5-Fluorouracil (5-FU) is a widely used chemotherapeutic drug for the treatment of a variety of solid tumors. The antitumor activity of 5-FU has been attributed in part to its ability to induce p53-dependent cell growth arrest and apoptosis. However, the molecular mechanisms underlying p53 activation by 5-FU remain largely obscure. Here we report that 5-FU treatment leads to p53 stabilization and activation by blocking MDM2 feedback inhibition through ribosomal proteins. 5-FU treatment increased the fraction of ribosome-free L5, L11, and L23 ribosomal proteins and their interaction with MDM2, leading to p53 activation and G1/S arrest. Conversely, individual knockdown of these ribosomal proteins by small interfering RNA prevented the 5-FU-induced p53 activation and reversed the 5-FU-induced G1/S arrest. These results demonstrate that 5-FU treatment triggers a ribosomal stress response so that ribosomal proteins L5, L11, and L23 are released from ribosome to activate p53 by ablating the MDM2-p53 feedback circuit.

The antimetabolite agent 5-fluorouracil (5-FU) is widely used in the treatment of many types of cancers, including colorectal carcinomas, breast cancers, and cancers of the aerodigestive tract (1). It has been believed that 5-FU achieves its therapeutic efficacy by two mechanisms. First, 5-FU is converted to 5-fluoro-dUMP (FdUMP) and 5-fluorodeoxyuridine triphosphate (FdUTP) in cells. FdUMP binds to the nucleotide-binding site of thymidylate synthase (TS), an enzyme that catalyzes the reaction from dUMP to dTMP, and inhibits its enzymatic activity, resulting in depletion or imbalance of the intracellular deoxynucleotide pool (2). Consequently, 5-FU suppresses DNA synthesis and repair and results in DNA damage (1). Second, 5-FU can also be converted to 5-fluorouridine triphosphate which incorporates into RNA molecules, particularly RNA, and leads to inhibition of rRNA processing (3–5). As a result, 5-FU induces cell cycle arrest and/or apoptosis.

The effect of 5-FU on cell growth arrest and apoptosis has been attributed to the ability of this drug to induce the level and activity of the tumor suppressor p53 (6, 7). Consistent with this statement is the fact that mutations or deletions of p53 result in the resistance of cells to 5-FU (6, 8–13). In unstressed cells, p53 level and activity are controlled by its physiological regulator MDM2, an E3 ubiquitin ligase that ubiquitinates and targets p53 for proteasome-mediated degradation (14–17), through a feedback mechanism (18, 19). This regulation ensures a proper low level of p53 in cells. In response to stress, cellular signaling pathways are activated to untie the MDM2-p53 feedback loop, consequently activating p53 (20, 21). For example, in response to DNA damage signals, phosphorylation of both MDM2 and p53 prevents the MDM2-p53 interaction and relieves p53 from MDM2 inhibition (22–28). Thus, 5-FU might induce p53 by triggering a DNA damage phosphorylation pathway. However, mutation of the N-terminal and DNA damage responsive phosphorylation sites does not prevent the induction of p53 by 5-FU (7). Also, a study showed that the cytotoxicity of 5-FU was not due to the inhibition of TS (8). Instead this cytotoxicity was due to the incorporation of 5-FU into RNAs, as uridine, which inhibits the incorporation of 5-FU into RNAs, but not thymidine, which prevents the inhibition of TS, relieves the toxicity of 5-FU to cells (6, 29, 30). Hence, it was later believed that 5-FU might activate p53 through an RNA processing-related mechanism (1). However, it remains completely unclear of the identity of this mechanism.

