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Acetylation-dependent regulation of MDM2 E3 ligase activity dictates its oncogenic function

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Abstract

Abnormal activation of the oncogenic E3 ubiquitin ligase murine double minute 2 (MDM2) is frequently observed in human cancers. By ubiquitinating the tumor suppressor p53 protein, which leads to its proteasome-mediated destruction, MDM2 limits the tumor-suppressing activity of p53. On the other hand, by ubiquitinating itself, MDM2 targets itself for destruction and promotes the p53 tumor suppressor pathway, a process that can be antagonized by the deubiquitinase herpesvirus-associated ubiquitin-specific protease (HAUSP). We investigated the regulation of MDM2 substrate specificity and found that acetyltransferase p300-mediated acetylation and stabilization of MDM2 are molecular switches that block self-ubiquitination, thereby shifting its E3 ligase activity toward p53. In vitro and in cancer cell lines, p300-mediated acetylation of MDM2 on Lys¹⁸² and Lys¹⁸⁵ enabled HAUSP to bind, presumably deubiquitinate, and stabilize MDM2. This acetylation within the nuclear localization signal domain decreased its interaction with the acidic domain, subsequently increased the interaction between the acidic domain and RING domain in MDM2, enabled the binding of HAUSP to the acidic domain in MDM2, and

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SUPPLEMENTARY MATERIALS

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Fig. S1. p300 promotes MDM2 acetylation largely at the Lys¹⁸² and Lys¹⁸⁵ residues.

Fig. S2. Sirtuin family of deacetylases governs MDM2 acetylation status in cells.

Fig. S3. MDM2 acetylation status affects its intra- and intermolecular interaction with MDM2 interacting proteins.

Fig. S4. Acetylation of MDM2 governs the intramolecular interaction between its functional domains, as well as the intermolecular interaction between MDM2 and HAUSP.

Fig. S5. HAUSP interaction with MDM2 is independent of nuclear translocation of MDM2.

Fig. S6. Acetylation of MDM2 enhances the oncogenic function of MDM2.

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shifted MDM2 activity from autoubiquitination to p53 ubiquitination. However, upon genotoxic stress through exposure to etoposide, the deacetylase sirtuin 1 (SIRT1) deacetylated MDM2 at Lys¹⁸² and Lys¹⁸⁵, thereby promoting self-ubiquitination and less ubiquitination and subsequent degradation of p53, thus increasing p53-dependent apoptosis. Therefore, this study indicates that dynamic acetylation is a molecular switch in the regulation of MDM2 substrate specificity, revealing further insight into the posttranslational regulation of the MDM2/p53 cell survival axis.

INTRODUCTION

The p53 tumor suppressor is indispensable for the cellular DNA damage response to maintain genomic stability, in part, by transactivating a large cohort of downstream target genes to control the induction of cell cycle arrest, DNA repair, and apoptosis (1). As such, the critical tumor-suppressive function of p53 is frequently compromised through *TP53* gene deletion or mutation in various types of human tumors. Most p53 mutations are found within the DNA binding domain and often abolish its transcriptional activity. In some tumors that express wild-type p53, p53 function is dysregulated as a result of abnormal expression of murine double minute 2 (MDM2) homolog, loss of *p14^{ARF}* gene, or viral infection (2). MDM2 is the major upstream E3 ubiquitin ligase for p53 that marks p53 for proteolysis by polyubiquitinating it on six lysine (Lys or K) residues within its C-terminal region (3). Notably, MDM2 also negatively regulates its own abundance largely by self-ubiquitination. In addition, MDM2 activity is regulated through protein interactions, such as interaction with the herpesvirus-associated ubiquitin-specific protease (HAUSP) and the tumor suppressor p14^{ARF} (4). Amplification or overexpression of *MDM2* has been reported in multiple tumor types, highlighting the clinical significance of aberrant *MDM2* expression in tumorigenesis (5, 6). As such, inhibition of the oncogenic activity or acceleration of the self-ubiquitination of MDM2 might suppress tumorigenesis. Thus, the precise regulatory mechanism that governs the oncogenic activity of MDM2 in cells may be of clinical relevance.

Modification of Lys residues on nonhistone proteins is often involved in the regulation of their subcellular localization and inter- and intramolecular interactions through neutralization of the positive charge of the Lys residue (7). Biochemically, acetylation is a reversible posttranslational modification performed by the opposing activities of protein acetyltransferases and deacetylases (7). The acetyltransferase p300 was originally identified as a global transcriptional coactivator, regulating transcriptional activation by interacting with sequence-specific transcriptional factors. Mechanistically, p300 acetylates histones as well as various transcription factors, including signal transducer and activator of transcription 3 (STAT3), nuclear factor κ B (NF κ B), and Forkhead box protein O (FOXO), and modulates their DNA binding affinity and intermolecular interaction (8). Hence, reversible Lys modification by acetyltransferase and deacetylase functions as a molecular switch to govern the cellular function of their substrates (9, 10).

Here, we investigated the acetylation-mediated regulation of MDM2 with regard to its substrate specificity, which dictates its oncogenic function. We found that p300 and the deacetylase sirtuin 1 (SIRT1) reciprocally modulated the function of MDM2 through shared

target Lys sites. Acetylation at Lys¹⁸² and Lys¹⁸⁵ in MDM2 by p300 promoted the interaction of MDM2 with the deubiquitinase HAUSP, thereby suppressing self-ubiquitination, as well as altered the conformation of MDM2, thereby enhancing its functional interaction with p53. However, under conditions of cellular stress, deacetylation of the same Lys residues in MDM2 by SIRT1 promoted its self-ubiquitination and degradation, subsequently enabling stabilized p53 abundance and increased p53-dependent apoptosis. Together, our findings reveal that this acetylation/deacetylation switch governs the oncogenic function of MDM2.

RESULTS

Acetyltransferase p300 acetylates MDM2 at Lys¹⁸² and Lys¹⁸⁵

The p300 protein acetyltransferase is reported to interact with and trigger p53 polyubiquitination (11), but its mechanistic link to MDM2 E3 ligase activity remains elusive. This prompted us to examine whether p300 directly acetylates MDM2 to enhance its p53 ubiquitination activity. Consistent with a previous report (11), we observed an interaction of p300 with MDM2 in U2OS and T47D cells (Fig. 1A and fig. S1A). Moreover, endogenous MDM2 was acetylated under physiological conditions in multiple cancer cell types (Fig. 1B and fig. S1, B and C). Among various acetyltransferases, which are reportedly involved in p53 regulation (12, 13), p300 specifically promoted the acetylation of MDM2 (Fig. 1C). In contrast, other acetyltransferases, including general control of amino acid synthesis protein 5-like 2 (GCN5) and Tat-interactive protein 60 kDa (TIP60α), failed to acetylate MDM2 in cells under our experimental conditions (Fig. 1C). Although PCAF [p300-CREB (3',5'-cyclic adenosine monophosphate response element-binding protein)-binding protein (CBP)-associated factor] also has the ability to acetylate MDM2, p300 appears to be the dominant acetyltransferase for MDM2 in 293T cells (Fig. 1C).

