Ribosomal proteins play a critical role in tightly coordinating p53 signaling with ribosomal biogenesis. Several ribosomal proteins have been shown to induce and activate p53 via inhibition of MDM2. Here, we report that S27a, a small subunit ribosomal protein synthesized as an 80-amino acid ubiquitin C-terminal extension protein (CEP80), functions as a novel regulator of the MDM2-p53 loop. S27a interacts with MDM2 at the central acidic domain of MDM2 and suppresses MDM2-mediated p53 ubiquitination, leading to p53 activation and cell cycle arrest. Knockdown of S27a significantly attenuates the p53 activation in cells in response to treatment with ribosomal stress-inducing agent actinomycin D or 5-fluorouracil. Interestingly, MDM2 in turn ubiquitinates S27a and promotes proteasomal degradation of S27a in response to actinomycin D treatment, thus forming a mutual-regulatory loop. Altogether, our results reveal that S27a plays a non-redundant role in mediating p53 activation in response to ribosomal stress via interplaying with MDM2.

The tumor suppressor protein p53 is stabilized and activated to induce cell cycle arrest or apoptosis in response to diverse stress, including DNA damage and oncogenic stress, thus preventing cell from genomic instability and malignant transformation (1–3). Studies over the past decade have revealed that p53 also plays a crucial role in mediating the cellular response to a newly defined stress called ribosomal stress (4), which is induced by perturbation of ribosomal biogenesis. By doing so, p53 coordinates ribosomal biogenesis (cell growth) with cell cycle progression (cell division). Ribosomal biogenesis includes coordinated synthesis of ribosomal RNA (rRNA) and ribosomal proteins (RPs), rRNA processing, and the assembly of the mature ribosome subunits in the nucleolus and their transport into the cytoplasm (4, 5). Disturbing any one of the steps perturbs ribosomal biogenesis and triggers ribosomal stress (4). Because ribosomal stress is often accompanied by the disruption of nucleolar integrity, it is also referred as to nucleolar stress (6). Examples of ribosomal stress include inhibition of RNA synthesis by treatment with a low dose of actinomycin D (Act D) (7–9) or mycophenolic acid (MPA) (10), inhibition of RNA processing by treatment with 5-fluorouracil (5-FU) (11), genetic disruption of the RNA polymerase I transcription initiation factor TIF-IA (12), and knockdown of individual RPs (13–15) or nucleolar factors such as nucleostemin (16).

Emerging evidence has established a critical role for a group of RPs, including L5, L11, L23, L26, S7, and S27 (8, 9, 17–24), in mediating p53 signaling in response to ribosomal stress. These RPs, when overexpressed or in response to ribosomal stress, bind to MDM2 and inhibit MDM2-mediated p53 ubiquitination and degradation, leading to stabilization and activation of p53. It is well known that MDM2 forms an elegant feedback regulatory loop with p53, as MDM2 is transcriptionally induced by p53 (25–27), whereas it in turn directly inhibits p53 transcriptional activity (28, 29) and mediates p53 ubiquitination and proteasomal degradation (30, 31). Interestingly, knockdown of certain RPs including L5, L11, and S7 attenuates p53 signaling after ribosomal stress (7–11, 18, 24), indicating that these RPs play a non-redundant role in ribosomal stress-induced p53 activation. Furthermore, mice with a knockin of the cancer-associated MDM2 mutant, C305F (MDM2C305F), which fails to bind to L5 and L11 (32), displayed a specific defect in p53 signaling in response to ribosomal stress but not DNA damage, compellingly validating the ribosomal stress-induced RPs-MDM2-p53 signaling pathway in vivo (33).

