Inhibition of HDM2 and Activation of p53 by Ribosomal Protein L23

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The importance of coordinating cell growth with proliferation has been recognized for a long time. The molecular basis of this relationship, however, is poorly understood. Here we show that the ribosomal protein L23 interacts with HDM2. The interaction involves the central acidic domain of HDM2 and an N-terminal domain of L23. L23 and L11, another HDM2-interacting ribosomal protein, can simultaneously yet distinctly interact with HDM2 together to form a ternary complex. We show that, when overexpressed, L23 inhibits HDM2-induced p53 polyubiquitination and degradation and causes a p53-dependent cell cycle arrest. On the other hand, knocking down L23 causes nucleolar stress and triggers translocation of B23 from the nucleolus to the nucleoplasm, leading to stabilization and activation of p53. Our data suggest that cells may maintain a steady-state level of L23 during normal growth; alternating the levels of L23 in response to changing growth conditions could impinge on the HDM2-p53 pathway by interrupting the integrity of the nucleolus.

The tumor suppressor protein p53 plays a pivotal role in preventing damaged and abnormal cells from becoming malignant, and its loss of function is associated with a majority of human cancers (26, 36, 37). The activity of p53 is not required for normal cell growth, and the protein is kept at low levels and inactive. This is accomplished by the proto-oncprotein HDM2 (human counterpart of MDM2 in mice) through ubiquitin-dependent p53 degradation in the cytoplasm (6, 7, 11) or repression of p53 transcriptional activity in the nucleus (17, 35). The HDM2 gene can, in turn, be transcriptionally activated by p53, constituting a feedback regulatory loop (1, 40). The current understanding of the major mechanisms of p53 activation includes one that is triggered by DNA damage and induces p53 phosphorylation through a cascade of protein kinases (5, 15, 32) and one that is triggered by aberrant onco-genesis signals and is mediated by p14ARF (p19ARF in mice) (31).

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In this study, we describe functional interactions of HDM2 with L23, a protein component in the 60S large ribosomal subunit. The interaction of HDM2 with L23 involves a domain in HDM2 that is distinct from that required for L11 binding, and L23 and L11 can simultaneously bind with HDM2 to form a ternary complex. Our data indicate that L23 reacts to inhibitions of rRNA biogenesis distinctively from that of L11 and suggest that multiple ribosomal proteins interact with HDM2 and that each may play a different role in regulating the HDM2-p53 pathway in response to perturbations of protein or ribosomal biogenesis.

MATERIALS AND METHODS

Plasmids and cell culture. Mutant HDM2 and L23 constructs were generated by PCR-mediated site-directed mutagenesis and confirmed by direct sequencing. U2OS, Saos2, and SJSA cells were obtained from the American Type Culture Collection. WI38 cells (PD 25) and E6 retroviruses were from Judith Campisi (Lawrence Berkeley National Laboratory, Berkeley, Calif.). All cells were routinely maintained in Dulbecco’s modified Eagle medium containing 10% fetal bovine serum in a 37°C incubator with CO2.

Immunoprecipitation, immunoblotting, and immunostaining. Mouse monoclonal antibody to human HDM2 (Ab-1; Oncogene Research Products), goat polyclonal antibody to human p53 (FL393; Santa Cruz), and mouse monoclonal antibody to human p53 (DO-1; NeoMarkers) were purchased commercially. Rabbit polyclonal antibody to human L23 was produced by a synthetic peptide based on a DNA sequence corresponding to amino acid residues 81 to 96 of human L23 (VIRQKSYRRKDGVFL). Procedures and conditions for immunoprecipitation, immunoblotting, and immunostaining were previously described (9).

Ad and retrovirus infections. Adenovirus (Ad) expressing human L23 was produced by subcloning full-length L23 into the vector pHuttle, and recombinant Ad was produced by overlap recombination. Sources of Ad expressing human p53, HDM2, and human ARF and procedures for Ad infection were described elsewhere (42, 44). E6 retroviruses were infected in WI38 cells and selected as previously described (10).

