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Auto-ubiquitination of Mdm2 Enhances Its Substrate Ubiquitin Ligase Activity*

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Background: Mdm2, the principal ubiquitin ligase for the tumor suppressor p53, also ubiquitinates itself, but the consequences are unclear.
Results: Auto-ubiquitination enhances Mdm2 binding to ubiquitin-conjugating enzymes (E2s) and its ability to ubiquitinate p53.
Conclusion: Increased E2 recruitment by auto-ubiquitinated Mdm2 may enable processivity of substrate ubiquitination.
Significance: Auto-ubiquitination may be a general mechanism for the activation of ubiquitin ligases.

The RING domain E3 ubiquitin ligase Mdm2 is the master regulator of the tumor suppressor p53. It targets p53 for proteasomal degradation, restraining the potent activity of p53 and enabling cell survival and proliferation. Like most E3 ligases, Mdm2 can also ubiquitinate itself. How Mdm2 auto-ubiquitination may influence its substrate ubiquitin ligase activity is undefined. Here we show that auto-ubiquitination of Mdm2 is an activating event. Mdm2 that has been conjugated to polyubiquitin chains, but not to single ubiquitins, exhibits substantially enhanced activity to polyubiquitinate p53. Mechanistically, auto-ubiquitination of Mdm2 facilitates the recruitment of the E2 ubiquitin-conjugating enzyme. This occurs through noncovalent interactions between the ubiquitin chains on Mdm2 and the ubiquitin binding domain on E2s. Mutations that diminish the noncovalent interactions render auto-ubiquitination unable to stimulate Mdm2 substrate E3 activity. These results suggest a model in which polyubiquitin chains on an E3 increase the local concentration of E2 enzymes and permit the processivity of substrate ubiquitination. They also support the notion that autocatalysis may be a prevalent mode for turning on the activity of latent enzymes.

Covalent conjugation to ubiquitin is a major post-translational modification that regulates protein stability, function, and localization (1). Ubiquitination takes place due to sequential actions of three enzymes: a ubiquitin-activating enzyme (E1),[2] a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3). The specificity and efficiency of ubiquitination are largely determined by the E3, which binds to both an E2 thioesterified with ubiquitin (E2∼Ub) and a substrate protein, and stimulates the transfer of ubiquitin from E2∼Ub to the substrate. The vast majority of the hundreds of known ubiquitin E3s contain a RING (really interesting new gene) domain (1, 2). Some RING proteins contain only the RING domain and function in multisubunit E3 complexes (*e.g.* Cullin-RING ligases or CRLs) (3), but most RING proteins are relatively large proteins with multiple domains and can function as single-molecule E3s.

A protein can be conjugated at one or multiple sites with a single ubiquitin or a polyubiquitin chain (1, 2). Formation of polyubiquitin chains is critical for protein degradation and various nondegradative processes including signal transduction. However, the mechanism for the processive assembly of ubiquitin chains remains poorly understood. For RING E3s, a major rate-limiting step for the formation of a polyubiquitin chain is the recruitment of E2∼Ub to the E3 (2, 4). Because an E2 interacts with both E1 and the E3 RING domain through overlapping regions (5), it needs to dissociate from the RING domain to be re-thioesterified with ubiquitin. Previous studies have shown that the cullin protein Cul1-based CRLs circumvent this rate-limiting step in part through rapid association and disassociation between an E2 and the E3 RING domain. This dynamic interaction is facilitated by a separate E2 binding site on the Cul1 subunit (6). Yet, the mechanism by which relatively large RING E3s achieve processive ubiquitination is not known. A notable trait of RING-containing E3s is their auto-ubiquitination (7). Despite being commonly regarded as a mechanism of autocatalytic degradation, the function of E3 automodification is not well defined.

The multidomain RING-containing protein Mdm2 is the principal ubiquitin ligase of the pre-eminent tumor suppressor p53 (8–11). p53 becomes activated in response to cellular stresses such as DNA damage, nutrient deprivation, and oncogene activation. The activation of p53 leads to potent anti-proliferative outcomes ranging from cell cycle arrest to senescence and apoptosis, making the control of p53 levels a central issue in mammalian cells (8). In unstressed cells, p53 is a short lived protein largely due to Mdm2-mediated ubiquitination and proteasomal degradation. Mdm2 also undergoes auto-ubiquitination. Although this was previously thought to cause Mdm2 to be degraded, subsequent studies have shown that auto-ubiquitination of Mdm2 is not responsible for Mdm2 degradation *in*

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[2] The abbreviations used are: E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin ligase; CRL, Cullin-RING ligase; Mdm2, mouse double minute 2; RING, really interesting new gene; Ub, ubiquitin; UBD, ubiquitin binding domain.

vivo (12). Here we seek to address the function of Mdm2 auto-ubiquitination. We show that auto-ubiquitination of Mdm2 can enhance its substrate ubiquitination activity. We also find that auto-ubiquitination of Mdm2 leads to strong recruitment of E2-conjugating enzymes, overcoming the rate-limiting step of E2 recruitment and increasing the processivity of ubiquitination.

EXPERIMENTAL PROCEDURES

Plasmids and Reagents—Plasmids for expressing p53 and Mdm2 in mammalian cells are in pRK5 vector with N-terminal FLAG, HA, or GST tags as described previously (13, 14). UbcH5c WT pET28a (plasmid 12643) and UbcH5c S22R pET28a (plasmid 12644) (15) were obtained from Addgene. The following reagents were purchased from Boston Biochem: ubiquitin E1 (E-305), UbcH5a (E2–616), Mg^{2+}-ATP (B-20), ubiquitin (U-100H), methylated ubiquitin (U-501), Lys^{48}-only ubiquitin (UM-K480), and I44A ubiquitin (UM-I44A). The antibodies for the following proteins were purchased from the indicated sources: p53 (DO-1, Santa Cruz Biotechnology); Mdm2 (Ab-1, Calbiochem); ubiquitin (P4D1, Santa Cruz Biotechnology); polyubiquitinated conjugates (FK1 clone, Enzo Life Sciences); UbcH5 (A-615, Boston Biochem); UbcH5c (ab58251, Abcam); and MdmX (A300-287A, Bethyl Scientific).