Multiple RPs are required for p53 activation in response to ribosomal stress, suggesting that they may form a multi-RP-MDM2 protein complex to optimally suppress MDM2 function. Supporting this notion, L5 and L11 synergistically inhibit MDM2, leading to robust activation of p53 compared with overexpression of L5 or L11 individually (34). Additionally, many of these RPs appear to bind to different regions within the central acidic and zinc finger domains of MDM2 (9, 18, 21–24). Although increasing numbers of RPs have been reported to target the MDM2-p53 pathway, we have recently shown that L29 and L30 (15) as well as S12 (this study) do not bind to MDM2 and do not suppress MDM2-mediated p53 degradation, demonstrating that RP regulation of the MDM2-p53 pathway is specific to some, but not all RPs. In this study we have identified RP S27a as another novel MDM2 regulator. S27a is one of two (the other is L41) RPs naturally synthesized as an ubiquitin (Ub) C-terminal extension protein (CEP) (35, 36).
S27a contains 80 amino acids, and thus it was previously also called CEP80. The Ub-S27a precursor protein, also called Ub80 (Ub-S27a is used hereafter), is rapidly processed to an individual Ub monomer and the S27a protein by hydrolysis in mammalian cells (37). Together with Ub52 (Ub fused with the 52-amino acid RP L41), Ub, and UbC, Ub-S27a contributes to the cellular Ub pool required for diverse cellular processes regulated by ubiquitination modification. Our data show that S27a, when overexpressed, significantly inhibits MDM2-mediated p53 suppression. Knockdown of S27a markedly attenuates p53 activation induced by treatment with a low dose of Act D or 5-FU. Interestingly, MDM2 in turn ubiquitinates S27a, leading to proteasomal degradation of S27a, in cells after treatment with Act D. Thus, S27a, the shortest natural MDM2 binding polypeptide (80 amino acids) identified thus far plays a non-redundant role in mediating p53 activation in response to ribosomal stress by interplaying with MDM2.

**MATERIALS AND METHODS**

**Cell Culture, Plasmids, and Antibodies—**Human p53-null lung non-small cell carcinoma H1299, p53-proficient osteosarcoma U2OS cells, and p53-null osteosarcoma Saos-2 cells were cultured under standard conditions as previously described (15). To generate human S27a expression construct (FLAG-S27a), the full-length S27a cDNA was amplified from HeLa cell mRNA using primers 5'-CCGGATCCATGCAGATTTTCGTGAAAACC-3' (P1) and 5'-CCGGAATTCCTCTTACTTG-TCTTCTGGTTTGTTG-3' (P2). The PCR product was cloned into the pcDNA3-FLAG vector at BamHI and EcoRI sites. To clone the full-length Ub-S27a (Ub80a) expression construct (V5-Ub-S27a-FLAG), the full-length Ub-S27a cDNA was amplified by RT-PCR from HeLa cells using primers 5'-CCGGATCCATGCAGATTTTCGTGAAAACC-3' (P1) and 5'-CCGGAATTCCTCTTACTTG-TCTTCTGGTTTGTTG-3' (P2). The PCR product was cloned into the pcDNA3-FLAG vector at BamHI and EcoRI sites. To clone the full-length Ub-S27a (Ub80a) expression construct (V5-Ub-S27a-FLAG), the full-length Ub-S27a cDNA was amplified by RT-PCR from HeLa cells using primers 5'-CCGGATCCATGCAGATTTTCGTGAAAACC-3' (P1) and 5'-CCGGAATTCCTCTTACTTG-TCTTCTGGTTTGTTG-3' (P2). The PCR product was cloned into the pcDNA3-FLAG vector at BamHI and EcoRI sites. To clone the full-length Ub-S27a (Ub80a) expression construct (V5-Ub-S27a-FLAG), the full-length Ub-S27a cDNA was amplified by RT-PCR from HeLa cells using primers 5'-CCGGATCCATGCAGATTTTCGTGAAAACC-3' (P1) and 5'-CCGGAATTCCTCTTACTTG-TCTTCTGGTTTGTTG-3' (P2). The PCR product was cloned into the pcDNA3-FLAG vector at BamHI and EcoRI sites. To clone the full-length Ub-S27a (Ub80a) expression construct (V5-Ub-S27a-FLAG), the full-length Ub-S27a cDNA was amplified by RT-PCR from HeLa cells using primers 5'-CCGGATCCATGCAGATTTTCGTGAAAACC-3' (P1) and 5'-CCGGAATTCCTCTTACTTG-TCTTCTGGTTTGTTG-3' (P2). The PCR product was cloned into the pcDNA3-FLAG vector at BamHI and EcoRI sites.

**Cell Cycle—**U2OS or Saos2 cells were transfected with GFP or GFP-S27a plasmid. The cells were harvested and stained in 500 μl of propidium iodide (PI, Sigma) stain buffer (50 μg/ml PI, 200 μg/ml RNase A, 0.1% Triton X-100 in phosphate-buffered saline) at 37 °C for 30 min and then analyzed for DNA content using a BD Biosciences FACScan flow cytometer. GFP-positive cells were gated for cell cycle analysis. Data were collected using CellQuest and analyzed with the ModFit software program.