Cell transfection and fluorescence-activated cell sorter analysis. Cell transfections were carried out by using either Effectene or Lipofectamine reagents (Invitrogen, Carlsbad, Calif.) according to the manufacturer’s instructions. For fluorescence-activated cell sorter analysis, cells were cotransfected with the indicated plasmid DNA, harvested by trypsinization, fixed in 70% ethanol, and analyzed by flow cytometry analysis. Green fluorescent protein (GFP) was used as a marker for analysis of transfected cells. Results for DNA content from at least 20,000 cells are presented in the DNA histograms.

siRNA interference. Purified and annealed duplex small interfering RNA (siRNA) oligonucleotides targeting nucleotides 363 to 383 relative to the translation initiation codon of human L23, siRNA oligonucleotides targeting nucleotides 147 to 168 relative to the translation initiation codon of human L11, and
control scrambled siRNA oligonucleotides were synthesized at Dharmacon (Lafayette, Colo.). Transfection was performed by using Lipofectamine and Plus reagents (Invitrogen) according to the manufacturer’s instructions.

Indirect immunofluorescence. Indirect immunofluorescence was previously described in detail (43). Antibodies to human p53 and HDM2 were previously described (42). Texas Red- and fluorescein isothiocyanate-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) were purchased commercially.

RESULTS

L23 interacts with HDM2. It was noticed in a previous study that a number of low-molecular-weight polypeptides were present in an HDM2 immunocomplex and that the presence of these polypeptides is independent of the interaction of HDM2 with ARF and/or p53 (44). To identify the nature of these HDM2-interacting polypeptides, we carried out large-scale co-immunoprecipitation and mass spectrometry experiments. Utilizing Ad-mediated overexpression of HDM2 and co-IP with an antibody (4B11) specific to the HDM2 protein, a number of polypeptides were detected only in the HDM2 immunocomplex, but not in the control sample infected with Ad expressing GFP (Ad-GFP) (Fig. 1A). The three most prominent bands on the silver-stained gel were subjected to mass spectrometry analysis. They were identified as ribosomal proteins L5 (34 kDa), L11 (20 kDa), and L23 (15 kDa), all of which are peptide components associated with the 60S large ribosomal subunit. The interaction of HDM2 with L5 (14) and L11 (13, 41) has been previously reported; the interaction of HDM2 with L23 has not been shown. Full-length L23, 140 amino acid residues with a calculated molecular mass of 14,865 Da (EMBL accession number X55954), was obtained by reverse transcription-PCR from a HeLa cell cDNA library. Polyclonal antibodies against a short peptide (VIRQRKSYRRKDGVFL) from L23 were raised. The in vivo interaction of HDM2 and L23 was confirmed by co-IP with ectopically expressed HDM2 and L23 (Fig. 1B) and by reciprocal co-IP with endogenous proteins at physiological conditions with SJSA cells (ATCC CRL-2098), which express a relatively high level of HDM2 (Fig. 1C).