Acetylation site prediction (14) revealed that the Lys¹⁸² and Lys¹⁸⁵ residues in the nuclear localization signal (NLS) domain and the Lys³⁴⁴, Lys⁴⁶⁹, and Lys⁴⁷⁰ residues in the C-terminal region may be putative acetylation sites that are targeted by p300 (Fig. 1D and fig. S1D). To test this prediction, we generated C-terminal truncation mutant of MDM2 (containing only 1 to 341 amino acids) for acetylation analysis. MDM2 acetylation by p300 in 293 cells was largely confined within amino acids 1 to 341 (fig. S1E). Moreover, the NLS deletion mutant of MDM2 (NLS) was deficient in p300-mediated acetylation in transfected cells (Fig. 1, E and F), suggesting that the Lys residues within the NLS motif, including Lys¹⁸² and Lys¹⁸⁵, might be the major acetylation sites for p300. Consistently, Arg-substituted mutants for these two Lys residues, K182R and K185R, failed to be acetylated by p300 upon transfection into 293 cells (Fig. 1G), supporting the notion that the Lys¹⁸² and Lys¹⁸⁵ residues are the major sites of p300-mediated acetylation in this experimental setting. Given that Lys¹⁸², but not Lys¹⁸⁵, is evolutionarily conserved and that expression of K182R, more so than K185R, inhibited p300-dependent acetylation of MDM2 in cells (Fig. 1G), we decided to focus our study largely on the contribution of Lys¹⁸² acetylation to the oncogenic role and E3 ligase activity of MDM2.

Thus, to further explore the biological significance of p300-dependent acetylation of MDM2 in cells, we generated and validated an antibody against acetyl-Lys¹⁸²-MDM2 (Ac-K182)

for further studies (Fig. 1H and fig. S1F). Using this antibody, we confirmed that p300 acetylated Lys¹⁸² MDM2 in solution (in vitro; fig. S1G). Phosphorylation of MDM2 at Ser¹⁸⁶ mediated by the kinase AKT promotes its oncogenic function and regulates subcellular distribution (15). Thus, we examined whether the phosphorylation of MDM2 by AKT affects Lys¹⁸² acetylation in cells. However, we found that p300-mediated Lys¹⁸² acetylation was also observed on an AKT1 phosphorylation-mimetic S186D MDM2 mutant in 293 cells (fig. S1H), indicating that AKT-mediated phosphorylation of MDM2 does not appear to substantially influence MDM2 acetylation, at least under these experimental conditions. To further evaluate MDM2 acetylation, this time under genotoxic stress conditions, we conducted acetylation analyses of ectopically expressed MDM2 in *p53*^{-/-} HCT116 cells treated with the genotoxic agent etoposide (ETO). Notably, acetylation of exogenous MDM2 was slightly decreased after treatment with ETO (fig. S1I), implying that MDM2 may be modified by deacetylation after genotoxic stress. Together, these data indicate that p300 acetylates MDM2 largely at the Lys¹⁸² and Lys¹⁸⁵ residues and that these sites might be subjected to deacetylation in response to genotoxic stimuli.

SIRT1 interacts with and deacetylates MDM2

Acetylation of nonhistone proteins is reversibly regulated by deacetylases (7). SIRT3 and SIRT1 deacetylate S-phase kinase-associated protein 2 (SKP2) and FOXO3, respectively, at the sites acetylated by p300 (10, 16). We found that p300-induced MDM2 acetylation was restored in 293 cells by treatment with NIC, an inhibitor of class III (sirtuin) deacetylases (fig. S2A), suggesting that a sirtuin family member (or members) may be involved in MDM2 deacetylation. To clarify the molecular mechanism for MDM2 deacetylation, we screened the sirtuin family of deacetylases for their interaction with MDM2. We found that SIRT1, SIRT6, and SIRT7 coimmunoprecipitated with MDM2 from 293T cells (Fig. 2A), indicating that these sirtuins may interact with MDM2. In vitro deacetylation assays using synthetic MDM2 peptides further showed that SIRT1, SIRT2, and SIRT3 efficiently deacetylated MDM2 (Fig. 2, B and C). Given that MDM2 is not localized to mitochondria, we further examined the deacetylation of MDM2 by sirtuins, excluding the mitochondria-localized paralogs SIRT3 and SIRT4. Notably, p300-induced MDM2 acetylation was significantly reduced in 293T cells by ectopic expression of SIRT1 (Fig. 2D). In contrast, SIRT2, SIRT6, and SIRT7 failed to deacetylate MDM2 in this experimental setting. Furthermore, in vitro deacetylation assay indicated that Lys¹⁸² acetylation was largely abolished by SIRT1 in an nicotinamide adenine dinucleotide (NAD)-dependent manner (fig. S2B). To further assess whether SIRT1 physiologically deacetylates MDM2, we analyzed MDM2 acetylation status in *SIRT1*-depleted U2OS and *Sirt1*^{-/-} mouse embryonic fibroblast (MEF) cells (Fig. 2E and fig. S2C). Knockdown of endogenous *SIRT1* led to an accumulation of endogenous MDM2 acetylation in U2OS cells (Fig. 2E). In keeping with this finding, the acetylation of MDM2 in *Sirt1*^{-/-} MEFs was markedly increased compared to wild-type MEFs (fig. S2C). Endogenous SIRT1 coimmunoprecipitated with MDM2 in MCF7 cells (Fig. 2F), indicating that the two proteins may interact. These data combined suggest that SIRT1 is the major deacetylase that physiologically regulates MDM2 acetylation in cells (Fig. 2G).