Protein Expression and Purification—For purifying Mdm2 and p53, the corresponding expression plasmids were transfected into HEK293T cells. Cells expressing Mdm2 were further treated with proteasome inhibitor MG132 for 4 h. Cells were rinsed with ice-cold 1× PBS and lysed in lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10% glycerol, 1 mM EDTA, 0.5% Triton X-100, 0.5% Nonidet P-40, 1 mM DTT, 1 mM PMSF, 100 mM NaF, and 1× Complete protease mixture). GST-Mdm2 was precipitated with glutathione-Sepharose 4B beads (GE Healthcare, 17-0756-01), and HA-Mdm2 with anti-HA affinity beads (Roche Applied Science). Bead-bound Mdm2 was sequentially washed twice with lysis buffer, once with lysis buffer plus 0.5 M KCl, once with lysis buffer plus 1 M KCl, and once with ubiquitination reaction buffer. Bead-bound Mdm2 was resuspended in ubiquitination reaction buffer and used for subsequent *in vitro* reactions. FLAG-tagged p53 was purified with M2 beads (Sigma) as described previously (13) and eluted from the beads with elution buffer (20 mM Tris-HCl, pH 8, 150 mM NaCl, 1 mM DTT, 10% glycerol) plus 3×FLAG peptide (Sigma, F4799).

To purify the Mdm2-MdmX complex, GST-Mdm2 and FLAG-MdmX were co-expressed in HEK293T cells. Cells were treated with proteasome inhibitor MG132 for 4 h. Lysates were incubated with M2 beads for 3 h at 4 °C. Beads were washed four times with lysis buffer and twice with elution buffer. Bound MdmX was eluted with 3× FLAG peptide for 1.5 h at 4 °C. Eluate was incubated with glutathione beads in lysis buffer overnight. Bead-bound Mdm2-MdmX complexes were washed as described for the purification of Mdm2 proteins.

To generate recombinant WT and S22R UbcH5c proteins, BL21 cells containing either WT UbcH5c pET28a or S22R UbcH5c pET28a were induced with 0.2 mM isopropyl β-D-1-thiogalactopyranoside for 4 h at 30 °C. Cells were resuspended in sonication buffer (20 mM HEPES, pH 6.0, 150 mM NaCl, 2.5 mM $MgCl_2$, 1 mM DTT) and lysed by sonication. Lysates were centrifuged at 13,000 rpm for 15 min. Supernatant was fractionated by gel filtration using a Superdex 200 10/300 GL column driven by an AKTA FPLC system (GE Healthcare). Fractions of 0.5 ml each were collected. Purified proteins were resolved by SDS-PAGE, stained by Coomassie, and quantified by densitometry against a BSA standard curve or by Western blotting against known protein standards. Fractions containing only UbcH5c were pooled and used for ubiquitination/binding reaction.

Western Blotting—Proteins in sample buffer containing 5% 2-mercaptoethanol were boiled at 95 °C for 5 min and resolved by 8% SDS-PAGE for Mdm2 and p53, 15% SDS-PAGE for E2, and 8–15% gradient for simultaneously detecting GST and GST-Mdm2. Stacking gels were retained for all ubiquitination reactions. Proteins were transferred onto nitrocellulose membrane. For ubiquitin blotting, membranes were boiled in water for 2 min using a microwave prior to blocking. Membranes were blocked with 5% nonfat dry milk in PBS-T and probed with the indicated antibodies.

In Vitro Ubiquitination—Auto-ubiquitination reactions consisted of 3–5 ng of bead-bound Mdm2, 100 nM E1, 500 nM UbcH5a, 2 mM Mg^{2+}-ATP, 2 mM DTT, and 2–5 μg of wild-type or mutant ubiquitin in final volume of 20 μl of ubiquitination reaction buffer (40 mM Tris-HCl, pH 7.6). In control reactions, either ubiquitin (see Fig. 2*B*) or Mg^{2+}-ATP (in the rest of figures) was omitted. Reaction mixtures were incubated at 37 °C on a microtube orbital shaker (Labnet, Shaker20) at 1400 rpm and were either stopped by addition of sample buffer or washed three times with ubiquitination reaction buffer and aliquotted in separate tubes for p53 ubiquitination. p53 ubiquitination was performed at 22 °C with 10 ng of FLAG-p53 for 5 min or the indicated times. Mdm2 and p53 ubiquitination was detected by Western blotting using anti-Mdm2 and anti-p53 antibody, respectively. To detect p53 polyubiquitination, FLAG-p53 (30 ng) was ubiquitinated by Mdm2 as described above. Reaction mixtures were denatured by adding SDS to 1% final concentration and boiling for 5 min, and diluted to reduce the SDS concentration to 0.1%. FLAG-p53 was pulled down with anti-FLAG M2 beads (Sigma) and analyzed by Western blotting with anti-polyubiquitin or anti-p53 antibodies.

Thioesterification of E2 was performed using 150 nM E1, 600 ng of WT or S22R E2, 100 mM NaCl, 5 mM Mg^{2+}-ATP, and 2 μg of ubiquitin in a final volume of 20 μl of ubiquitination reaction buffer. Reactions were incubated at 22 °C for indicated times and analyzed by nonreducing SDS-PAGE and Western blotting.

In Vitro Binding Assays—For p53 and Mdm2 binding, GST-Mdm2 (unmodified or auto-ubiquitinated) immobilized on glutathione beads was first blocked with 3% BSA for 1 h at 4 °C. Beads were incubated with 30 ng of p53 in lysis buffer for 1 h at 4 °C. Beads were washed five times with lysis buffer, and the bound proteins were analyzed by Western blotting.