To determine the domains in HDM2 involved in the L23 interaction, we constructed deletion mutants of the HDM2 protein and tested their binding activity to L23 in transiently transfected U2OS cells (Fig. 2A). Our results mapped a sequence between amino acids 216 and 284 in HDM2 that is required for L23 binding. A deletion mutant of HDM2 con-
FIG. 2. Mapping of the HDM2 domain for L23 binding and the L23 domain for HDM2 binding. (A) Mapping of the HDM2 domain for L23 binding. Extracts from U2OS cells transfected with the indicated plasmid DNA encoding deletion mutants of HDM2 were immunoprecipitated with HDM2 antibodies (4B11 for lanes 1, 6, and 7; SMP14 for lane 2; 2A10 for lanes 3 and 5), and the precipitates were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and blotted with a mixture of two rabbit anti-HDM2 (N20 and H228; Santa Cruz) antibodies. A diagram of each deletion mutant is shown. WT, wild type; /H9251-myc, anti-myc; WB, Western blotting; /H11001, present; /H11002, absent. (B) Mapping of the L23 domain for HDM2 binding. Extracts from U2OS cells transfected with the indicated plasmid DNA encoding deletion mutants of L23 were immunoprecipitated with HDM2 antibody 4B11, and the precipitates were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and blotted with antibodies to HDM2 (N20) and myc (A14) as indicated. A diagram for the deletion mutants is shown.
ternary complex. Recent studies have shown that the ribosomal protein L23 interacts (Fig. 2B). Between positions 43 and 70 in L23 was necessary for HDM2 binding. Our results showed that a short stretch of amino acids 216 to 244 in HDM2 (amino acids 216 to 284) appears to overlap the sequences required for ARF binding (amino acids 216 to 374) of HDM2 (lanes 5 and 12) of HDM2 containing sequences from amino acids 284 to 491 (the C terminus of HDM2) also appeared to have a weak binding activity, suggesting that other sequences in HDM2 downstream of amino acid 284 also contribute to L23 binding (Fig. 2A, lane 7). The deletion mutants containing the C terminus (amino acids 216 to 491 and 284 to 491) (lanes 6, 7, 13, and 14) or the middle part (amino acids 216 to 374) (lanes 5 and 12) of HDM2 migrated slightly slower on the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel than they were supposed to according to their calculated sizes. The reason for this slow migration is not clear. Notably, the L23 binding domain in HDM2 (amino acids 216 to 284) appears to overlap with the sequences required for ARF binding (amino acids 212 to 244 in HDM2) (16). Whether L23 and ARF antagonize each other for HDM2 binding remains to be determined. We also mapped the sequences in L23 that are required for HDM2 binding. Our results showed that a short stretch of amino acids between positions 43 and 70 in L23 was necessary for HDM2 interaction (Fig. 2B).

L23 and L11 simultaneously interact with HDM2 to form a ternary complex. Recent studies have shown that the ribosomal protein L11 interacts with HDM2, and the L11-binding site on HDM2 was mapped at amino acids 212 to 347 in one study (13) and at amino acids 284 to 374 in another study (41). Our data indicate that the major site in HDM2 for L23 binding is between amino acids 216 and 284. Thus, the HDM2 binding sites for L11 and L23 appear to be nonoverlapping, and therefore, it is possible that L11 and L23 can bind with HDM2 simultaneously to form ternary complexes. To determine whether L23 and L11 can bind HDM2 simultaneously to form a multi-ribosomal-protein–HDM2 complex or, alternatively, whether the two ribosomal proteins may compete with each other for HDM2 binding, we carried out co-IP to test the possibility of a complex formation among HDM2, L11, and L23. Endogenous L23 was immunoprecipitated with an anti-L23 antibody from U2OS cells infected with Ad-HDM2 and blotted with antibodies to HDM2, L23, and L11, respectively. As shown in Fig. 3, when precipitated with an L23 antibody, L11 was detected only in the presence of HDM2 but not in the control cell lysate lacking overexpressed HDM2, indicating that (i) L23 does not directly interact with L11 even though they are both components of the 60S large ribosomal subunit and both exist abundantly in cells and (ii) HDM2 is able to interact with both L11 and L23 simultaneously to form a ternary complex.