**In Vivo Ubiquitination Assay—**An in vivo ubiquitation assay was conducted as previously described using either the Ni2+ -NTA purification method under denaturing conditions (9, 16) or co-IP assays (15) using anti-FLAG or anti-S27a antibodies.

**RNA Interference (RNAi)—**RNAi-mediated gene knockdown was performed essentially as previously described (9, 16). The control scramble sequence was described (9). The target sequences for S27a were 5'-TTAGTCCCTTCTGCGAGA-GA-3' (S27a si-1) and 5'-CAGACATTATTTGCGAAA-3' (S27a si-2). All the 21-nucleotide siRNA duplexes with a 3'-dTdT overhang were synthesized (Dharmacon). These siRNA duplexes (100 nM) were introduced into cells using SilentFect (Bio-Rad) following the manufacturer’s protocol.

**RESULTS**

S27a Interacts with MDM2 in Cells and in Vitro—To determine whether S27a regulates the MDM2-p53 loop, we first examined whether S27a interacts with MDM2 using co-IP followed by IB assays. H1299 cells were transfected with MDM2...
together with RP S7, S12, or S27a. S7, a known MDM2 binding RP (17, 24), was used as a positive control. As shown in Fig. 1 A, ectopically expressed MDM2 was co-immunoprecipitated with FLAG-tagged S27a (FLAG-S27a) and S7, but not S12, using anti-FLAG antibody. Conversely, FLAG-S27a, but not FLAG-S12, was co-immunoprecipitated with MDM2 by anti-HA, but not control antibodies (Fig. 1 B). Endogenous MDM2 was also specifically co-immunoprecipitated with endogenous S27a by polyclonal anti-S27a antibodies, but not preimmune sera, in SJSA cells (Fig. 1 C). These results suggest that S27a specifically interacts with MDM2 in cells. To determine whether the binding between MDM2 and S27a is direct, we performed GST fusion protein-protein association assays. As shown in Fig. 1 D, purified His-MDM2 was bound by purified GST-S27a protein, but not GST alone, in vitro, indicating that S27a directly binds to MDM2.

**S27a Forms a Complex with MDM2 and p53 in Cells**—To determine whether S27a associates with p53, we co-transfected p53-null H1299 cells with different combinations of p53, MDM2, and S27a followed by co-IP assays. As shown in Fig. 2 A, p53 was co-immunoprecipitated with S27a when MDM2 was also co-expressed using anti-FLAG antibody (lane 7), whereas this interaction was significantly decreased in the absence of ectopic HA-MDM2 (lane 8), suggesting that p53 indirectly binds to S27a through MDM2. The weak interaction between FLAG-S27a and p53 (lane 8) may be due to the presence of endogenous MDM2. To further verify this notion, similar co-IP assays were performed in p53<sup>−/−</sup>mdm2<sup>−/−</sup> MEF cells (Fig. 2 B). Indeed, p53 was co-immunoprecipitated with S27a only in the presence of the ectopic expression of MDM2 (compare lane 6 to lane 4). These data suggest that S27a forms a complex with MDM2 and p53 and that expression of S27a does not disrupt the interaction between MDM2 and p53 (compare lane 10 with lane 11, Fig. 2 A).

**S27a Binds to the Central Acidic Domain of MDM2**—To determine where S27a binds within MDM2, H1299 cells were transfected with FLAG-S27a alone or together with a set of deletion mutants of MDM2 (Fig. 3 B) followed by co-IP using anti-V5 antibody. As shown in Fig. 3 A, wild-type (wt) and the
central acidic domain (amino acids 221–274) containing mutants (lanes 4 and 5) of MDM2 interacted with S27a, whereas neither the N-terminal (lane 3) nor the C-terminal (lanes 6–8) fragments interacted with S27a. Thus, the central acidic domain is required for MDM2 to interact with S27a (Fig. 3B). It has been shown that L5 and L11 bind to MDM2 at the zinc finger domain and a tumor-derived MDM2 mutation of the residue Cys-305 at this domain disrupts the interaction of MDM2 with L5 and L11 in cell culture and in vivo (32, 33). Thus, we tested whether mutation of Cys-305 would affect the binding of MDM2 to S27a. As shown in Fig. 3C, the C305S point mutation of MDM2 does not affect its binding to S27a. H1299 cells were transfected with FLAG-S27a in the absence or presence of wt MDM2 or the MDM2C305S mutant. The cell lysates were immunoprecipitated with anti-FLAG antibody followed by IB using antibodies as indicated. D, MDM2 binds to the N-terminal domain of S27a in cells. H1299 cells were transfected with HA-MDM2 together with FLAG-S27a or its N-terminal fragment. Cell lysates were immunoprecipitated with the anti-FLAG antibody followed by IB using anti-MDM2 and anti-FLAG antibodies. E, shown is a schematic diagram of S27a protein with indication of the N-terminal (NT) domain where MDM2 binds and a conserved putative zinc finger motif.