For binding between Mdm2 and E2, ~100 ng of immobilized GST or GST-Mdm2 (unmodified or auto-ubiquitinated) was washed with 50 mM HEPES and incubated with 1 μg of UbcH5c in a 50-μl final volume of lysis buffer at 4 °C for 2 h. Samples were treated with 15 mM dithiobis[succinimidyl propionate], a thiol-cleavable cross-linker (Thermo Scientific), at 22 °C for 2 min. Cross-linking was quenched with 50 mM (final concentra-

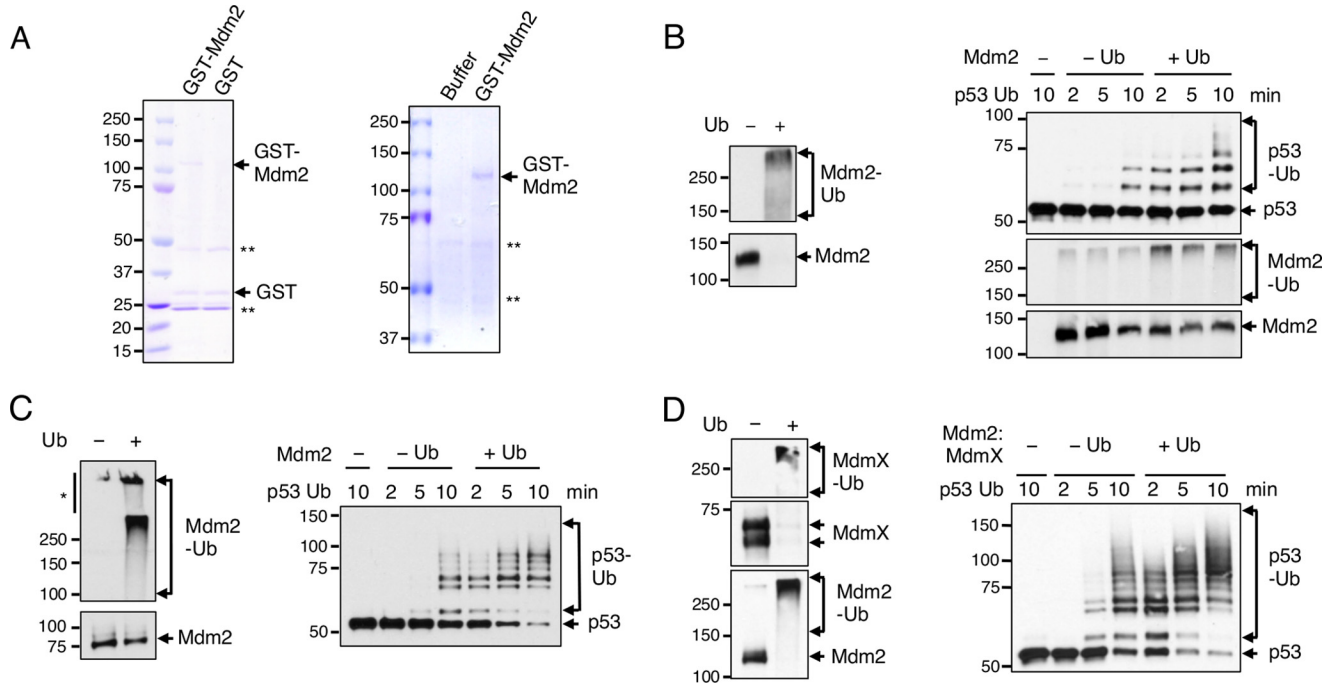

FIGURE 1. **Auto-ubiquitination of Mdm2 and Mdm2-MdmX enhances ability to modify p53.** *A*, GST alone or GST-Mdm2 was pulled down from 293T lysate and immobilized on glutathione beads. Purified GST and GST-Mdm2 were resolved by SDS-PAGE on an 8–15% gradient (*left*), and purified GST-Mdm2 was resolved on an 8% SDS-PAGE, and both were visualized by Coomassie Blue staining. **, nonspecific bands. *B* and *C*, *left*, Western blot depicting immobilized GST-Mdm2 (*B*) or HA-Mdm2 (*C*) without (−) or with (+) auto-ubiquitination immunoblotted with anti-Mdm2 (*bottom left*) and anti-ubiquitin (*top left*). *B* and *C*, *right*, p53 ubiquitination performed in the presence of no Mdm2 (−), unmodified Mdm2 (−Ub), or auto-ubiquitinated Mdm2 (+Ub) and Western blot probed with anti-p53 antibody. *D*, Mdm2-MdmX complex that was co-purified from 293T cells unmodified or automodified and analyzed with anti-MdmX (*top left* and *middle left*) or anti-Mdm2 (*bottom left*) antibodies. Ubiquitination of p53 by previously unmodified or automodified Mdm2-MdmX complex was detected with anti-p53 antibody (*right*). Molecular mass markers (in kDa) are shown on the *left*. This and all subsequent results are representative of at least three independently repeated experiments.

tion) Tris-HCl, pH 7.5, for 15 min. After extensive washing, the bead-bound proteins were boiled in sample buffer containing 5% 2-mercaptoethanol to reverse the cross-linking and analyzed by Western blotting.

RESULTS

Auto-ubiquitination Enhances the Substrate E3 Activity of Mdm2 and the Mdm2-MdmX Complex—To examine how Mdm2 auto-ubiquitination may affect its ability to covalently modify p53, we used an *in vitro* system where auto-ubiquitination could be readily separated from the subsequent p53 ubiquitination. Glutathione *S*-transferase (GST)-tagged Mdm2 was immobilized on beads and incubated with E1, E2, and ubiquitin, in the presence or absence of ATP to permit or prevent Mdm2 auto-ubiquitination (Fig. 1*A* and 1*B*, *left*). Afterward, the ubiquitination reaction components were washed away, and the unmodified and automodified Mdm2 was used to conjugate p53 with ubiquitin in the presence of fresh reaction components. Of note, compared with the unmodified Mdm2, automodified Mdm2 exhibited a noticeably stronger ability to ubiquitinate p53 (Fig. 1*B*, *right*). To exclude any GST tag-specific effects, we performed a similar experiment using hemagglutinin (HA)-tagged Mdm2. Automodified HA-Mdm2 also showed a strongly enhanced ability to ubiquitinate p53 (Fig. 1*C*).