Overexpression of L23 inhibits HDM2-mediated p53 polyubiquitination and degradation and induces a p53-dependent cell cycle arrest. To investigate the functional consequences of L23-HDM2 interaction, we examined whether L23 could affect the function of HDM2 in degrading p53. Plasmid DNA expressing HDM2, p53, and myc-tagged L23 were used to transfect U2OS cells, and the steady-state levels of each protein were determined by Western blotting. As shown in Fig. 4A, cotransfection of plasmid DNA expressing myc-L23 restored the level of p53 that had been reduced by HDM2 (compare lanes 3 and 4), indicating that L23, like two other HDM2-binding proteins, ARF and L11, can inhibit HDM2-mediated p53 degradation. Similar inhibition of HDM2-mediated p53 degradation was also observed in normal human fibroblast WI38 cells infected with Ad expressing a myc-tagged L23 (Ad-myc-L23) (Fig. 4B). L23 overexpression not only stabilized p53 but also stabilized HDM2—a self-ubiquitinated fast-turnover protein. The ability of L23 to inhibit HDM2-induced p53 degradation could be explained, at least in part, by its ability to inhibit HDM2-mediated p53 polyubiquitination (Fig. 4C). The ability of L23 to inhibit HDM2’s E3 ligase function appeared to be at a level comparable to that of ARF and L11 (Fig. 4C, compare lane 3 with lanes 4 and 5). To distinguish whether the increased HDM2 level is from p53 activation or from L23-inhibited HDM2 degradation, we established a WI38 derivative cell line, WI38-E6, in which p53 is under negative regulation by the oncogene E6 of the human papillomavirus. The product of the E6 gene targets p53 by inducing its degradation by the ubiquitin proteolytic pathway (30). As shown in Fig. 4D, when the WI38-E6 cells were singly infected with Ad-HDM2 or doubly infected with Ad-myc-L23 and Ad-HDM2, the HDM2 level was significantly higher in the double infection, indicating that L23 stabilizes HDM2 in the absence of p53.

To determine whether the L23-stabilized p53, in the presence of an elevated level of HDM2, is transcriptionally active, we infected WI38 cells with Ad-myc-L23 and determined the endogenous level of p53 as well as the endogenous levels of HDM2 and p21—two proteins whose genes can be transactivated by p53. In cells infected with Ad-myc-L23, the levels of p53, HDM2, and p21 were all increased, whereas, by comparison, p21 and HDM2 were not increased in Ad-myc-L23-infected WI38-E6 cells, indicating that this function of L23 to induce HDM2 and p21 is dependent on its ability to activate p53 (Fig. 5A and B). The activation of p53 by L23 was also determined by analysis of cell cycle distributions in WI38 and WI38-E6 cells infected with Ad-myc-L23. As expected, L23 induced cell cycle arrest only in WI38, but not in WI38-E6, cells (Fig. 5C and D).

To examine the cellular localization of L23-HDM2 interaction, we infected U2OS cells with Ad-myc-L23 and examined the localization of myc-L23 and endogenous HDM2 by immunofluorescence staining. As shown in Fig. 5E, in cells expressing myc-L23, HDM2 was detected primarily in the nucleoplasm but also was visible in the cytoplasm. myc-L23, on the
other hand, was seen highly concentrated in the nucleolus, with a clear accumulation in the nucleoplasm, and to a lower extent in the cytoplasm. Thus, the interaction between L23 and HDM2 could occur in both the nucleus and the cytoplasm. Together, our data show that overexpression of L23 leads to inhibition of HDM2-mediated p53 polyubiquitination, increased protein levels of both HDM2 and p53, and an increased transactivation activity of p53 and induces a cell cycle arrest.