S27a Inhibits MDM2-mediated p53 Ubiquitination and Degradation—Next, we examined whether S27a, like other MDM2-interacting RPs, regulates MDM2-mediated p53 ubiquitination and degradation. We performed transient transfection followed by IB analysis in H1299 cells. As shown in Fig. 4A, overexpression of MDM2 markedly reduced the p53 levels as expected (30, 31, 39). Further overexpression of S27a or S7 (17, 24), but not S12, partially rescued MDM2-mediated p53 degradation. Similarly, overexpression of S27a, but not S12, stabilized MDM2 as well (top panel, Fig. 4A), which is independent of p53 (supplemental Fig. S1). These results suggest that overexpression of S27a blocks MDM2-mediated degradation of p53 and itself. In vivo ubiquitination assays showed that S27a (fourth lane, Fig. 4B), but not S12 (data not shown), markedly inhibited MDM2-mediated p53 ubiquitination. The N-terminal fragment of S27a (S27a-NT) also suppressed MDM2-mediated p53 ubiquitination (fifth lane, Fig. 4B), suggesting that this fragment is sufficient to mediate MDM2 inhibition by S27a. To determine whether overexpression of S27a induces and activates endogenous p53, we introduced S27a into wild-type p53-containing U2OS cells. As shown in Fig. 4C and as in the case of S7 (17, 24), overexpression of S27a, but not S12, markedly induced p53 levels. Correspondingly, the levels of the p53 targets p21 and MDM2 were also induced (Fig. 4C). Con-
Overexpression of S27a significantly stabilized endogenous p53 as determined by half-life assay (Fig. 4D). In contrast, overexpression of S27a did not further increase the half-life of exogenous p53 in p53<sup>−/−</sup> mdm2<sup>−/−</sup> MEF cells (supplemental Fig. S1B). Together, these results demonstrate that overexpression of S27a inhibits MDM2-mediated p53 ubiquitination and degradation in cells.

**Overexpression of S27a Stimulates p53-dependent Transcription and Cell Cycle Arrest**—To determine whether overexpression of S27a affects p53 transactivation activity, we performed luciferase reporter assay in H1299 cells. As shown in Fig. 4E, overexpression of S27a significantly rescued MDM2-mediated suppression of p53-driven luciferase activity in a dose-dependent manner. In contrast, overexpression of S27a did not significantly increase the p53-driven luciferase activity in p53<sup>−/−</sup> mdm2<sup>−/−</sup> MEF cells (supplemental Fig. S1C), further suggesting that S27a regulates p53 activity by suppressing MDM2.

To test the effect of S27a overexpression on p53-dependent suppression of cell proliferation, we conducted cell cycle analysis. U2OS cells or p53-null Saos2 cells were trans-
fected with GFP or GFP-S27a. GFP-positive cells were then gated for cell cycle analysis. As shown in Fig. 4F, overexpression of GFP-S27a significantly suppressed cell proliferation in U2OS, but not Saos2, cells. These data suggest that overexpression of S27a induces p53-dependent G1 cell cycle arrest.

**S27a Derived from the Ub-S27a Fusion Protein Stabilizes and Activates p53**—Because S27a is naturally synthesized as an Ub-S27a fusion protein precursor, we cloned the full-length Ub-S27a cDNA into mammalian expressing vector (Fig. 5A) and observed whether Ub-S27a could also affect the MDM2-p53 pathway. As shown in Fig. 5B, transfection of V5-Ub-S27a-FLAG vector generated V5-Ub (top panel) and S27a-FLAG (bottom panel). The V5-Ub derived from this vector can be used as Ub resource to ubiquitinate cellular proteins as efficiently as V5-Ub alone (compare lane 4 to lane 3, Fig. 5B). We then tested whether overexpression of Ub-S27a could affect the MDM2-p53 loop. Again, overexpression of Ub-S27a, but not V5-Ub alone, significantly rescued MDM2-mediated p53 degradation in H1299 cells (Fig. 5C) and induced the levels of endogenous p53 and its targets p21 and MDM2. This effect was not an off-target effect, as both siRNAs against S27a failed to induce p53 (second and third lanes).