MDM2 acetylation blocks self-ubiquitination via recruitment of the deubiquitinase HAUSP

Because MDM2 elicits self-ubiquitination in response to DNA damage (13), we further evaluated the biological consequences of MDM2 acetylation on its self-ubiquitination. To this end, we generated acetylation-mimetic (K182Q/K185Q, denoted as KQKQ) and acetylation-deficient (K182R/K185R, denoted as KRKR) mutant forms of MDM2. Notably, self-ubiquitination was readily observed in cells expressing wild-type MDM2, whereas the acetylation-mimetic KQKQ mutant failed to self-ubiquitinate in 293T cells (Fig. 3A). K182Q, but not the K185Q mutant, phenocopied the KQKQ mutant (Fig. 3A), indicating that acetylation of Lys¹⁸² residue versus Lys¹⁸⁵ residue might provide different biological outputs, at least with regard to MDM2 self-ubiquitination. Given that Lys¹⁸², but not the Lys¹⁸⁵ residue, is evolutionarily conserved, our results suggest that Lys¹⁸² acetylation might play a more important role than Lys¹⁸⁵ to govern MDM2 E3 ligase activity in cells. Consistent with this result, the half-life of the KQKQ mutant was prolonged compared to wild-type MDM2 in doxycycline (Dox)-inducible U2OS cells (Fig. 3, B and C), suggesting that MDM2 acetylation blocks self-ubiquitination to stabilize MDM2.

Furthermore, depletion of *SIRT1* from U2OS cells also led to a decrease in the ubiquitination of endogenous MDM2 (Fig. 3D), presumably as a result of the accumulation of acetylated MDM2 (Fig. 2E and fig. S2C). To further define the molecular mechanism by which MDM2 acetylation attenuates its self-ubiquitination, we examined the interaction of MDM2 with various proteins previously reported to modulate MDM2 function (4, 17). Notably, MDM2 acetylation status did not appear to dictate its dimer formation (fig. S3A), which is critical for the activation of MDM2 E3 ligase activity. In addition, the interaction with MDMX, p14^{ARF}, AKT1, or p53 was not altered among various MDM2 acetylation mutants (fig. S3, B to H). However, the deubiquitinase HAUSP interacted with acetylation-mimetic mutants of MDM2 with greater affinity than it did with wild-type MDM2 in 293T cells (Fig. 3E), which might in part explain the reduced autoubiquitination status for the acetylation-mimetic MDM2 mutants.

Given that previous studies revealed acetylation-mediated neutralization of positively charged Lys residues to be involved in regulating protein-protein interaction in various cellular processes (18), we speculated that p300-mediated acetylation might change inter- and/or intramolecular interaction of MDM2. In this regard, MDM2 is largely composed of four major functional domains: N-terminal p53 binding domain, NLS domain, acidic domain, and C-terminal catalytic RING domain (Fig. 3F). Biologically, the acidic domain is a core domain for the intramolecular binding between the acidic and RING domains, which also mediates the interaction with HAUSP (19, 20). Because the KQKQ-MDM2 mutant bound HAUSP more strongly than did the wild-type MDM2 (Fig. 3E), we hypothesized that acetylation within the NLS may promote HAUSP recruitment on the acidic domain.

In support of this hypothesis, in vivo competition assays in 293T cells revealed that increasing dosage of wild-type NLS domain attenuated the association between the acidic domain and HAUSP, indicating that both HAUSP and the nonacetylated NLS motif harboring several positively charged lysine residues might compete for interaction with the negatively charged acidic domain (Fig. 3, F and G). On the other hand, upon neutralization of the positively charged lysine residues, including Lys¹⁸² and Lys¹⁸⁵, via p300-dependent

acetylation events, the acetylation-mimetic KQKQ-NLS displayed a reduced ability to interact with the negatively charged acidic domain, thereby compromising its ability to compete with HAUSP for binding to the MDM2 acidic domain (Fig. 3G). These findings suggest that p300-mediated MDM2 acetylation within the NLS domain enables HAUSP to be recruited to the acidic domain, leading to deubiquitination of MDM2 (Fig. 3J).

In further support of our model, KQKQ-NLS exhibited less binding with the acidic domain compared to wild-type NLS (Fig. 3G), indicating that NLS acetylation led to an alteration in the intramolecular interaction of MDM2 in part via binding to acidic domain. A previously reported study shows that the intramolecular interaction of the acidic domain with the RING domain among MDM2 enables the RING domain to gain access to the N-terminally bound p53, thereby enhancing p53 ubiquitination (20). On the basis of these findings, we further examined whether acetylation of MDM2 in the NLS motif affects the intramolecular interaction between its acidic and RING domains. In agreement with the previous report (20), we found that the acidic domain (126 to 380 amino acids) interacted with wild-type MDM2 (full length) in 293T cells, whereas deletion of the acidic domain (210 to 298 amino acids) largely abolished this association (Fig. 3, H and I). Notably, the K182Q and KQKQ, but not the K185Q, mutants of MDM2 abrogated binding with the acidic domain of MDM2 (Fig. 3I), suggesting that NLS acetylation at a specific site, such as Lys¹⁸², hampers the intramolecular interaction between the acidic domain and the NLS motif to indirectly promote MDM2 association with HAUSP (Fig. 3J).

Acetylation of MDM2 enhances its ubiquitination ability toward p53

Next, we examined whether acetylation within the NLS domain affects the intramolecular interaction between the acidic and the RING domains. Coimmunoprecipitation assays using 293T cells revealed that expression of the wild-type NLS motif diminished the association of the acidic domain with the RING domain, whereas expression of the acetylation-mimetic KQKQ-NLS failed to affect the interaction (fig. S4A). Although further studies are required, these data suggest that the NLS motif and the C-terminal RING domain might compete for binding with the acidic domain, and acetylation-mediated neutralization of the positive charges in Lys¹⁸² and Lys¹⁸⁵ may reduce NLS interaction with the acidic domain (Fig. 3I). As such, acetylation within the NLS of MDM2 may subsequently lead to the release of the acidic domain from the NLS to facilitate the interaction between the acidic and the RING domains, a prerequisite for MDM2-mediated ubiquitination of p53 (Fig. 3J) (20). To further extend these findings, we assessed whether MDM2 acetylation affected p53 ubiquitination. In contrast to the observed role for acetylation in blocking MDM2 self-ubiquitination (Fig. 3A), the acetylation-mimetic KQKQ mutant have an enhanced ubiquitination activity toward p53 in cells (Fig. 3K). Together, these results indicate that p300-mediated MDM2 acetylation might be involved in fine-tuning its substrate specificity by modulating its intra- and intermolecular interaction, rather than grossly affecting its E3 ligase activity. These data also suggest that acetylation within the NLS domain also enhances the RING domain accessibility to the N-terminally bound p53 through altering the intramolecular interaction, thereby increasing MDM2-mediated ubiquitination of p53 (Fig. 3, J and K).