In vivo Mdm2 is present predominantly as a heterodimer with MdmX, which possesses minimal E3 activity of its own, but stimulates the E3 activity of Mdm2 (16–18). To generate the Mdm2-MdmX complex, we co-expressed GST-tagged Mdm2 and FLAG-tagged MdmX in cells and performed sequential pulldowns with anti-FLAG antibody-conjugated beads and glutathione beads. Upon auto-ubiquitination, the purified Mdm2-MdmX complex showed markedly enhanced E3 activity toward p53 (Fig. 1*D*). These results suggest that auto-ubiquitination enhances the E3 activity of both Mdm2 and the Mdm2-MdmX complex.

Auto-ubiquitination of Mdm2 Promotes p53 Polyubiquitination—A polyubiquitin chain linked through the Lys^{48} residue on ubiquitin is the canonical signal for proteasomal degradation (1). We examined whether p53 polyubiquitination was enhanced by Mdm2 auto-ubiquitination. When automodified Mdm2 was used, polyubiquitinated p53 species, which were indicated by their reactivity to a polyubiquitin-specific antibody as well as by their extremely high molecular masses, appeared rapidly (within 2 min). In contrast, when unmodified Mdm2 was used, polyubiquitinated p53 species appeared relatively slowly (in ~20 min) (Fig. 2*A*). Automodified Mdm2 also exhibited an enhanced ability to conjugate p53 with Lys^{48}-only ubiquitin, in which all Lys residues except for Lys^{48} were mutated to Arg residues (Fig. 2*B*, *lanes 1–7*). These results suggest that auto-ubiquitination of Mdm2 enhances its ability to conjugate p53 with Lys^{48}-linked polyubiquitin chains.

Substrate E3 Activity of Mdm2 Is Enabled by Varying Extents of Polyubiquitination but Not by Mono-ubiquitination—To examine whether the extent of Mdm2 auto-ubiquitination influences its substrate E3 activity, we performed a time course

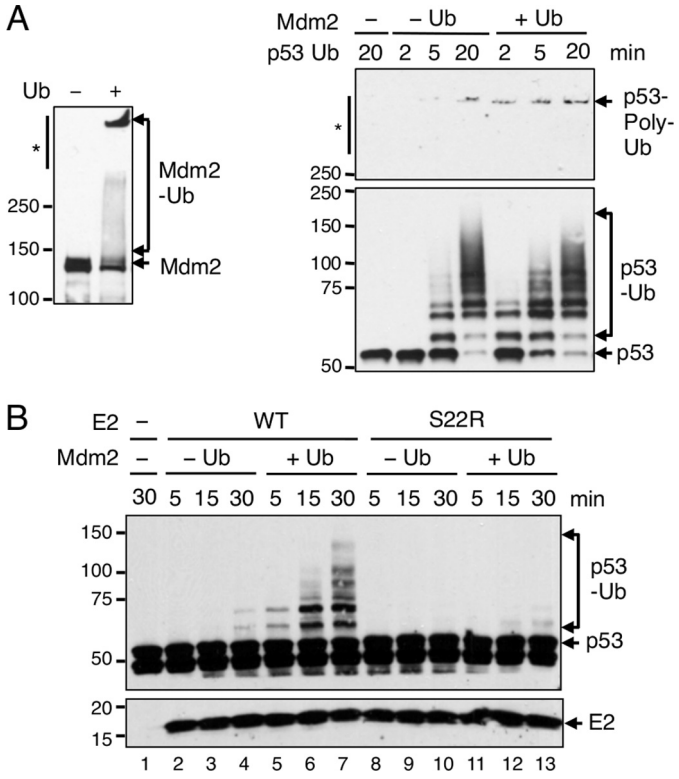

FIGURE 2. **Mdm2 auto-ubiquitination promotes Lys^{48}-linked polyubiquitination of p53.** *A*, GST-Mdm2 auto-ubiquitination was probed with anti-Mdm2 (*left*), and p53 ubiquitination equivalent to 5% input for immunoprecipitation (*bottom right*) was analyzed with anti-p53 antibody. A portion of the p53 ubiquitination reaction was used to immunoprecipitate p53 and analyzed with a polyubiquitin-specific antibody (FK1) for p53 polyubiquitination (*top right*). *, stacking gel. *B*, GST-Mdm2 that was unmodified or auto-ubiquitinated with wild-type ubiquitin was used to ubiquitinate p53 using Lys^{48}-only ubiquitin in the presence of no E2, WT UbcH5c, or S22R UbcH5c (in which Ser^{22} of UbcH5c was mutated to Arg). Antibodies for Western blotting were anti-p53 (*top*) and anti-UbcH5 (*bottom*).

experiment for Mdm2 auto-ubiquitination. The ability of GST-Mdm2 to ubiquitinate p53 initially increased in relation to more auto-ubiquitination, but later declined with higher levels of auto-ubiquitination (Fig. 3*A*). A similar result was observed using HA-Mdm2 (Fig. 3*B*). However, regardless of the extent of auto-ubiquitination, automodified Mdm2 was consistently more active than unmodified Mdm2 at ubiquitinating p53. To determine whether the increase in Mdm2 substrate E3 activity is due to polyubiquitination, we used methylated ubiquitin, which permits only mono-ubiquitination at one or multiple sites (19). The substrate E3 activity of methyl-ubiquitinated Mdm2 was comparable with that of unmodified Mdm2 (Fig. 3*C*), indicating that mono-ubiquitination did not enhance Mdm2-mediated p53 ubiquitination.