Next, we transfected U2OS cells with scrambled or one of the two S27a siRNAs followed by treatment with a low dose of Act D (5 nM), which specifically inhibits RNA polymerase I activity and inhibits rRNA synthesis. As shown in Fig. 6B, knockdown of S27a using either S27a siRNAs significantly attenuated Act D-induced p53 induction and the levels of p21 and MDM2 (compare lanes four to three and eight to seven). Similarly, knockdown of S27a also drastically inhibited p53 activation by treatment with 5-FU, which blocks the late-stage of rRNA processing (40) (compare lane four to three, Fig. 6C). However, knockdown of S27a did not significantly inhibit p53 activation by treatment with DNA damage-inducing agent etoposide (Fig. 6D). Consistently, knockdown of S27a significantly inhibited the cell cycle arrest induced by Act D treatment (Fig. 6, E and F).

**S27a Is Critical for p53 Activation in Response to Ribosomal Stress**—To elucidate the physiological relevance of S27a-MDM2 interaction, we asked whether ablation of S27a by siRNA could affect p53 signaling in response to ribosomal stress. It has been shown that knockdown of RP L23 (9, 18), L29, L30 (15), L9 (14), or S6 (13), triggers ribosomal stress and induces p53, whereas knockdown of L5, L11, or S7 fails to do so as these RPs are required for p53 activation (non-redundant) in response to ribosomal stress (7, 8, 23, 24). Thus, we were interested in determining whether S27a is a redundant or non-redundant MDM2 inhibitor. As shown in Fig. 6A, knockdown of S27a did not induce p53, whereas consistently with the previous report (15), knockdown of L30 induced p53 and its target gene p21 and MDM2. This effect was not an off-target effect, as both siRNAs against S27a failed to induce p53 (second and third lanes).

**FIGURE 5. Overexpression of Ub-S27a (CEP80) induces p53 levels and activity.** A, shown is a schematic diagram of FLAG-S27a, V5-Ub, and V5-Ub-S27a-FLAG plasmids. B, shown is expression of the Ub and S27a from the above plasmids using IB with anti-V5 (top panel) and anti-FLAG (middle panel) antibodies. C, overexpression of Ub-S27a suppresses MDM2-mediated p53 degradation. H1299 cells were transfected with plasmids as indicated and assayed for protein expression by IB with antibodies as indicated. D, overexpression of Ub-S27a induces endogenous p53. U2OS cells were transfected with the plasmids indicated followed by IB with antibodies as indicated on the left.
interaction of S27a with MDM2 and p53 in cells (Fig. 6G). These results suggest that S27a plays a critical role in p53 activation specifically in response to ribosomal stress.

Of note, as Ub-S27a is encoded by a single mRNA, knockdown of S27a presumably also reduces the levels of Ub portion of Ub-S27a mRNA. Indeed, knockdown of Ub-S27a greatly reduced the levels of full-length mRNA, as evidenced by similar reduced mRNA levels measured with RT-quantitative PCR using primers located within the coding region of either Ub or S27a (supplemental Fig. S2A). However, the total levels of cellular Ub were not significantly changed by siRNA-mediated knockdown of S27a (supplemental Fig. S2B). This observation can be explained by several possibilities. The cellular Ub pool may be stable (36), and thus, transient knockdown would presumably not affect the total levels of Ub significantly. Alternatively, a compensation mechanism provided by other Ub resources (Uba52, Ubb, and Ubc) may overcome the reduction of Uba80. Nevertheless, these data support that the observed effect of S27a knockdown on p53 signaling in response to ribosomal stress is not due to a change in the levels of Ub.