Knocking down L23, but not L11, activates p53 and induces cell cycle arrest. To examine the effect of down-regulation of L23 on the function of HDM2 and p53, we carried out an RNA interference experiment to knock down endogenous L23 in U2OS cells and examined the endogenous protein level and the transcriptional activity of p53 by both Western blotting and cell cycle analysis. When L23 was knocked down to approximately half of its original level, there was a moderate but
FIG. 5. L23 induces a p53-dependent cell cycle arrest. (A and B) L23 overexpression stabilizes and activates p53. Normal human fibroblast WI38 cells and isogenic mutant WI38-E6 cells were infected with the indicated Ad for 2 days. Western blotting was performed as described above. α-myc, anti-myc; α-HDM2, anti-HDM2; α-p53, anti-p53; α-p21, anti-p21; α-actin, anti-actin. (C and D) L23 induces a p53-dependent cell cycle arrest. WI38 and WI38-E6 cells were infected with the indicated viruses. Cells were harvested 2 days after infection, fixed with 70% ethanol for 2 h, and stained with propidium iodide for 1 h, and the cell cycle distribution was determined by flow cytometry. Cell populations in the S phase...
reproducible increase of the protein levels of p53 (Fig. 6A, lane 3). Accompanied by an increased p53 protein level, its transcriptional activity was also increased, as shown by the increased protein levels of HDM2 and p21, the downstream targets of p53's transcriptional function (Fig. 6A). In contrast, however, knocking down L11 to approximately half of its original level did not increase the protein level or the transactivation activity of p53, but rather it appeared to decrease the level of p53 (Fig. 6B). Thus, L23 and L11 apparently employ different mechanisms in regulating HDM2-p53 function. Consistent with the Western blotting results, knocking down L23 also induced a cell cycle arrest in U2OS cells (Fig. 6C), whereas knocking down L11 did not apparently affect the cell cycle distribution (Fig. 6D).

To determine whether the cell cycle arrest by down-regulation of L23 was dependent on the function of p53, we chose to use the normal human fibroblast WI38 cells and their p53-deficient derivatives, WI38-E6 cells, to perform the knocking down experiments. When L23 was knocked down in WI38 cells, there was a small but reproducible increase in the protein level of p53 (Fig. 7A). In contrast to the marginal increase in the level of p53, knocking down L23 considerably increased the levels of HDM2 and p21, indicating an increase of the transactivation activity of p53. In WI38-E6 cells, knocking down L23 did not affect the level of p21, and the levels of p53 and HDM2 remained undetectable (Fig. 7B). Consistent with the activation of p53, knocking down L23 also induced cell cycle arrest in WI38 cells but not in WI38-E6 cells (Fig. 7C and D).

To gain an insight into the mechanism by which down-regulation of L23 activates p53, we performed immunofluorescence staining of the nucleolar protein B23 in U2OS cells. B23 has been shown to possess multiple cellular functions, including ribosomal protein assembly and transport (23), centrosome duplication (22), molecular chaperone activity in preventing protein aggregation (33), regulation of the stability and activity of p53 (2), and an endoribonuclease activity in the processing of the 32S rRNA precursor into 28S rRNA (9, 29). B23 dislocation from the nucleolus after cellular stress has recently been linked to p53 activation (27). We found that knocking down L23 caused the release of B23 from the nucleolus and accumulated it in the nucleoplasm (Fig. 7E). Thus, a potential mechanism of p53 activation by down-regulation of L23 could be to induce a nucleolar stress and to release the nucleolar B23 as well as other constituents of the nucleolus into the nucleoplasm, where B23 could interact with p53 to cause cell cycle arrest (2).

Inhibition of ribosomal biogenesis down-regulates the protein level of L23. Actinomycin D has been used as a chemotherapeutic drug in the treatment of a variety of human cancers (4). At high concentrations (e.g., >30 nM), actinomycin D causes DNA damage and inhibits transcription from all three classes of RNA polymerase promoters, whereas at low concentrations (e.g., <10 nM), actinomycin D does not cause DNA damage but selectively inhibits RNA polymerase I-dependent transcription and, therefore, rRNA biogenesis (8, 24). To examine whether inhibition of rRNA biogenesis by actinomycin D may affect the level and/or localization of L23, we first determined the minimal dosage of actinomycin D that is able to induce p53 in U2OS cells. When U2OS cells were treated with an increasing amount of actinomycin D for 24 h, endogenous p53 was stabilized and activated at a concentration as low as 1.2 nM (Fig. 8A). Surprisingly, we also noticed a clear decrease in endogenous L23 when the cells were treated with actinomycin D, and the decrease is in an inverse correlation with the increase in p53. We further determined the incubation time of actinomycin D with cells that could cause the decrease of L23. At 5 nM, actinomycin D caused a significant decrease in L23 by 24 h of incubation but did not have much effect by 8 h (Fig. 8B). We consider the down-regulation of L23 to not be the consequence of cell cycle arrest, since cell cycle arrest induced by UV irradiation or serum starvation maintained normal levels of L23 (data not shown). Nor was it a result of inhibition of protein synthesis, since in actinomycin D-treated cells, p53 and HDM2 accumulated to high levels, indicating that protein synthesis remained active (Fig. 8A, lanes 5 and 6, and B, lanes 2 and 3).