Ribosomal RNA (rRNA) processing is a critical step for ribosomal biogenesis, which converts rRNA precursors into mature rRNAs (31). Emerging evidence suggests that perturbation of ribosomal biogenesis by the inhibition of rRNA processing, synthesis, and ribosome assembly causes ribosomal stress, leading to p53 activation (32, 33). Examples for such stress include treatment of cells with a low dose of actinomycin D (33), loss-of-function mutations of the rRNA processing factor Bop1 (32), or serum starvation (22). This type of stress has been demonstrated using a number of reagents that disrupt the structure of the nucleolus (34). Recently, our laboratory and others have shown that several ribosomal proteins, including L5, L11, and L23, target the MDM2-p53 feedback loop in response to such nucleolar or ribosomal stress (22–28). These ribosomal proteins directly bind to MDM2 and inhibit MDM2-mediated ubiquitylation of p53, thus stabilizing and activating p53 (22–28). Because the 5-FU active metabolite 5-fluorouridine triphosphate...
could incorporate into rRNA and inhibit rRNA processing (3–5), we hypothesized that 5-FU treatment might also trigger nucleolar/ribosomal stress and consequently turn on the ribosomal protein-MDM2 pathway to activate p53.

This study is aimed to test this hypothesis. In our study, we found that indeed 5-FU treatment stabilized p53 by enhancing the interaction of MDM2 with L5, L11, and L23. Also, individually knocking down these L proteins markedly reduced the 5-FU-dependent induction of p53 and its target genes MDM2 and p21, and attenuated the ability of 5-FU to induce G1/S phase arrest. Furthermore, 5-FU treatment induced the release of more ribosome-free ribosomal proteins. Therefore, these results reveal ribosomal proteins L5, L11, and L23 as critical players in 5-FU-mediated p53 activation.

**MATERIALS AND METHODS**

**Cell Culture and 5-FU Treatment**—Human osteosarcoma U2OS and human p53-null lung non-small cell carcinoma H1299 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 50 units/ml penicillin, and 0.1 mg/ml streptomycin at 37 °C in a 5% CO2 humidified atmosphere as previously described (23). The cells were treated with different doses of 5-FU (Sigma) and harvested at the indicated time courses for immunoblot and co-immunoprecipitation (co-IP) assays.

**Immunoblot and Co-immunoprecipitation Analyses**—Cells were lysed in lysis buffer consisting of 50 mM Tris/HCl (pH 8.0), 0.5% Nonidet P-40, 1 mM EDTA, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 0.25 mM leupeptin, 0.25 μg/ml pepstatin A, and 1 mM leupeptin. Equal amounts of clear cell lysate were used for immunoblot analysis, as described previously (23). Co-IP assays were conducted as described previously (23). Beads were washed twice with lysis buffer, once with SNNT buffer (50 mM Tris/HCl (pH 7.4), 5 mM EDTA, 1% Nonidet P-40, 500 mM NaCl, and 5% sucrose), and once with RIPA buffer (50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.1% SDS, and 1% (w/v) sodium deoxycholate). Bound proteins were detected by immunoblot using antibodies as indicated in the figure legends. Anti-L5 (24), anti-L23 (24), and anti-MDM2 (2A10 and 4B11) (23, 24) antibodies have been coated in the figure legends. Anti-L5 (24), anti-L23 (23), and anti-p53 (DO-1, Santa Cruz), and anti-nucleophosmin (B23) (Zymed Laboratories Inc.) were purchased. Rabbit polyclonal anti-L11 was generated using purified His-tagged full-length L11 protein (24) expressed in *Escherichia coli* as an antigen.

**Cell Transfection and in Vivo Ubiquitylation Assays**—H1299 cells were transfected with plasmids, as indicated in each figure legend using TransFectin Reagent following the manufacturer’s protocol (Bio-Rad). Plasmids encoding p53 and HA-tagged MDM2 were described (23). Twenty-four hours post-transfection, the cells were treated with or without 5-FU for 12 h. The cells were harvested at 36 h post-transfection and assayed for protein expression by immunoblot analysis. For *in vivo* ubiquitylation assays, the transfected cells were also treated with 20 μM MG132 for 6 h prior to harvest. *In vivo* ubiquitylation assays were conducted as described previously (23). Eluted proteins were analyzed by immunoblot with anti-p53 (DO-1) antibodies.