**MDM2 Ubiquitinates S27a in Cells**—Ribosomal proteins are subjected to posttranslational modifications such as ubiquitination and NEDDylation (41), and MDM2 has been shown to ubiquitinate S7 and L26 in cells (20, 24), although the exact function of this ubiquitination is not clear. Thus, we next examined whether MDM2, in turn, ubiquitinates S27a. To this end, H1299 cells were transfected with FLAG-S27a and His-Ub together with control and wild-type MDM2, an E3 activity-deficient mutant MDM2 (MDM2C464A). An in vivo ubiquitination assay was conducted using Ni²⁺-NTA purification methods under denaturing conditions. As shown in Fig. 7A, wild-type MDM2, but not the MDM2C464A mutant, drastically increased the ubiquitinated species of S27a. A straight Western blot also showed enhanced ubiquitination in wild-type MDM2- but not MDM2C464A-transfected cells (second panel). A similar result was also observed using V5-Ub-S27a-FLAG, as both a substrate (S27a) and an Ub resource (V5-Ub) followed by IP with anti-FLAG antibody and IB with anti-V5 antibody (Fig. 7B). To test whether MDM2 ubiquitinates endogenous S27a, S27a was immunoprecipitated with anti-S27a antibodies in cells transfected with V5-Ub together with control, wild-type MDM2, or MDM2C464A plasmid followed by IB with anti-V5 antibody. Again as shown in Fig. 7C, wild-type MDM2, but not MDM2C464A, significantly increased the ubiquitinated species of endogenous S27a. These results clearly suggest that S27a is subjected to MDM2-mediated ubiquitination in cells.

**Ubiquitinated S27a by MDM2 Is Subjected to Proteasomal Degradation**—Next, we tested whether MDM2-mediated ubiquitination of S27a could be targeted for proteasomal degradation. We compared the levels of ubiquitinated species of S27a in the presence or absence of proteasome inhibitor MG132. As shown in Fig. 7D, MG132 treatment significantly increased the levels of ubiquitinated species of S27a (compare sixth to second lanes). Also, the ubiquitinated species of S27a mediated by MDM2 was also significantly increased by the treatment of MG132 (compare the seventh lane to the third lane). These results suggest that ubiquitinated S27a by MDM2 is subjected to proteasomal degradation.

**Act D Treatment Induces Proteasomal Degradation of S27a**—As MDM2 is induced by treatment with Act D, we wondered whether S27a stability is affected by ribosomal stress and whether MDM2 plays a role in regulating S27a stability. U2OS cells were treated with 5 nM Act D for different time courses, and S27a protein levels were examined using IB. As reported, treatment of cells with Act D significantly induced the levels of p53 as well as p21 and MDM2 (9, 18). Interestingly, the levels of S27a began to decrease 12 h after treatment, whereas the levels of the nucleolar protein nucleophosmin (B23) were not significantly changed, and L11 was decreased 24 h after Act D treatment (Fig. 8A). Similar results were also observed in human primary fibroblast WI38 cells (Fig. 8B). Furthermore, Act D-induced S27a reduction was completely rescued by treatment with MG132 (compare the fourth lane to the second lane, Fig. 8C). These results indicate that treatment of cells with Act D results in the proteasome-mediated degradation of S27a.

**MDM2 Mediates Ubiquitination and Proteasomal Degradation of S27a in Response to Ribosomal Stress**—Next, we asked whether Act D treatment induces S27a ubiquitination. U2OS cells transfected with V5-Ub plasmid were treated with Act D for different time courses followed by IP assays using anti-S27a antibody. As shown in Fig. 8D, treatment with Act D markedly increased polyubiquitination of endogenous S27a over the time course. To examine whether MDM2 mediates S27a ubiquitination in response to ribosomal stress, we transfected U2OS cells with either scrambled or MDM2 siRNA followed by treatment with or without Act D. As shown in Fig. 8E, Act D-mediated reduction of S27a was significantly rescued by knockdown of MDM2 (compare the fourth lane to the third lane). Consistently, knockdown of MDM2 significantly reduced the ubiquitination.