Auto-ubiquitination Facilitates the Interaction of Mdm2 with the UbcH5 E2 Enzyme—Next we investigated the mechanisms by which auto-ubiquitination of Mdm2 stimulates the substrate E3 activity of Mdm2. A possible explanation is that auto-ubiquitination might enhance the ability of Mdm2 to interact with p53. However, an *in vitro* pulldown assay showed that unmodified and automodified Mdm2 were comparable in their binding to p53 (Fig. 4*A*).

Additionally, we considered the possibility that Mdm2 may transfer ubiquitin chains assembled on itself to p53. A previous study showed that the RING domain ubiquitin ligase gp78, which is involved in degradation of misfolded endoplasmic reticulum proteins, could transfer to the substrate protein a polyubiquitin chain that is attached via a thioester bond to the active site Cys residue of the E2 Ube2g2 (20). For a polyubiquitin chain attached via an isopeptide bond to a Lys residue on an E3, such a transfer mechanism has not been reported. To distinguish between *de novo* chain synthesis and ubiquitin transfer, we incubated auto-ubiquitinated GST-Mdm2 with p53 and ubiquitination components but omitted ubiquitin from the reaction. p53 was ubiquitinated in the absence of fresh ubiquitin, but the amount of ubiquitination was very small and accounted for only a minute fraction of total p53 ubiquitination mediated by automodified Mdm2 (Fig. 4*B*, *lanes 2–4 versus lanes 5–7*). This result suggests that auto-ubiquitin transfer is unlikely to be the mechanism by which auto-ubiquitination enhances the ability of Mdm2 to stimulate p53 ubiquitination.

Of note, members of the UbcH5 family, which are the cognate E2s for an array of E3s including Mdm2 (21), contain a ubiquitin binding domain (UBD) (15, 22). The UBD is required for the processivity of UbcH5-mediated auto-ubiquitination of the RING domain ligase BRCA1 (15). We reasoned that the polyubiquitin chains on Mdm2 could simultaneously recruit multiple E2~Ubs through binding to their UBDs, thereby circumventing the requirement for multiple rounds of E2 recharging. To test this possibility, we compared the interactions of ubiquitinated and unmodified Mdm2 with recombinant UbcH5c (Fig. 4*C*) in an *in vitro* pulldown assay. Because of the transient nature of the E2-E3 interaction (2, 4), we employed chemical cross-linking to stabilize the binding. The interaction of UbcH5c with ubiquitinated Mdm2 could be readily detected under these conditions, but the interaction between UbcH5c and unmodified Mdm2 could not (Fig. 4*D*, *lanes 1–3*). In accordance with the lack of a stimulating effect of mono-ubiquitination on Mdm2 substrate E3 activity (Fig. 3*D*), the interaction between UbcH5c and mono-ubiquitinated Mdm2 could not be detected under these conditions (Fig. 4*E*). These results suggest that polyubiquitin chains on Mdm2 may enhance the recruitment of E2 enzymes.

The Noncovalent Interaction between the UBD on E2s and Ubiquitin Is Required for the Enhancement of E2 Recruitment and Substrate E3 Ligase Activity of Mdm2—To examine the functional importance of the noncovalent E2-ubiquitin interaction, we used E2 and ubiquitin mutations that impair the noncovalent interaction. Mutation of Ser^{22} within the UBD to Arg (S22R) impaired the interaction of UbcH5c with ubiquitin (15), whereas it did not affect the overall structure of UbcH5c (15) or its thioesterification with ubiquitin (Fig. 5*A*). Unlike WT UbcH5c, the UbcH5c S22R mutant showed no enhanced binding to automodified Mdm2 (Fig. 4*D*, *lanes 4–6*). Moreover, in the presence of S22R, automodified Mdm2 became ineffective at conjugating p53 with WT ubiquitin (Fig. 5*B*) and even less effective at conjugating p53 with Lys^{48}-only ubiquitin (Fig. 2*B*, *lanes 8–13*).

Most UBDs contact a hydrophobic surface on ubiquitin that is centered on Ile^{44} (23). Mdm2 conjugated with I44A ubiquitin showed no increase in binding to UbcH5c (Fig. 6*A*). We performed Mdm2 auto-ubiquitination using either I44A ubiquitin