Although our results suggest that acetylation of MDM2 at Lys¹⁸² could lead to the recruitment of HAUSP in part by releasing the NLS–acidic domain interaction, given that previous studies revealed the possibility of acetylation and ubiquitination occurring on the same set of lysine residues (21), we went on to explore whether the major sites for acetylation, Lys¹⁸² or Lys¹⁸⁵, also served as sites of ubiquitination. To this end, and to exclude possible contribution of ubiquitination signals from MDM2-associated proteins, we performed ubiquitination assays using 293T cells transfected with wild-type or acetylation-deficient mutant MDM2 and His-tagged ubiquitin and subsequently lysed under guanidine-denatured conditions for Western blotting (fig. S4B). In this experimental setting, we found that self-ubiquitination of MDM2 was largely reduced by Arg substitution for Lys residue(s), Lys¹⁸² and/or Lys¹⁸⁵, suggesting that these two Lys residues might serve as sites of posttranslational modifications, including acetylation and ubiquitination, depending on the upstream signal cues (fig. S4C).

MDM2 acetylation modulates its subcellular distribution

Given that these acetylation sites are located within the NLS domain (Figs. 1, D and E, and 4A), we next evaluated whether acetylation of Lys¹⁸² and/or Lys¹⁸⁵ may affect MDM2 subcellular distribution. Whereas, consistent with previous report (22), wild-type MDM2 predominantly localized to the nucleus, the acetylation-mimetic mutants resided in both the nucleus and the cytoplasm (Fig. 4B). Endogenous acetylation of MDM2 in MCF7 cells was observed in both the nucleus and the cytoplasm (fig. S5A). SIRT1 coexpression did not affect the observed localization of wild-type or KQKQ mutant MDM2 in 293T cells (fig. S5, B and C). Previous studies showed that nucleus-localized proteins harboring an NLS sequence directly interact with the Importin α subunit to translocate to the nucleus (23). Our in vitro pull-down assays showed that Importin α 5 and α 7 interact with nonacetylated MDM2 (Fig. 4C). Lys¹⁸²-acetylated but not Lys¹⁸⁵-acetylated peptides of MDM2 blocked the interaction of MDM2 with Importin α 5 and α 7 in vitro (Fig. 4C), suggesting that Lys¹⁸² acetylation inhibits Importin α binding to the NLS sequence. Consistently, wild-type and K185Q mutant, but not K182Q or KQKQ mutant MDM2, coimmunoprecipitated with Importin α 7 in 293T cells (Fig. 4D), indicating a physical interaction. These data support our model in which p300 spatially and temporally regulates MDM2 subcellular distribution in part by blocking MDM2 interaction with the Importin complex subunit α 5 or α 7.

HAUSP interaction with MDM2 was enhanced by Lys¹⁸² acetylation (Fig. 3E). This finding led us to wonder whether HAUSP interaction and nuclear translocation of MDM2 are linked or separately regulated. To this end, we conducted cell fractionation assays to determine the subcellular localization where HAUSP interacts with MDM2 in 293T cells. As we expected, wild-type MDM2 and the KRKR-MDM2 mutant, both of which dominantly express in the nucleus, interacted with HAUSP largely in the nucleus (fig. S5D). On the contrary, compared to wild-type and the KRKR mutant, the KQKQ mutant exhibited increased interaction with HAUSP in both the nucleus and the cytoplasm, suggesting that nuclear translocation of MDM2 is not required for HAUSP interaction with MDM2, at least in this experimental setting (fig. S5D). Therefore, HAUSP interaction with and nuclear translocation of MDM2 are possibly independent events from that of MDM2 acetylation.

MDM2 acetylation/deacetylation cycle governs the p53 “pulse” after DNA damage

Given the critical role for p53 in governing cell fate after determination of genotoxic stress to further investigate the biological significance of MDM2 acetylation on p53 function, we analyzed caspase-3 activation in cells expressing wild-type or acetylation-mimetic mutant MDM2 under genotoxic stress conditions (cultured with ETO). Because Lys¹⁸⁵ is also acetylated in cells (Fig. 1G) and K185Q mutant strongly promoted p53 ubiquitination (Fig. 3K), the acetylation-mimetic or acetylation-deficient mutant for both Lys¹⁸² and Lys¹⁸⁵ was also used in these subsequent biological analyses to ascertain the contribution of both acetylation events toward MDM2 function in this experimental setting. Notably, ectopic expression of the acetylation-mimetic KQKQ mutant in U2OS cells attenuated DNA damage–triggered activation of caspase-3 (Fig. 5A). Although the protein abundance of wild-type and KRKR mutant MDM2 slightly decreased after DNA damage, there was no significant reduction in the protein abundance of the KQKQ-MDM2 mutant (Fig. 5A), presumably as a result of reduced self-ubiquitination. As a result, compared to expression of wild-type or KRKR MDM2, ectopic expression of the KQKQ mutant largely blocked p53 stabilization in response to DNA damage (Fig. 5A), an occurrence that we expect in part as the result of increased ubiquitination of endogenous p53 under this experimental condition (Fig. 5B).

Consistently, upon genotoxic stress, there was a reduction of p53 ubiquitination in cells expressing wild-type or KRKR mutant MDM2, whereas p53 ubiquitination was sustained in KQKQ-expressing cells (Fig. 5B). These results suggest that acetylation of MDM2 leads to the fine-tuning of MDM2 E3 ligase activity more toward p53 ubiquitination than self-ubiquitination. To this end, we further examined the effect of acetylation of MDM2 on its association with its upstream deubiquitinase HAUSP. We found that DNA damage–induced HAUSP dissociation from MDM2 was largely diminished in *SIRT1*-depleted U2OS cells (Fig. 5C), supporting our model that MDM2 acetylation triggers a stable interaction of MDM2 and HAUSP.

Biologically, p53 exerts its tumor suppressor role in part by evoking apoptotic cell death through the induction of various proapoptotic genes (24). *PUMA*, a well-characterized p53 target gene encoding the p53–up-regulated modulator of apoptosis protein, collapses mitochondrial membrane potential and releases cytochrome c, resulting in activation of caspase-3 (25). To evaluate the effects of MDM2 acetylation on transcriptional activity of p53, we analyzed *PUMA* induction in U2OS cells expressing wild-type, KRKR, or KQKQ MDM2 using reporter assays. Notably, *PUMA* transactivation was induced by DNA damage in wild-type MDM2– or KRKR-MDM2–expressing U2OS cells as reported previously (Fig. 5D) (26). However, ectopic expression of the KQKQ-MDM2 mutant largely suppressed *PUMA* induction (Fig. 5D), presumably as the result of decreased abundance of p53 through ubiquitin-mediated proteolysis (Fig. 5A). Consistent with these results, compared to that in U2OS cells expressing wild-type MDM2 or KRKR-MDM2, induction of apoptosis after DNA damage was largely attenuated in KQKQ-MDM2 mutant–expressing cells (Fig. 5, E and F).