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**FIGURE 6. Knockdown of S27a attenuates p53 activation in response to ribosomal stress.** A, knockdown of S27a does not evoke p53 activation. U2OS cells transfected with scrambled (Scr) or siRNAs against S27a or L30 were examined for protein expression using IB. B, knockdown of S27a attenuates p53 activation by treatment with Act D. U2OS cells were transfected with scrambled or one of the two S27a siRNAs. The cells were treated with 5 nm Act D for 8 h before harvesting. Cell lysates were then examined for expression of the indicated protein using IB. C, knockdown of S27a attenuates p53 activation by treatment with 5-FU. U2OS cells were transfected with scrambled or S27a siRNAs. The cells were treated with 10 μg/ml of 5-FU for 8 h before harvesting. Cell lysates were then examined for the expression of indicated protein using IB. D, knockdown of S27a does not attenuate p53 activation by treatment with etoposide (Eto). U2OS cells transfected with scrambled or S27a siRNAs were treated with 20 μM etoposide for 5 h before harvesting. Cell lysates were then examined for the expression of indicated protein using IB. E and F, knockdown of S27a attenuates Act D-induced cell cycle arrest. U2OS cells transfected with scrambled or S27a siRNA were treated with 5 nm Act D for 16 h. The cells were trypsinized, stained with PI, and analyzed by flow cytometry. The percentage of cells in different cell cycle phases is indicated, * , p < 0.05; **, p < 0.01, compared with both scrambled RNA-transfected cells and S27a siRNA-transfected and Act D-treated cells. G, treatment of Act D enhances the interaction of S27a with MDM2 and p53. U2OS cells were treated with 5 nm Act D and harvested at different time points as indicated on top. Cell lysates were subjected to co-IP with anti-S27a antibodies followed by IB with anti-MDM2, anti-p53, or anti-S27a antibodies.
Altogether, these results suggest that MDM2 mediates ubiquitination and proteasomal degradation of S27a in response to treatment with the ribosomal stress-inducing agent Act D.

**DISCUSSION**

The Uba80 gene encodes the Ub-S27a precursor protein that contributes to two opposing cellular processes. The derivative Ub monomer is used in ubiquitin modification, primarily as signals for protein degradation, whereas S27a, a component of ribosome, is involved in executing protein synthesis. The exact function of S27a in translation and whether it has other extraribosomal functions are currently unknown. In this study we found that S27a is a novel regulator of the MDM2-p53 pathway. S27a directly binds to MDM2 both in cells and in vitro. Overexpression of S27a significantly suppressed MDM2-mediated p53 ubiquitination and degradation, leading to p53 activation and cell cycle...
Knockdown of S27a significantly attenuated p53 activation in response to treatment with Act D or 5-FU. Thus, S27a plays a non-redundant role in mediating p53 activation in response to ribosomal stress.

S27a binds to the acidic domain of MDM2, a region also bound by L23, L26, and S27 (9, 18, 21, 22). By contrast, L5, L11, and S7 bind to the adjacent zinc finger domain of MDM2 and play an indispensable role in p53 activation in response to ribosomal stress (8, 24, 32, 33). Mutation of Cys 305 at the zinc finger domain of MDM2 abolishes its binding to L5 and L11 in cells (32) and specifically abrogates p53 signaling in response to ribosomal stress in vivo (33), emphasizing the importance of the central zinc finger domain in RP regulation of the MDM2-p53 loop. Because S27a binds to the acidic domain of MDM2 and plays a non-redundant role in ribosomal stress-induced p53 activation, our data suggest that the acidic domain of MDM2 might also be essential for RP regulation of the MDM2-p53 loop in response to ribosomal stress. Interestingly, it has been shown that the acidic domain is essential for MDM2-mediated p53 ubiquitination and degradation in cells (42, 43). Thus, it is most likely that the entire central region, containing both acidic and zinc finger domains, is required for optimal regulation of MDM2 activity by RPs. In fact, the binding of non-redundant MDM2 binding RPs to different central regions of MDM2 suggests that these RPs may form a multi-RP-MDM2 complex and collaboratively suppress MDM2 E3 activity toward p53. This is supported by the finding that L5 and L11 synergistically regulate the MDM2-p53 loop (34). Also, this may explain why multiple RPs are required for optimal suppression of MDM2. Knockdown of individual RPs may interfere with the formation of a multiprotein complex and attenuate the p53 activation after ribosomal stress. It will be interesting to test whether disruption of the central acidic domain would also abolish p53 response to ribosomal stress in vivo.