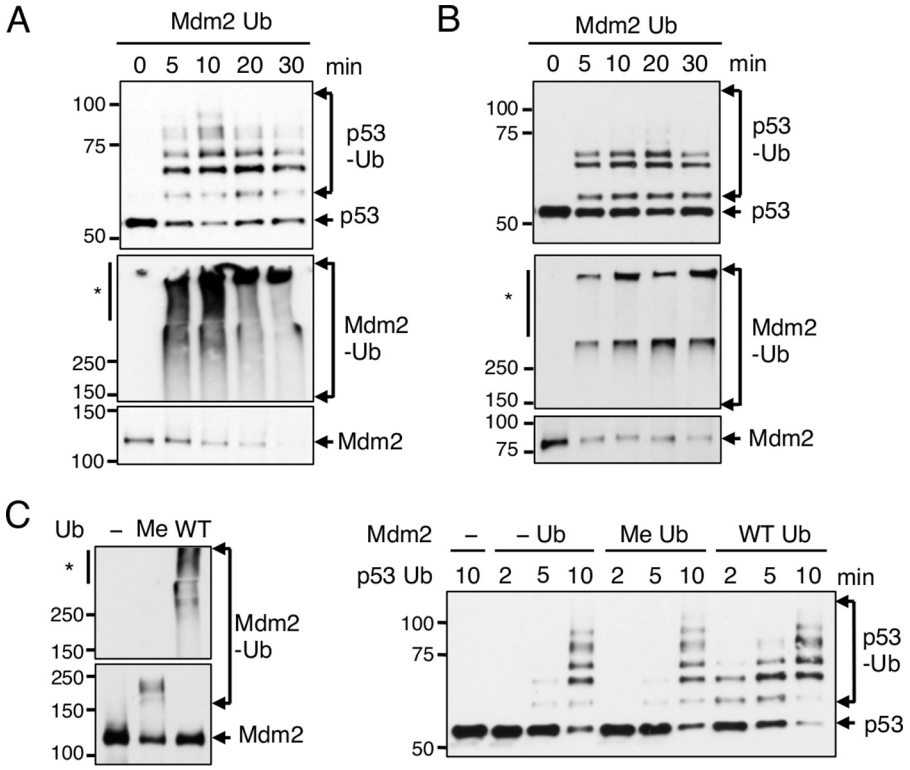

FIGURE 3. **The extent of polyubiquitination, not monoubiquitination, regulates Mdm2 E3 activity.** *A* and *B*, ubiquitination of p53 with GST-Mdm2 (*A*) and HA-Mdm2 (*B*) that has been auto-ubiquitinated for the indicated times. Ubiquitination of p53 was detected with anti-p53 antibody (*top*), and Mdm2 auto-ubiquitination was analyzed with anti-Mdm2 (*bottom*) and anti-ubiquitin (middle) antibodies. *C*, ubiquitination of p53 in the presence of no Mdm2 (−), unmodified Mdm2 (−Ub), mono-ubiquitinated Mdm2 (*Me Ub*), or polyubiquitinated Mdm2 (*WT Ub*). Mdm2 auto-ubiquitination was probed with anti-Mdm2 (*bottom left*) and anti-ubiquitin (*top left*). Ubiquitinated p53 was detected with anti-p53 antibody (*right*).

for different times (30 and 45 min) or WT ubiquitin for a shorter time (10 min) (Fig. 6*B*). Mdm2 conjugated with I44A ubiquitin showed noticeably reduced activity compared with Mdm2 conjugated with WT ubiquitin, especially at early time points (2 and 5 min) (Fig. 6*C*). Mdm2 with longer I44A ubiquitination (45 min) had even less activity compared with Mdm2 with shorter I44A ubiquitination (30 min). Therefore, when the E2 and ubiquitin chains on Mdm2 cannot bind to each other, auto-ubiquitination becomes ineffective at stimulating Mdm2 substrate E3 activity.

DISCUSSION

The current study shows that auto-ubiquitination of Mdm2 results in an enhanced substrate ubiquitin ligase activity. It also suggests a model where the polyubiquitin chains on an E3 act as "landing pads" for UbcH5∼Ub through the noncovalent ubiquitin-UbcH5 interaction (Fig. 7). The noncovalent ubiquitin-UbcH5 interaction has been shown to facilitate the assembly of UbcH5∼Ub into homo-oligomeric complexes (15), which may further enrich UbcH5∼Ub in the proximity of the E3-bound target protein. The increased local concentration of E2∼Ub may overcome the rate-limiting step of E2 recruitment and permit processive ubiquitination of the substrate.

Auto-ubiquitination is a general feature of RING domain E3 ligases. We speculate that auto-ubiquitination may also augment the activity of other multiple-domain RING ligases that use members of the UbcH5 family as their cognate E2s. In principle, auto-ubiquitination can accelerate other steps of ubiquitination and could be an activating event for multiple-domain RING ligases that employ E2s without an UBD. Of note, for Cul1-based CRLs, the rapid E2-E3 association and dissociation, albeit facilitating substrate ubiquitination, cannot fully account for the high processivity of the reaction (6). It would be interesting to determine whether auto-ubiquitination also enhances the substrate E3 activity of these CRLs.

Certain enzymes can be activated through autocatalytic action, as exemplified by the activation of receptor tyrosine kinases by autophosphorylation (25) and of apoptotic proteases (caspases) by autoproteolytic cleavage (26). The results presented here further support the notion that autocatalytic action is a prevalent mechanism for switching on enzymatic activity. Like receptor tyrosine kinases and caspases, ubiquitin ligases catalyze a post-translational modification that has profound effects on various target proteins and that, if not controlled properly, can have deleterious consequences to the cell and the organism. Thus, it is vital to synthesize these enzymes with minimal or no activity and to activate them in a controlled manner. Autocatalytic activation, as opposed to trans-activation by molecules of the same class or a different class of enzymes, would offer important advantages. It is highly efficient because of the reduced reliance on other enzymes. From an evolutionary point of view, autocatalytic activation might also be a necessity. When a new class of enzyme emerged, other regulatory proteins might not initially be able to perform the task, or might not even exist. Perhaps more importantly,