As a result, in comparison to wild-type MDM2–expressing cells, KQKQ mutant–expressing U2OS cells had greater resistance to ETO-induced cell death (Fig. 5, G and H, and fig.

S6A). These results combined suggest that MDM2 acetylation may serve as a switch to promote MDM2 oncogenic function under genotoxic stress conditions. In this regard, previous reports demonstrated that p53 expression fluctuates after neocarzinostatin (NCS)–induced DNA damage by MDM2-mediated negative feedback regulation (27, 28). We confirmed the oscillation of MDM2 and p53 after DNA damage in MCF7 cells (fig. S6B). Consistent with this result, endogenous MDM2 acetylation also pulsates in response to DNA damage (Fig. 5I). To assess the regulation for the oscillation of MDM2 acetylation after DNA damage, we examined the interaction of MDM2 with p300 or SIRT1. Notably, the binding with p300 and MDM2 in 293T cells was slightly attenuated in response to DNA damage (fig. S6C). However, the SIRT1/MDM2 complex was relatively stable even after ETO treatment (fig. S6C). These data suggest that the decrease in MDM2 acetylation after DNA damage is likely in part the result of its dissociation from p300. MDM2 acetylation and HAUSP interaction in U2OS cells declined 4 hours after treatment with ETO (Fig. 5, C and I), supporting a model in which HAUSP associates with MDM2 possibly in an MDM2 acetylation–dependent manner.

We further investigated whether MDM2 acetylation influences the p53 oscillation in MCF7 cells upon genotoxic stress. In keeping with previous reports (27), expression of endogenous MDM2 increased 4 hours after DNA damage, presumably because of p53-dependent transcriptional activity, representing negative feedback to cause p53 decline after 5 to 7 hours of exposure to NCS, thereby creating a pulsatile nature of p53 abundance (Fig. 5J). In agreement with our finding that SIRT1 depletion stabilized MDM2 protein abundance by increasing its acetylation, we observed that, in SIRT1-deficient MCF7 cells, the second peak of p53 was largely diminished in response to NCS stimulation and was coupled with a prolonged stabilization of endogenous MDM2 (Fig. 5J and fig. S6, D and E). Likewise, compared to wild-type MDM2, the protein abundance of ectopic acetylation-mimetic MDM2-KQKQ mutant was much higher in MCF7 cells after DNA damage, resulting in attenuation of the p53 pulse in KQKQ-MDM2–expressing cells that phenocopies depletion of endogenous SIRT1 (fig. S6F). Furthermore, p300 depletion abolished endogenous MDM2 acetylation (fig. S6G) and MDM2 pulse upon DNA damage (fig. S6H). Together, these data suggest that the p53 pulse is also in part controlled by MDM2 acetylation, indicating that MDM2 acetylation might be a molecular switch for p53-dependent cell fate decisions.

DISCUSSION

p53 is the most well-characterized tumor suppressor, and its tumor-suppressive functions are dysregulated in more than 50% of human tumor tissues (29). The *MDM2* oncogene is frequently amplified in many tumor tissues to functionally inactivate p53 (5), suggesting that inhibition of MDM2 activity might provide a robust method for cancer prevention. Previous studies have demonstrated that p300 cooperates with MDM2 and triggers p53 polyubiquitination as a ubiquitin E4 ligase (11, 30). Here, we propose a novel regulatory mechanism for p300 via acetylation-dependent control of the oncogenic function of MDM2. Specifically, p300 directly acetylates MDM2, a process that channels that E3 ligase activity away from MDM2 autoubiquitination to concentrate on promoting p53 ubiquitination, in part because of acetylation-induced alteration of intramolecular interaction of MDM2. Thus, p300 may regulate p53 ubiquitination through multiple regulatory mechanisms.

Furthermore, we identified that acetylation of MDM2 by p300 triggers a cytoplasmic localization of MDM2 (Fig. 4B), suggesting that p300 spatiotemporally regulates MDM2 function via acetylation (Fig. 5K). Given that MDM2 constitutively ubiquitinates p53 in the cytoplasm (31), cytoplasmic localization of acetylated MDM2 might be essential for earmarking p53 for degradation. To support this notion, the acetylation-mimetic MDM2 has stronger E3 ligase activity toward p53 in cells (Fig. 3K). Our previous study showed that oncogenic function of SKP2, which is a component of SCF (SKP1/cullin/F-box protein)–type E3 ubiquitin ligase complex, is also regulated by p300, and the acetylation by p300 affects subcellular distribution of SKP2 (27). Besides MDM2 and SKP2, the adenovirus-transforming protein E1A is also acetylated by p300 to regulate its interaction with Importin (32). Collectively, p300-mediated acetylation may function as a universal regulatory mechanism for governing subcellular distribution of its acetylation substrates.

Many upstream regulators of MDM2 have been recently discovered to be involved in its oncogenic function in part via direct physical interaction with MDM2. For example, Wip1 contributes to MDM2 stabilization (33), and p14^{ARF} triggers MDM2 self-ubiquitination (34). Akt1 also regulates MDM2 activity toward p53 degradation by phosphorylating MDM2 at Ser¹⁶⁶ and Ser¹⁸⁶ to trigger MDM2 nuclear translocation and p53 ubiquitination (15). Here, we examined whether acetylation regulates the cross-talk between MDM2 and Akt1. However, MDM2 acetylation was observed even in Akt1 phosphorylation–mimetic S186D mutant (fig. S1H). In addition, our results reveal that MDM2 acetylation did not affect its binding with Akt1 (fig. S3, E and F). Furthermore, phosphorylation-mimetic S186D mutants exhibited stronger ubiquitination ability toward p53 and MDM2 itself (Fig. 3, A and J). These data suggest that Akt1 phosphorylation is required for MDM2 activation but is not involved in the acetylation-dependent switch for MDM2 function. Therefore, we concluded that Akt regulates MDM2 E3 ligase activity in an acetylation-independent manner.