FIGURE 8. MDM2 mediates ubiquitination and proteasomal degradation of S27a in response to Act D treatment. A and B, S27a is reduced upon treatment with Act D. U2OS (A) or WI38 (B) cells were treated with Act D (5 nM) for different time points. The cell lysates were examined for the expression of indicated proteins using IB. C, treatment with Act D results in proteasome-mediated degradation of S27a. U2OS cells were treated with 5 nM Act D for 12 h with or without MG132 for 6 h. The cell lysates were assayed for the expression of the indicated proteins using IB. D, Act D treatment increases the ubiquitination of S27a. U2OS cells transfected with V5-Ub were treated with or without 5 nM Act D for the indicated times. The cell lysates were immunoprecipitated with anti-S27a antibodies followed by IB with anti-V5 or anti-S27a antibodies. The ubiquitinated species of S27a are indicated. E, knockdown of MDM2 blocked Act D-induced reduction of S27a. U2OS cells transfected with scrambled (scr) or MDM2 siRNA were treated with or without 5 nM of Act D for 12 h. The cell lysates were assayed for the expression of indicated proteins using IB. F, endogenous MDM2 ubiquitmates S27a. U2OS cells transfected with scrambled or MDM2 siRNA together with V5-Ub as indicated. The cells were treated with MG132 for 6 h followed by IP assays with anti-S27a antibodies. The ubiquitinated species of S27a are indicated. The expression of MDM2, S27a, and total ubiquitinated proteins were shown in the left panels.

FIGURE 9. Schematic model for the interplay between S27a and MDM2 in p53 signaling in response to ribosomal stress. Bars indicate inhibition, and arrows indicate activation.
On the other hand, we have found that MDM2 in turn ubiquitinates S27a and mediates its proteasomal degradation, adding S27a to the list of RPs, including L26, S7, and S27 (20, 21, 24), which are the substrates of MDM2. Although subjected to MDM2-mediated ubiquitination, the proteasomal degradation of endogenous L26, S7, and S27 by MDM2 is not documented (20, 21, 24). Our data showed that treatment of cells with a low dose of Act D resulted in S27a degradation as early as 12 h after treatment (Fig. 8, A and B). This reduction can be rescued by either knockdown of endogenous MDM2 or treatment of cells with a proteasome inhibitor (Fig. 8, C and E), suggesting that S27a is a physiological substrate of MDM2. We also observed that overexpressed MDM2 ubiquitinates L11 (data not shown). However, L11 degradation by Act D treatment occurs at a later time point (Fig. 8, A and B). Thus, S27a appears to be sensitive to MDM2-mediated ubiquitination and degradation early in the response to ribosomal stress. This mutual inhibitory regulation between MDM2 and S27a suggests that RP-MDM2 regulation is dynamic and that down-regulation of RPs such as S27a by MDM2 might be important for cell recovery after stress stimuli (Fig. 9). RPs are synthesized at higher levels than needed for ribosomal assembly. Free RPs that are not incorporated into ribosomes are constantly being degraded within cells (44). Our observations in this study, in addition to those made by several others (20, 21, 24), suggest that MDM2 might be one Ub E3 ligase that mediates ubiquitination and proteasomal degradation of RPs. It will be interesting to test whether MDM2 regulates ribosomal biogenesis and translation through its action on RPs.

It should be mentioned that although S27a is derived from the Ub-S27a fusion precursor, our data suggest that the function of S27a in regulating MDM2 occurs after the post-translational processing of Ub-S27a. First, Ub-S27a fusion protein is rapidly processed to individual Ub and S27a in mammalian cells (Fig. 5B). Second, overexpression of Ub-S27a suppresses MDM2-mediated p53 ubiquitination and degradation as efficiently as overexpression of S27a alone, whereas overexpression of Ub alone did not significantly change the levels of p53 (Fig. 5, C and D). Third, knockdown of S27a using a siRNA against the Ub-S27a gene decreased the entire Ub-S27a mRNA (supplemental Fig. S2A). However, the cellular pool of Ub did not significantly change (supplemental Fig. S2B). This suggests that the attenuation of p53 activation in response to ribosomal stress by S27a knockdown is not due to a change in cellular Ub levels. The maintenance of a stable cellular Ub pool could be due to the transient knockdown in our assays and the stability of Ub itself (36). Alternatively, a possible compensation mechanism by other Ub resources (Uba, Ubb, and Uba52) (36) may contribute to the stable levels of Ub. Altogether, our observed S27a function in regulating the MDM2-p53 loop is independent of the Ub synthesized in cis. However, MDM2 can ubiquitinate S27a using Ub derived from the Ub-S27a precursor (Fig. 7C). Thus, future topics include how the Ub-S27a gene product is regulated in response to growth signals and stress stimuli, how the two opposite processes of protein degradation and synthesis contributed by the Ub-S27a gene are physiologically and physically coordinated in cells, and how this coordination contributes to the regulation of the ribosomal biogenesis and translation and whether its deregulation contributes to tumorigenesis.

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