A previous study has shown that treating cells with 5 nM actinomycin D for 24 h did not alter the endogenous protein level of L11 (41). To determine whether actinomycin D-induced down-regulation of L23 is specific for L23, we compared the levels of L23 and L11 under the same conditions. As shown in Fig. 8C, only the level of L23, but not that of L11, was decreased by actinomycin D treatment, indicating that the down-regulation of L23 by actinomycin D treatment is a specific response of L23 and suggesting that distinct mechanisms are employed by L11 and L23 in response to perturbations of ribosomal biogenesis. The outcomes of down-regulating L23 by actinomycin D treatment and by siRNA are quite similar in that, in both experiments, a decreased level of L23 is correlated with activated p53. These results prompted us to hypothesize that cells may have a mechanism for maintaining a constant level of L23. Perturbations of rRNA biogenesis and/or other types of growth inhibition may cause cell cycle arrest through down-regulation of the level of L23, which will consequently activate p53 function. To determine whether expression of exogenous L23 may suppress the expression of endogenous L23, we infected cells with Ad expressing myc-L23 and determined the level of both exogenous myc-L23 and endogenous L23 with an anti-L23 antibody. Consistently, U2OS cells expressing myc-L23 exhibited a substantially lower level of endogenous L23, indicating a repression of the expression of endogenous L23 by ectopically expressed L23 (Fig. 8D, lane 2). In contrast, cells expressing myc-L11 did not show a decrease of endogenous L11 (Fig. 8D, lane 4), indicating that this is an L23-specific reaction of the cell. The repression of endogenous L23 by exogenous L23 was not due to a potential effect of a...
changed cell cycle distribution, since the reduction of endogenous L23 was not observed when cells were promoted into S phase with Ad-HDM2 infection or were arrested at G1 phase with Ad-p53 infection (Fig. 8E, lanes 3 and 5). Furthermore, the effect of exogenous myc-L23 on the suppression of expression of endogenous L23 was not affected by coexpression of L23 with HDM2 or with p53 (Fig. 8E, lanes 4 and 6), indicating that the repression of endogenous L23 by exogenous L23 is independent of the function of p53. Together, these results suggest that a normal level of L23 is essential for maintaining normal cell growth and proliferation.