Accumulating evidence has revealed that p53 ubiquitination by MDM2 is regulated primarily by posttranslational modifications of p53. The interaction of MDM2 with p53 is primarily regulated by N-terminal p53 phosphorylation by ataxia telangiectasia mutated (ATM) (1). Here, we propose a regulatory mechanism for p53 ubiquitination at the level of MDM2 acetylation. Notably, p300-mediated acetylation alters the tertiary structure of MDM2 and enhances p53 ubiquitination without affecting its interaction with p53 (Fig. 3K and fig. S3H). Consistent with a previous report (35), we observed the p53 pulse after DNA damage (fig. S6B). p53 oscillation was reported to be derived from two-phase dynamics of ATM activity (36). ATM increases p300 activity (37, 38), suggesting that p300 activity also oscillates after DNA damage. ATM activates homeodomain-interacting protein kinase 2 (HIPK2) and dual specificity tyrosine-phosphorylation–regulated kinase 2 (DYRK2), which induce p53-dependent apoptosis (39–41). The second ATM activation is required for proapoptotic function of DYRK2 (40), suggesting that the second peak of p53 is essential for its ability to induce apoptosis. Here, we demonstrated that the second peak of p53 was largely diminished in both KQKQ-MDM2–expressing cells and *SIRT1* knockdown cells with increased MDM2 acetylation (Fig. 5J and fig. S6, D and E). As a consequence, the acetylation-mimetic KQKQ-MDM2 mutant failed to provoke apoptosis (Fig. 5, E and F). Therefore, it is plausible that apoptosis induction by the KQKQ-MDM2 mutant was

attenuated in part because of its acquired ability to diminish the second peak of p53. Our results showed that SIRT1 expression level upon DNA damage does not correlate with p53 expression (Fig. 5J), suggesting that SIRT1 expression might be independent of p53 as reported previously (42). To clarify how MDM2 deacetylation by SIRT1 is alternately regulated after DNA damage, further investigation into the molecular regulation for p300 and SIRT1 activity after DNA damage is required to clarify the p53 oscillation machinery regulated by the MDM2 acetylation event.

A previous study reported that a p300 inhibitor, I-CBP112, shows strong cytotoxicity for acute myeloid leukemia cells (43). It is conceivable that not only the chemical compound targeting for p300 activity but also competitive p300-MDM2 interaction inhibitor might be a promising drug for cancer therapy.

MATERIALS AND METHODS

Cell culture, transfection, virus infection, and chemical treatments

Human embryonic kidney (HEK) 293T, HEK293, HCT116 (p53^{-/-}), PC3, MCF7, T47D, ZR-75, A549, and U2OS cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U of penicillin, and streptomycin (100 mg/ml). Transfection was performed using polyethylenimine (PEI) (Polysciences). ETO (E1383), Dox (D9891), and NCS (N9162) were purchased from Sigma-Aldrich. MG-132 (MBL-PI102) was purchased from Enzo Life Sciences. For lentiviral infection, 293T cells were transfected with packaging vectors [delta 8.9 and vesicular stomatitis virus G (VSVG)] and pTRIPZ-HA-MDM2, GFP-shRNA, SIRT1-shRNA, MDM2 shRNA, or p300 shRNA pLKO plasmids (sourced below), and then the virus particles were collected for infection. Cells were incubated with virus in the presence of polybrene, followed by selection with medium containing puromycin.

Plasmids, proteins, and shRNA

Synthesis of HA-tagged human MDM2 was described previously (27). HAUSP complementary DNA (cDNA) was amplified by polymerase chain reaction (PCR) and cloned into the pcDNA3-Flag and pcDNA3-HA vectors. SIRT1, SIRT2, SIRT6, and SIRT7 cDNA was amplified and cloned into the pcDNA3-Flag vector. MDM2 RING domain was amplified by PCR and cloned into the pcDNA3-HA vector. MDM2 acidic domain was cloned into the pCMV-GST vector. Site-directed mutagenesis was performed by PCR and verified by DNA sequencing. To construct the Dox-inducible MDM2 expressing vector, MDM2 cDNA was subcloned into the pTRIPZ-puro vector described previously (44). Cells were transfected with the indicated pTRIPZ lentiviral vectors that enable ectopic expression of the indicated MDM2 cDNAs under the control of Dox. SIRT1 and MDM2 lentiviral shRNA constructs were purchased from Open Biosystems. Myc-p300, Flag-GCN5, Flag-PCAF, and Flag-TIP60α were purchased from Addgene.

Immunoblotting, immunoprecipitation, and immunofluorescence

Cells were washed with phosphate-buffered saline (PBS) and then lysed in lysis buffer [50 mM tris (pH 7.5), 120 mM NaCl, and 0.5% NP-40] supplemented with protease inhibitors

(cOmplete Mini, Roche), phosphatase inhibitors (phosphatase inhibitor cocktail set, Calbiochem), 2 μ M TSA (T8552, Sigma-Aldrich), and 10 mM NIC (N0636, Sigma-Aldrich) as described previously (45). Cell extracts were separated by SDS–polyacrylamide gel electrophoresis and transferred to nitro-cellulose membranes. The membranes were incubated with indicated antibodies: acetylated Lys [#9814, Cell Signaling Technology (CST)], MDM2 (sc-965, Santa Cruz Biotechnology), Sirt1 (#8469, CST), cleaved caspase-3 (#9661, CST), GST (#2625, CST), p53 (sc-126, Santa Cruz Biotechnology), tubulin (#T-5168, Sigma-Aldrich), HA (sc-805, Santa Cruz Biotechnology), Flag (#F-3165, Sigma-Aldrich), or GFP (#632381, Clontech). We generated and validated an antibody against Ac-K182 in collaboration with CST. Immune complexes were incubated with secondary antibodies and visualized using Immobilon (Millipore). For immunoprecipitation, cell lysates were incubated with agarose-conjugated MDM2 antibody (sc-965, Santa Cruz Biotechnology), agarose-conjugated Flag antibody (#A-2220, Sigma-Aldrich), agarose-conjugated p300 antibody (sc-584, Santa Cruz Biotechnology), or an antibody against HAUSP (#BL851, Bethyl). Immune complex was washed five times with NETN buffer [20 mM tris (pH 8.0), 100 mM NaCl, 1 mM EDTA, and 0.5% NP-40]. For immunofluorescence, HA-MDM2 constructs were transfected into 293T cells, which were then fixed in 4% paraformaldehyde for 10 min, permeabilized with 0.5% Triton X-100 for 15 min, washed once with PBS, and blocked with 5% bovine serum albumin for 30 min. Cells were then stained with an antibody against HA (sc-805, Santa Cruz Biotechnology) or Flag (#F3165, Sigma-Aldrich) for 1 hour at room temperature, washed once with PBS, and then incubated with a goat anti-mouse secondary antibody conjugated to Alexa Fluor 594 for 1 hour. Nuclei were counterstained with 4',6-diamidino-2-phenylindole.

