation at 4°C for 1 h with shaking, the mixture was centrifuged to separate the beads (bound material) and supernatant (released material). The beads were washed once with PreScission buffer. Beads and supernatant were boiled in Laemmli sample buffer and subjected to SDS-PAGE for Western blot analysis of MDM2 fragments.

GST pulldown and dissociation assay. Bacterial lysates expressing different GST-p53 fragments or the GST-R175H mutant construct were incubated with glutathione-agarose beads to capture the fusion proteins. The loaded beads (20 μl packed volume) were incubated with H1299 cell lysate containing MDM2 and deletion mutants for 2 h at 4°C. The beads were washed with lysis buffer and incubated in 1 ml lysis buffer for up to 6 h with constant rotation. The beads were recovered and boiled in Laemmli sample buffer. The fraction of MDM2 remaining on the beads was analyzed by Western blotting.

***In vivo* ubiquitination assay.** H1299 cells were transfected with His_6-ubiquitin and plasmids indicated in the figures using the PEI method. Twenty-four hours after transfection, cells were collected into two aliquots. One aliquot (10%) served as input. The remaining cells (90%) were used for purification of His_6-tagged proteins by Ni^{2+}-nitrilotriacetic acid (NTA) beads. The cell pellet was lysed in buffer A (6 M guanidinium-HCl, 0.1 M Na_2HPO_4/NaH_2PO_4, 0.01 M Tris-HCl, pH 8.0, 5 mM imidazole, 10 mM β-mercaptoethanol) and incubated with Ni^{2+}-NTA (Qiagen) for 4 h at room temperature. The beads were washed with buffer A, buffer B (8 M urea, 0.1 M Na_2PO_4-NaH_2PO_4, 0.01 M Tris-HCl, pH 8.0, 10 mM β-mercaptoethanol), buffer C plus (8 M urea, 0.1 M Na_2PO_4-NaH_2PO_4, 0.01 M Tris-HCl, pH 6.3, 10 mM β-mercaptoethanol, 0.2% Triton X-100), and buffer C (8 M urea, 0.1 M Na_2PO_4-NaH_2PO_4, 0.01 M Tris-HCl, pH 6.3, 10 mM β-mercaptoethanol), and beads were boiled in Laemmli sample buffer containing 200 mM imidazole. The samples were analyzed by Western blotting for ubiquitin-conjugated p53 and MDM2.

Ubiquitin release assay. UbcH5c charged with ubiquitin was produced in a reaction mixture (20 μl) containing 0.2 μg His_6-E1, 0.2 μg His_6-UbcH5c, and 2 μg ubiquitin (Biomol) in a buffer containing 10 mM HEPES (pH 7.5), 100 mM NaCl, 40 μM ATP, and 2 mM $MaCl_2$ as previously described (38). The reaction mixture was incubated for 20 min at room temperature, and 0.04 unit/μl apyrase (Sigma) was added and the mixture incubated for 10 min at room temperature to deplete ATP. The MDM2-p53 complex or MDM2-R175H mutant complex was captured from transiently transfected H1299 cells using anti-p53 antibody Pab421 and protein A beads. The beads, loaded with similar amounts of MDM2 in complex with p53, were added to the reaction mixtures containing ubiquitin-charged UbcH5c. Samples were incubated for different times at 23°C, and the ubiquitin release reaction was stopped by adding nonreducing Laemmli sample buffer. Boiled samples were fractionated by SDS-PAGE and blotted with anti-UbcH5 antibody (Boston Biochem).

ACKNOWLEDGMENTS

This work was supported in part by grants from the National Institutes of Health (grants number CA141244 and CA186917) and by NCI Cancer Center support grant number P30-CA076292.

We declare no conflict of interest.

Experimental work was done by L.Y., T.S., Q.C., and L.C. Experimental design was done by L.Y., T.S, Q.C., L.C., and J.C. The manuscript was written by L.Y. and J.C. The study was conceived by J.C.

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