**RNA Interference**—RNA interference-mediated knockdown of endogenous L5, L11, and L23 was performed as described previously (23). The 21-nucleotide siRNA duplexes with a 3′ dTdT overhang were synthesized by Dharmacon (Lafayette, CO). The target sequence for L11 was 5′-AAAGGTCCGGGGAGTATGATTA-3′ (35). The target sequence for L5 and L23 as well as the control scrambled siRNA were described previously (23, 24). These siRNA duplexes (0.2 μM) were introduced into cells using siLentFect (Bio-Rad), following the manufacturer’s protocol. The transfected cells were treated with or without 10 μg/ml of 5-FU for 12 h before harvest. Cells were harvested 48 h after transfection for immunoblot, real-time reverse transcriptase-PCR, and cell cycle analyses.

**Reverse Transcriptase-Polymerase Chain Reaction and Real-time PCR Analyses**—Total RNA was isolated from cells using Qiagen RNeasy Mini Kits (Qiagen, Valencia, CA) and treated with DNase I (Invitrogen). Reverse transcriptions were performed as described (24). Quantitative real time PCR was performed on an ABI 7300 real time PCR system (Applied Biosystems) using SYBR Green Mix (Applied Biosystems). All reactions were carried out in triplicate. Relative gene expression was calculated using the ΔΔCT method following the manufacturer’s instructions. The following primers were used: p21, 5′-CTGGAACAGTTTTTCTCTCGGTC-3′ and 5′-TGTATA-TTCAGCATGTGGGAGGA-3′; mdm2, 5′-ATGAAATCCC-CCTTTCCAT-3′ and 5′-CAGGAAGCCAATTCTCAACGGA-3′; and glyceraldehyde-3-phosphate dehydrogenase, 5′-GATTTC-ACCAGGCAAATTC-3′ and 5′-AGCATGCCCACTTGATT-3′.

**Cell Cycle Analysis**—Cells were transfected with siRNAs as described above and treated with 10 μg/ml of 5-FU for 12 h. Cells were fixed with a solution of 95% ethanol and 5% acetic acid and stained in 500 μg/ml propidium iodide, 30 μg/ml polyethylene glycol 8000, 200 μg/ml RNase A, 0.1% Triton X-100, 0.38 mM NaCl, pH 7.2) at 37 °C for 30 min. The cells were then analyzed for DNA content using a BD Biosciences FACScan flow cytometer. Data were analyzed using the CellQuest and Modfit software programs.

**Immunofluorescence Staining**—Cells treated with 5-FU or mock Me2SO were fixed and stained with monoclonal anti-B23 antibody followed by staining with Alexa Fluor 546 (red) goat anti-mouse antibody (Molecular Probes, OR) as well as 4′,6′-diamidino-2-phenylindole for DNA staining. Stained cells were analyzed under a Zeiss Axiosvert 25 fluorescent microscope.

**Polyosome Profile Analysis**—Postmitochondrial supernatant extractions, sucrose gradient sedimentation of polysomes, and analysis of the polyosomes/mRNPs distribution of proteins were carried out as previously described (23). Briefly, cells were incubated with 100 μg/ml of cycloheximide for 15 min. The cells were homogenized in polysome lysis buffer containing 30 mM Tris-HCl (pH 7.4), 10 mM MgCl2, 100 mM KCl, 0.3% Nonidet P-40, 50 μg/ml cycloheximide, 30 units/ml RNasin inhibitor, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.25 μg/ml pepstatin A. After incubation on ice for 5 min, the lysates were centrifuged at 12,000 × g at 4 °C for 8 min. Supernatants were subjected to sedimentation centrifugation in a 15–47%
sucrose gradient solution containing 30 mM Tris-HCl (pH 7.4), 10 mM MgCl₂, 100 mM KCl in a Beckman SW41 rotor at 37,000 rpm at 4 °C for 2 h. Fourteen fractions were collected from each tube and 30 µl of each fraction was assayed for protein levels by immunoblot. RNAs were extracted from the fractions by phenol/chloroform extraction and ethanol precipitation.