Ubiquitination assays

Ubiquitination assays were performed as described previously (46). Briefly, cells were treated overnight with 10 mM MG-132. Cells were then lysed in buffer A [6 M guanidine-HCl, 0.1 M Na₂HPO₄/NaH₂PO₄, and 10 mM imidazole (pH 8.0)] and sonicated for 7 s. The lysates were incubated with Ni-NTA matrices (Qiagen) for 4 hours at room temperature. The precipitates were washed twice with buffer A, twice with buffer A/TI (1 volume of buffer A and 3 volumes of buffer TI), and once with buffer TI [25 mM tris-HCl and 20 mM imidazole (pH 6.8)].

Luciferase assays

Inducible U2OS cells were transfected with pGL3-puma vector and then treated with Dox. Cells were either untreated or pretreated with ETO for 24 hours. Luciferase activity was measured with the Bright-Glo Luciferase Assay System (Promega) according to the manufacturer's instructions.

Cell fractionation assays

Cells were lysed with 0.1% NP-40/PBS, and cell lysates were centrifuged for 10 s. The supernatant was collected as a whole-cell lysate. Pellet was resuspended with 0.1% NP-40/PBS and centrifuged. The supernatant was collected as a cytoplasmic fraction. Pellets for nuclear fraction were washed with 0.1% NP-40 once and suspended with EBC buffer.

Cell viability assays

Inducible U2OS cells were seeded on 96-well plates for 24 hours and then treated with Dox and ETO for 24 hours. Cell viability was then measured by the CellTiter-Glo Luminescent Cell Viability Assay Kit (Promega) according to the manufacturer's instructions.

Colony formation assays

Inducible U2OS cells were seeded on six-well plates and cultured for 1 week in the presence of Dox. Cells were washed with PBS and fixed with 10% acetic acid/10% methanol for 20 min. Colonies were stained with 0.4% crystal violet in 20% ethanol for 20 min. After staining, plates were washed gently with running water and allowed to dry. Colony numbers were counted manually.

In vitro binding assays

In vitro binding assays were performed as described previously (47). Briefly, 293T cells were transfected with the constructs indicated in the figures using PEI. Lysates were incubated first with biotinylated MDM2 peptides and then with streptavidin agarose beads for pull-down assays.

In vitro acetylation assays

GST-MDM2 (purified from bacteria) and p300 (SRP2079, Sigma-Aldrich) were incubated in acetylation reaction buffer [50 mM tris-HCl (pH 7.5), 50 mM KCl, 100 nM TSA, 5% glycerol, 1 mM dithiothreitol (DTT) and/or 1 mM acetyl-coenzyme A] for 1 hour at 30°C.

In vitro deacetylation assays

In vitro deacetylation assays were performed as described previously (10). GST-MDM2 and Myc-p300 were cotransfected into 293T cells and then pulled down with GST Sepharose. The immunoprecipitates were washed with NETN buffer for five times and then with deacetylation reaction buffer [50 mM tris-HCl (pH 8.8), 4 mM NaCl, and 0.2 mM DTT]. Precipitates were incubated with recombinant SIRT1 protein (7714-DA-050, R&D Systems) and/or 1 mM NAD (N0636, Sigma-Aldrich) for 2 hours at room temperature with constant agitation.

Fluorescence-activated cell sorting analyses

For sub-G₁ analyses, cells were fixed and stained with propidium iodide (Roche). For Annexin V analysis, cells were stained with Annexin V antibody (Roche) according to the manufacturer's instructions. Stained cells were analyzed using a FACS Aria (BD Biosciences) at the Beth Israel Deaconess Medical Center Core (BIDMC) Facility.

Statistical analyses

All quantitative analyses were presented as means $\pm$ SD as indicated, at three independent experiments by Student's *t* test among group differences. *P* < 0.05 was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. p300 acetylates MDM2 largely at the Lys¹⁸² and Lys¹⁸⁵ residues within the NLS domain
(A) Western blotting (IB) after immunoprecipitation (IP) of endogenous MDM2 from U2OS whole-cell lysate (WCL). Immunoglobulin G (IgG) immunoprecipitation served as a negative control. Blots are representative of $n = 2$ biological replicates. **(B)** Western blotting analysis after immunoprecipitation of endogenous MDM2 from T47D or ZR75 whole-cell lysate. $n = 2$ biological replicates. Ac-K, Ac-K182. **(C)** Western blotting analysis after immunoprecipitation with hemagglutinin (HA) antibody from whole-cell lysate from 293T cells transfected with HA-MDM2 and Myc-p300, Flag-GCN5, Flag-PCAF, or Flag-TIP60 α and then treated with 5 mM nicotinamide (NIC) and 1 μ M trichostatin A (TSA). $n = 2$ biological replicates. EV, empty vector. **(D)** Sequence alignment of the putative acetylation sites of MDM2 in various species. **(E)** Schematic diagram of the functional domains of human MDM2 protein. **(F and G)** Western blotting analysis after immunoprecipitation with HA antibody (F) or MDM2 antibody (G) from whole-cell lysate from 293 cells transfected with HA-MDM2 constructs and/or Myc-p300 and treated with 5 mM NIC and 1 μ M TSA. $n = 2$ or 3 biological replicates. WT, wild-type. **(H)** Same as in (F) using 293T cells. $n = 2$ biological replicates.

Fig. 2. SIRT1 interacts with and deacetylates MDM2 both in cells and in vitro

(**A**) Western blotting after immunoprecipitation with HA antibody in whole-cell lysate from 293T cells cotransfected with HA-MDM2 and the indicated Flag-tagged SIRT construct. *n* = 2 biological replicates. (**B** and **C**) Schematic of biotinylated MDM2 peptides (**B**) used subsequently in (**C**) in vitro deacetylation assays of biotinylated MDM2 peptide incubated with lysates from 293T cells expressing the indicated Flag-SIRT. *n* = 2 biological replicates. (**D**) Western blotting analysis after immunoprecipitation with HA antibody in whole-cell lysate from 293T cells transfected with HA-MDM2, Myc-tagged p300, and/or the indicated Flag-SIRT and then treated with 5 mM NIC and 1 μ M TSA. *n* = 2 biological replicates. (**E**) Western blotting analysis after pulldown of endogenous MDM2 from whole-cell lysate from U2OS cells infected with control [green fluorescent protein (GFP)–] or SIRT1-targeted short hairpin RNA (shRNA) and then treated with 5 mM NIC and 1 μ M TSA. *n* = 2 biological replicates. (**F**) Western blotting analysis after immunoprecipitation with SIRT1 antibody in whole-cell lysate from MCF7 cells treated with 10 μ M MG-132 and 5 mM NIC. *n* = 2 biological replicates. (**G**) Model of MDM2 acetylation regulated by p300 and SIRT1.