**DISCUSSION**

Although recent studies have shown considerable interest in understanding the link between ribosomal biogenesis and cell cycle progression, the molecular mechanism that controls such a link remains obscure (28, 34). Previous studies have identified the interaction of the ribosomal protein L5 with HDM2; the functional significance of this interaction, however, remains unclear (14). Recently, it has been shown that ribosomal protein L11 interacts with HDM2, and through the interaction, L11 stabilizes and activates p53 and induces a cell cycle arrest.
FIG. 7. Down-regulation of L23-induced cell cycle arrest is dependent on the function of p53. (A and B) Normal human fibroblast WI38 cells and isogenic mutant WI38-E6 cells were transfected with either a control scrambled RNA duplex (siScr) or L23 siRNA (siL23) for 2 days, and cell extracts were analyzed by Western blotting with the indicated antibodies. α-HDM2, anti-HDM2; α-p53, anti-p53; α-p21, anti-p21; α-L23, anti-L23; α-actin, anti-actin. (C and D) WI38 and WI38-E6 cells were transfected siRNA as described for panels A and B. Cells were harvested 2 days after infection and stained with propidium iodide, and their cell cycle distribution was determined by flow cytometry. Percentages of cells in S phase are shown. (E) Down-regulation of L23 releases nucleolar B23. U2OS cells were transfected with the indicated siRNA for 2 days. The cells were then fixed and stained with a mouse anti-B23 (α-B23) antibody (Zymed) and an fluorescein isothiocyanate-conjugated anti-mouse secondary antibody (Jackson ImmunoResearch). Fluorescence images were captured with a cooled charge-coupled device color digital camera (model 2.2.0; Diagnostic) on an Olympus IX70 inverted microscope equipped with the appropriate fluorescence filters.
FIG. 8. Inhibition of ribosomal biogenesis decreases the protein level of L23. (A) Low concentrations of actinomycin D induce p53-dependent cell cycle arrest. U2OS cells were treated with the indicated concentrations of actinomycin D (Act D) for 24 h, and the cell lysates were analyzed by Western blotting as described above. α-HDM2, anti-HDM2; α-p53, anti-p53; α-L23, anti-L23; α-actin, anti-actin. (B) Time required for actinomycin D treatment to suppress L23. U2OS cells were treated with 5 nM actinomycin D for the indicated times, and the protein levels were analyzed as described above. (C) Inhibition of ribosomal biogenesis by 5 nM actinomycin D down-regulates L23 but not L11. U2OS cells were treated with 5 nM actinomycin D for 24 h before lysing, the cell lysates were resolved by SDS-PAGE, and Western blotting was performed as described above. α-L11, anti-L11. (D) Ectopic expression of L23 suppresses endogenous L23. U2OS cells were infected with the indicated viruses for 2 days, and cell extracts were harvested and resolved by SDS-PAGE. The proteins were transferred onto a nitrocellulose membrane and blotted with the indicated antibodies. α-myc, anti-myc. (E) Suppression of endogenous L23 by ectopically expressed myc-L23 was independent of HDM2 and p53. Normal human fibroblast WI38 cells were infected with the indicated viruses for 2 days. Cell extracts were harvested and resolved by SDS-PAGE, and the proteins were analyzed as described above. +, present; −, absent.
In this study we identified the interaction of the ribosomal protein L23 with HDM2. L23-HDM2 interaction, like the interaction of L11-HDM2 and ARF-HDM2, inhibits the E3 ligase function of HDM2 and stabilizes and activates p53. The evidences that HDM2 interacts with multiple ribosomal proteins suggest that the ribosomal protein-HDM2 interaction may represent a link by which cells coordinate growth with proliferation. It is presently unclear why multiple ribosomal proteins interact with HDM2. However, our evidence suggests that multiple ribosomal proteins may sense different growth inhibitory signals and may activate p53 through different mechanisms. The distinctive responses of L23 and L11 to a low level of actinomycin D treatment support this notion. The multiple ribosomal proteins that interact with HDM2 could also have a synergistic effect to bring about a quick and strong inhibition of HDM2 when ribosomal damage occurs.

On the other hand, each of the HDM2-interacting ribosomal proteins may transmit different ribosomal biogenesis signals to the HDM2-p53 pathway so that multiple steps of ribosomal biogenesis could be tightly monitored. Our data indicated that the sites in HDM2 for L23 binding are different from those required for L11 binding and that L23 and L11 can bind HDM2 simultaneously to form a ternary complex (Fig. 3). Independent interaction with HDM2 and an ability to form a multi-ribosomal-protein--HDM2 complex support the notion that the different HDM2-binding ribosomal proteins could act independently to monitor different ribosomal stresses. Our study showed that the endogenous L23 and L11 reacted differently to actinomycin D treatment. We found that L23 drastically decreases in response to 24 h of actinomycin D treatment while the L11 level appears to be constant under the same treatment (Fig. 8C). The basis of the difference is not yet clear, but it further indicates that L23 and L11 react to different ribosomal stresses and use different mechanisms to inhibit HDM2. Ribosomal biogenesis is a highly coordinated process involving many important cellular functions. It is conceivable that perturbations of ribosomal function can occur at different stages of ribosomal biogenesis, and each of the HDM2-interacting ribosomal proteins may preferentially recognize a different ribosomal stress. This way, the cells ensure that the entire ribosomal biogenesis pathway is correctly and efficiently monitored. The two HDM2-binding ribosomal proteins, L11 and L23, clearly perform extraribosomal functions. There is previous evidence that a number of ribosomal proteins have secondary functions apart from both ribosome complex formation and protein synthesis, such as regulation of polymerase III transcription by S20, participation in DNA repair by S3, regulation of development by L19, and tumor suppression by S6 (39).