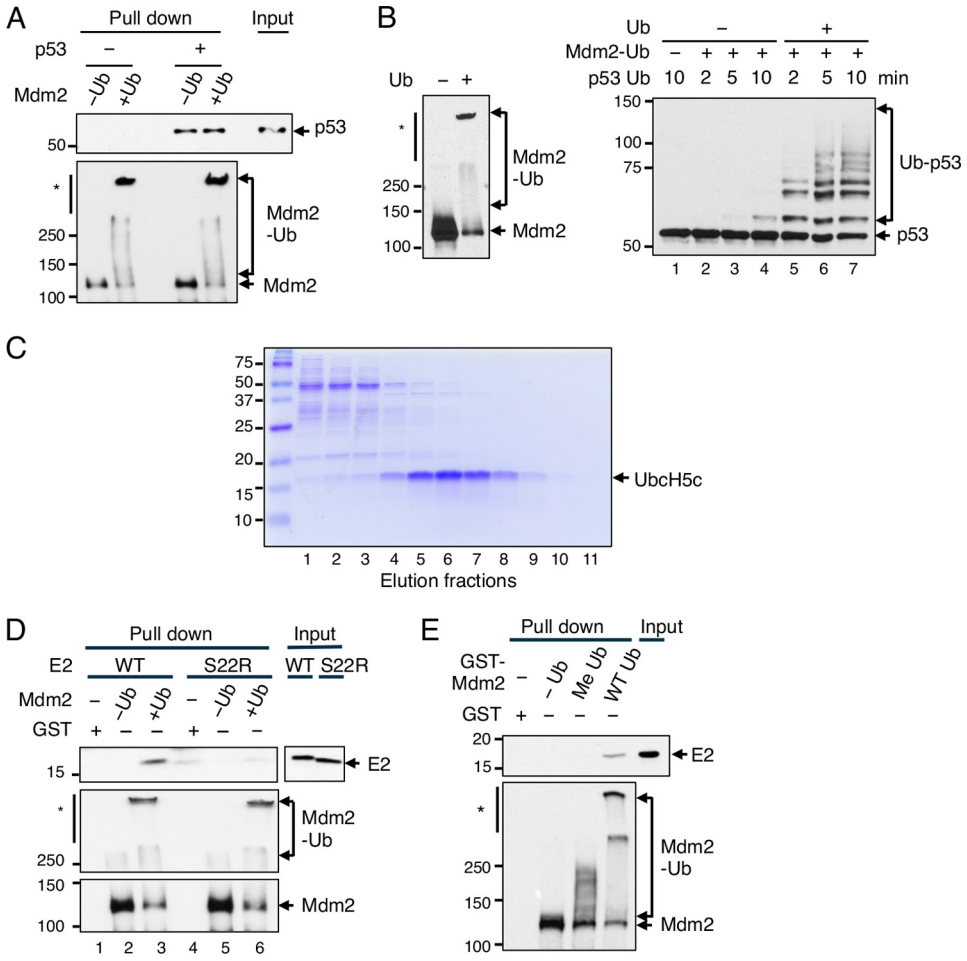

FIGURE 4. **Auto-ubiquitination of Mdm2 does not change affinity for p53 but enhances its recruitment of UbcH5c.** *A*, immobilized GST-Mdm2 with or without auto-ubiquitination incubated alone or with p53. The bound proteins were analyzed by Western blotting with anti-p53 (*top*) and anti-Mdm2 (*bottom*) antibodies. The p53 input shown is equivalent to 2.5% of total p53. *B*, immobilized GST-Mdm2 auto-ubiquitinated (*left*) and used for p53 ubiquitination without (−) or with ubiquitin (+) in the reaction (*right*). *C*, purification of WT UbcH5c. Extracts of BL21 cells expressing UbcH5c were fractionated on a Superdex 200 gel filtration column. Fractions were resolved by SDS-PAGE and stained with Coomassie Blue. The elution profile of S22R UbcH5c was similar. *D*, *in vitro* binding of WT UbcH5c or S22R UbcH5c with GST, unmodified GST-Mdm2, or auto-ubiquitinated GST-Mdm2 with minimal reversible cross-linking. Input is 1% of total UbcH5c used for binding. Western blot was analyzed with anti-UbcH5c (*top*) and anti-Mdm2 (*middle* and *bottom*). *E*, *in vitro* binding of UbcH5c with GST (*lane 1*), unmodified GST-Mdm2 (*lane 2*), mono-ubiquitinated GST-Mdm2 (*lane 3*), or polyubiquitinated GST-Mdm2 (*lane 4*) with minimal cross-linking. Input is 0.5% of total UbcH5c used for binding; immunoblotted with anti-UbcH5c (*top*) and anti-Mdm2 (*bottom*).

autocatalytic activation, as opposed to autocatalytic inhibition, engenders a built-in quality control mechanism: proteins that cannot fulfill the intended function would not become activated.

Nevertheless, if it is influenced by concentrations of E3 ligases, autocatalytic activation also enables a negative regulatory mechanism of these ligases. As demonstrated for the receptor tyrosine kinases and for the precursors of caspase, the autocatalytic activation can be induced by dimerization or oligomerization (25, 27). Activation of Mdm2 is also likely induced by its homo-oligomerization or hetero-oligomerization with MdmX mediated by the RING domains on these proteins, especially the C-terminal amino acids of these domains (28–30). Mdm2 oligomers exhibit enhanced E3 activity compared with Mdm2 monomers (29), indicating an important role of oligomerization in Mdm2 activation. Oligomerization also facilitates the auto-ubiquitination of Mdm2 or the Mdm2-MdmX complex. In a heterodimer formed by the RING domains of these proteins, self-ubiquitination occurs *in trans*, with Mdm2 ubiquitinating MdmX but not itself (31). The reasons for this selective ubiquitination are not completely clear, as the RING domains of Mdm2 and MdmX in this complex appear to adapt nearly identical structures. It is proposed that in an Mdm2 RING homodimer, one Mdm2 molecule might take on the role as a substrate, whereas the other one as the enzyme (31). Still, it is possible that in the complex formed by full-length Mdm2 or Mdm2 and MdmX proteins, auto-ubiquitination may occur *in cis*, as well as *in trans*. Also, the auto-ubiquitination may occur between different complexes instead of within the same complex. A precedent for the latter is shown for the activation of caspases, where the activating cleavage events occur between dimeric caspase precursors (24). This scenario would make auto-ubiquitination especially sensitive to the abundance of Mdm2.

Recent results from mouse models point to the importance of Mdm2-MdmX hetero-oligomerization for the ability of Mdm2 to restrain p53 *in vivo*. Mutation of the conserved cysteine residue in the MdmX RING domain, C462A, disrupts dimerization with Mdm2 and allows for p53 activation, leading to embryonic lethality by day 9.5 (17). Notably, in the