Fig. 3. MDM2 acetylation triggers p53 ubiquitination but blocks MDM2 self-ubiquitination (A) Western blotting after pulldown by nickel-nitrilotriacetic acid (Ni-NTA) beads in whole-cell lysates from 293T cells transfected with wild-type (WT) or mutant His-Ub and HA-MDM2 and treated with 10 μ M MG-132. $n = 2$ biological replicates. (B and C) Western blotting analysis of whole-cell lysate from Dox-inducible U2OS cells expressing WT or mutant MDM2 and treated with cycloheximide (CHX; 150 μ g/ml) in the presence of Dox (1 μ g/ml) (B). Quantification of signal intensity of HA-MDM2 normalized to that at $t = 0$ (C). Data are means $\pm$ SD from three independent experiments. $*P < 0.05$, unpaired Student's t test. (D) Western blotting analysis after immunoprecipitation with MDM2 antibody in whole-cell lysate from U2OS cells infected with one of two SIRT1 shRNAs. $n = 2$ biological replicates. (E) Western blotting analysis after immunoprecipitation with Flag antibody in

whole-cell lysate from 293T cells cotransfected with HA-MDM2 constructs and Flag-HAUSP and treated with 10 μ M MG-132. $n = 2$ biological replicates. **(F and G)** Schematic of the MDM2 mutants (F) used subsequently in (G) Western blotting analysis of precipitates after pulldown by glutathione *S*-transferase (GST)–Sepharose beads in whole-cell lysate from 293T cells transfected with the GST-tagged MDM2 acidic region construct, HA-tagged HAUSP, and/or the GFP-tagged NLS domain. Lysates were pulled down with GST–Sepharose beads. The precipitates and whole-cell lysate was subjected to Western blotting analysis. $n = 3$ biological replicates. **(H and I)** Schematic of the MDM2 mutants (H) used subsequently in (I) Western blotting analysis of precipitates after pulldown by GST–Sepharose beads in whole-cell lysate from 293T cells transfected with HA-MDM2 and the indicated GST-tagged MDM2 acidic region construct. $n = 2$ biological replicates. **(J)** Schematic models of intramolecular regulation of MDM2 by acetylation to direct its E3 ubiquitin ligase activity toward itself or p53. **(K)** Western blotting analysis of precipitates after pulldown by Ni-NTA beads in whole-cell lysate from 293T cells cotransfected with WT or mutant HA-MDM2, His-Ub, and Flag-p53 and treated with MG-132. $n = 2$ biological replicates.

Fig. 4. Acetylation of MDM2 regulates its subcellular distribution

(A) Sequence alignment of MDM2, p27, FOXO1, and SKP2 to show sequence similarity in the nuclear localization sequence (NLS) region. (B) Acetylation of MDM2 promoted its cytoplasmic localization. Immunofluorescence analysis of HA staining (to assess MDM2 localization) in 293T cells transfected with HA-MDM2. Nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI). Scale bars, 20 μ m. $n = 2$ biological replicates. (C) In vitro interaction of synthetic biotinylated MDM2 peptides acetylated or not at the Lys¹⁸² and/or Lys¹⁸⁵ residues with Importin α 5 or Importin α 7. $n = 2$ biological replicates. (D) Western blotting analysis after immunoprecipitation with Flag antibody in whole-cell lysate from 293T cells transfected with Flag-Importin α 7 and/or HA-MDM2. $n = 2$ biological replicates.

Fig. 5. MDM2 acetylation destabilizes p53 and subsequently inhibits cellular apoptosis in response to DNA damage

(A) Western blotting analysis of whole-cell lysate from U2OS cells transfected with HA-MDM2 and either untreated or treated with 25 μM ETO for 24 hours. $n = 2$ biological replicates. (B) Western blotting analysis after immunoprecipitation with p53 antibody in whole-cell lysate from U2OS cells transfected with the indicated HA-MDM2 constructs and treated with 1 mM MG-132 and/or 25 μM ETO. $n = 2$ biological replicates. (C) Western blotting analysis after immunoprecipitation with HAUSP antibody in whole-cell lysate from U2OS cells infected with shRNA targeting *GFP* or *SIRT1* and either untreated or treated with 25 μM ETO. $n = 2$ biological replicates. (D) Luciferase activity in Dox-inducible U2OS cells transfected with pGL3-puma-luc and treated with Dox (1 μg/ml) and/or 25 μM ETO for 24 hours. Data were normalized to luciferase activity from untreated cells and are presented relative to that in pGL3 vector-transfected controls. Data are means $\pm$ SD from three

independent experiments. $*P < 0.05$, unpaired Student's t test. DMSO, dimethyl sulfoxide. **(E and F)** Apoptosis assessed by flow cytometry in Dox-inducible U2OS cells treated with or without 25 μM ETO and stained for propidium iodide (E) or Annexin V (F). Data are means $\pm$ SD from three independent experiments. $*P < 0.05$, unpaired Student's t test. **(G)** MTS assay for cell viability in Dox-inducible U2OS cells either untreated or treated with 25 μM ETO overnight in the presence of Dox (1 $\mu\text{g/ml}$). Data are means $\pm$ SD from three independent experiments. $*P < 0.05$, unpaired Student's t test. **(H)** Colony formation in Dox-inducible U2OS cells treated with ETO in the presence of Dox and stained with crystal violet. Data are means $\pm$ SD from three independent experiments. $*P < 0.05$, unpaired Student's t test. **(I)** Western blotting analysis after immunoprecipitation with MDM2 antibody in whole-cell lysate from U2OS cells treated with 15 μM MG-132 and 25 μM ETO. $n = 2$ biological replicates. **(J)** Western blotting analysis of whole-cell lysate from MCF7 cells infected with GFP- or SIRT1-shRNA and treated with NCS (100 ng/ml) for the indicated time (hours). $n = 3$ biological replicates. **(K)** Proposed model for acetylation-dependent regulation of MDM2 E3 ligase activity and substrate selectivity by p300. Under basal conditions, MDM2 is acetylated by p300 and then interacts with its upstream deubiquitinase HAUSP. HAUSP blocks MDM2 self-ubiquitination and stabilizes MDM2, subsequently resulting in p53 ubiquitination. On the other hand, under conditions of genotoxic stress, MDM2 is deacetylated by SIRT1, which triggers self-ubiquitination in part by its dissociation from HAUSP.