Our data have shown that cells maintain a constant level of L23 during growth. An abnormally high level of L23, such as that provided by Ad-mediated expression, causes a p53-dependent cell cycle arrest. An abnormally low level of L23, for example, that generated by L23 siRNA, also causes a p53-dependent cell cycle arrest. However, the mechanisms employed by an abnormally low level of L23 and an abnormally high level of L23 to induce p53 appeared to be very different. At a high level, the extra L23 interacts with and inhibits the E3 ligase function of HDM2 to stabilize and activate p53. At a low level, it induces nucleolar stress and releases B23 (and perhaps many other nucleolar components) into the nucleoplasm, and the nucleoplasmic B23, in turn, can induce p53-dependent cell cycle arrest (2, 27). We contemplate that one of the functions of L23-HDM2 interaction could be to coordinate regulations of cell growth and cell division. For example, DNA damage-induced p53 activation could, on one hand, stop cell cycle progression through activation of p21 and, on the other hand, inhibit ribosomal biogenesis through activation of HDM2, and the high level of HDM2 could take away newly synthesized L23 to inhibit ribosomal biogenesis.

Ribosomal biogenesis consumes a major part of the cell’s energy and resources and plays a key role in the cell’s life cycle (3, 20, 38). It is conceivable that the status of ribosomal biogenesis is constantly monitored in cells. Once abnormal activities of ribosomal function are detected and cells must stop growth, it is necessary to transmit the signals to the cell cycle regulators to simultaneously stop proliferation. The tumor suppressor p53, as it does in many other circumstances of cellular stress, plays a key role in integrating ribosomal stress signals and transmitting them to the cell cycle regulators. The involvement of p53 in the monitoring of ribosomal biogenesis has been shown in an early study in which depletion of ribonucleotide pools induces a p53-dependent cell cycle arrest that does not involve DNA damage (12). In a recent study, functional inactivation of Bop1, a nucleolar protein involved in rRNA processing and ribosomal assembly, led to a p53-dependent cell cycle arrest (25). Although unclear about the molecular mechanisms, these studies have demonstrated that, in multicellular higher-order organisms, the ribosome status is continuously monitored by thus far unknown mechanisms that involve the function of p53. The interaction of HDM2 with multiple ribosomal proteins provides a potential link between ribosomal biogenesis and p53 function, so the disturbance of ribosomal function can impinge on cell cycle progression.

The involvement of ribosomal proteins in regulating p53 function rekindles interest in the possibility that some ribosomal proteins may act as tumor suppressors. Considering the abundance of ribosomal proteins in proliferating cells, it is likely that a proportion of these proteins participate in extraribosomal functions (18). Previous studies have shown that deregulated expression of ribosomal proteins induces cell cycle arrest and apoptosis (19, 21). These studies suggest that, when not participating in the translational machinery, the ribosomal proteins may contribute to other cellular functions. It is tempting to postulate that the interaction of ribosomal proteins with the HDM2-p53 pathway constitutes a surveillance system, which may have developed during evolution, to safeguard the integrity of ribosomal biogenesis in higher-order eukaryotic cells and to coordinate growth with proliferation. In this regard, the idea that mutations either in the ribosomal protein genes (e.g., L11 or L23) or in HDM2 that prohibit their interaction could have developed in certain human cancers seems not too far fetched and deserves to be further explored.

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