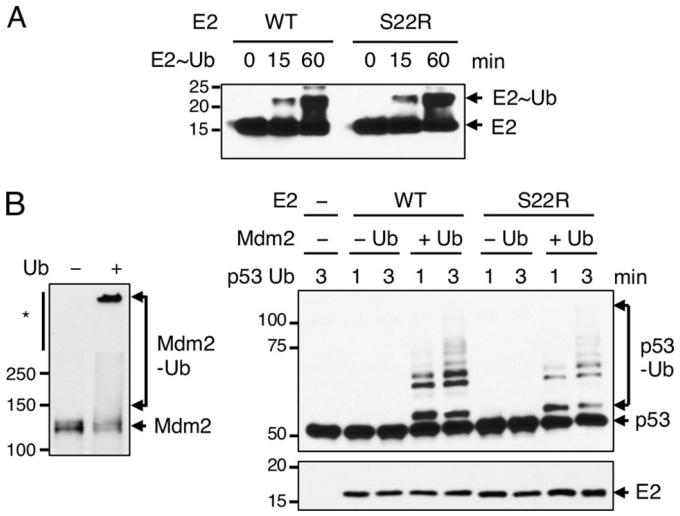

FIGURE 5. **The UbcH5c S22R mutant renders auto-ubiquitination ineffective in stimulating Mdm2 substrate E3 activity.** *A*, WT and S22R UbcH5c was thioesterified with ubiquitin for different durations. The reaction was analyzed by nonreducing SDS-PAGE and Western blotting with anti-UbcH5c antibody. *B*, GST-Mdm2 was unmodified (−) or auto-ubiquitinated (+) with WT UbcH5c, and the Western blot was analyzed with anti-Mdm2 antibody (*left*). Ubiquitination of p53 by unmodified or auto-ubiquitinated Mdm2 in the presence or absence of WT or S22R UbcH5c was analyzed with anti-p53 (*top*) and anti-UbcH5c (*bottom*).

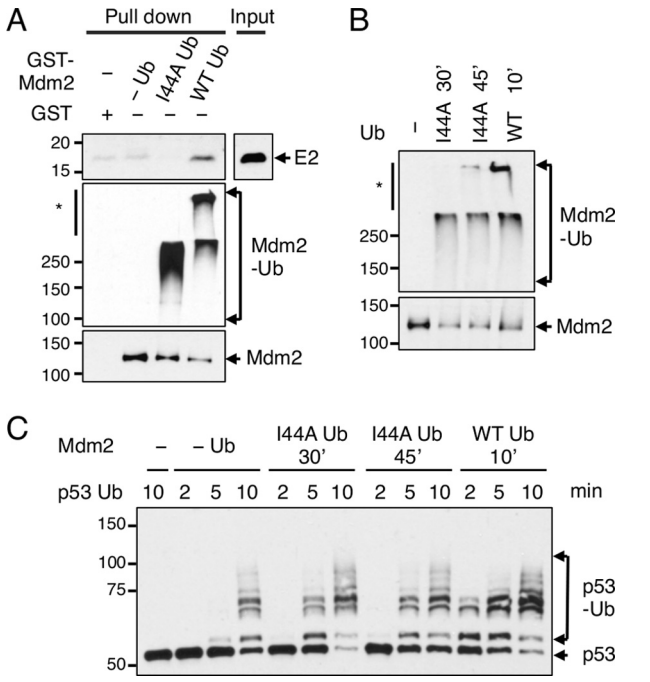

FIGURE 6. **Ubiquitin mutant I44A impairs E2 recruitment to and the substrate E3 activity of auto-ubiquitinated Mdm2.** *A*, *in vitro* binding of UbcH5c with GST, unconjugated Mdm2, or Mdm2 conjugated with I44A or WT ubiquitin. Input is 0.5% of total UbcH5c used for binding. Western blot was analyzed with anti-UbcH5c (*top*), anti-ubiquitin (*middle*), anti-Mdm2 (*bottom*). *B* and *C*, unconjugated Mdm2, or Mdm2 conjugated with I44A or WT ubiquitin for the indicated times (*B*) were used to ubiquitinate p53 for different durations (*C*). Mdm2 auto-ubiquitination was analyzed with anti-ubiquitin (*top*) and anti-Mdm2 (*bottom*). Ubiquitination of p53 was probed with anti-p53.

MdmX^{C462A} mouse model, disruption of heterodimerization results in less Mdm2 ubiquitination and higher levels of p53 and Mdm2. We envision a scenario where under physiological settings Mdm2 alone is unable to function as a potent E3 ligase

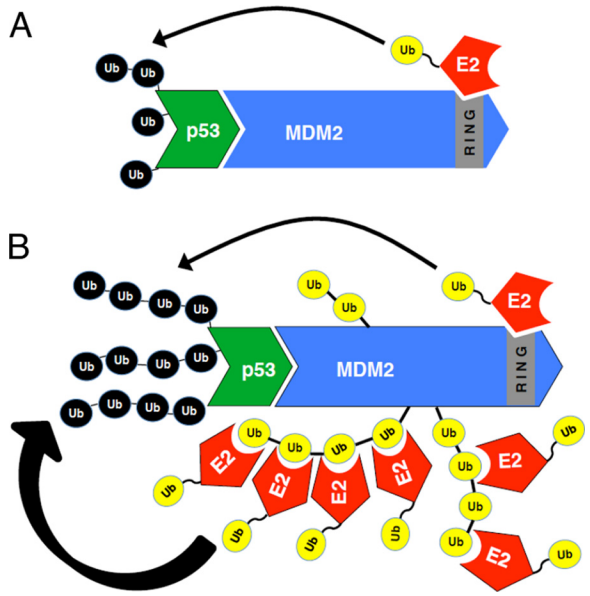

FIGURE 7. **Model for the enhanced substrate E3 activity of auto-ubiquitinated Mdm2.** *A*, unmodified Mdm2 recruits a single E2~Ub through the RING domain for each round of substrate ubiquitination. *B*, the polyubiquitin chains on Mdm2 may act as landing pads to recruit multiple E2~Ub molecules via noncovalent interactions between ubiquitin and the UBD on E2s. The increased local concentration of E2~Ub molecules allows for processive ubiquitination of p53.

probably due to its low levels and the relatively weak self-association. In comparison, the Mdm2-MdmX association may occur more readily, which triggers the formation of auto-ubiquitin chains that recruit multiple E2s to processively polyubiquitinate p53.

Regardless of the precise mechanism, the activation of RING domain ubiquitin ligases such as Mdm2 likely follows a similar mode to the dimerization/oligomerization-induced activation of receptor tyrosine kinases and caspases. In this case, auto-ubiquitination likely rids the cell of excessive E3s when the concentration of an E3 reaches a threshold while no substrates are around, thereby allowing a homeostatic control of the levels of these ligases.

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