RESULTS

5-FU Treatment Stabilizes p53 in Cells—It has been shown that 5-FU treatment induces p53 level and activity (6, 7). To determine the minimal dose that is necessary for activating p53, we conducted 5-FU dose-dependent p53 induction assays using human osteosarcoma U2OS cells. Cells were harvested at 12 h after 5-FU treatment for immunoblot analysis. As shown in Fig. 1A, 5-FU induced the levels of p53, MDM2, and p21 in a dose-dependent fashion, with the lowest activation dose for p53 being 0.1 µg/ml. Because 10 µg/ml of 5-FU led to peak induction, we decided to use this dose for the following experiments. To determine the kinetics of 5-FU-induced p53 activation, we also examined the time-dependent response after treatment with 10 µg/ml of 5-FU. As shown in Fig. 1B, the p53 induction was observed at 4 h post-treatment and reached a plateau from 8 to 12 h. Thus, we chose 12 h as a time point for the following experiments.

To test whether the induction of p53 by 5-FU is due to the stabilization of p53, we performed half-life assays. U2OS cells were treated with or without 10 µg/ml of 5-FU for 12 h. The cells were then incubated with 50 µg/ml of cycloheximide (CHX) was added into the media. The cells were harvested at different time points as indicated and assayed for levels of p53 and tubulin by immunoblot. The bands were quantified and normalized with loading controls determined by tubulin expression and plotted in D. E, 5-FU treatment inhibits MDM2-mediated p53 degradation. H1299 cells were transfected with p53 (0.2 µg) and HA-MDM2 (2 µg) plasmids as indicated. 24 h post-transfection, the cells were treated with or without 10 µg/ml of 5-FU for 12 h. Clear cell lysates were assayed for expression of p53 and MDM2 by immunoblot using antibodies as indicated. C and D, 5-FU treatment stabilizes p53. U2OS cells were treated with 10 µg/ml of 5-FU for 12 h and then 50 µg/ml of cycloheximide (CHX) was added into the media. The cells were harvested at different time points as indicated and assayed for levels of p53 and tubulin by immunoblot. The bands were quantified and normalized with loading controls determined by tubulin expression and plotted in D.

E, 5-FU treatment inhibits MDM2-mediated p53 ubiquitylation. H1299 cells were transfected with combinations of p53 (0.2 µg), HA-MDM2 (2 µg), and His-ubiquitin (His-Ub) (1 µg) plasmids as indicated. Twenty-four hours post-transfection, the cells were treated with 10 µg/ml of 5-FU for 12 h (lane 5). All transfected cells were treated with MG132 (20 µM) for 6 h before harvesting. Ubiquitylated proteins were detected by immunoblot with the anti-p53 (DO-1) antibody (upper panel). Ubiquitylated p53 (p53-(Ub)n) is indicated. The expression of total p53 and MDM2 is shown in the lower panels. * indicates nonspecific anti-HA antibody-reacting bands.

FIGURE 1. 5-FU treatment stabilizes p53 by inhibiting MDM2-mediated p53 ubiquitylation and degradation. A, dose response of p53 induction and activation by 5-FU. U2OS cells were treated with different doses of 5-FU as indicated for 12 h. Clear cell lysates were assayed for expression of p53, p21, and MDM2 by immunoblot using antibodies as indicated. B, time-dependent effect of 5-FU on p53 induction and activation. U2OS cells were treated with 10 µg/ml of 5-FU for different time courses as indicated. Clear cell lysates were assayed for expression of p53, p21, and MDM2 by immunoblot using antibodies as indicated. C and D, 5-FU treatment stabilizes p53. U2OS cells were treated with 10 µg/ml of 5-FU for 12 h and then 50 µg/ml of cycloheximide (CHX) was added into the media. The cells were harvested at different time points as indicated and assayed for levels of p53 and tubulin by immunoblot. The bands were quantified and normalized with loading controls determined by tubulin expression and plotted in D. E, 5-FU treatment inhibits MDM2-mediated p53 degradation. H1299 cells were transfected with p53 (0.2 µg) and HA-MDM2 (2 µg) plasmids as indicated. 24 h post-transfection, the cells were treated with or without 10 µg/ml of 5-FU for 12 h. Clear cell lysates were assayed for expression of p53 and MDM2 by immunoblot using antibodies as indicated. * indicates nonspecific anti-HA antibody-reacting bands. F, 5-FU treatment inhibits MDM2-mediated p53 ubiquitylation. H1299 cells were transfected with combinations of p53 (0.2 µg), HA-MDM2 (2 µg), and His-ubiquitin (His-Ub) (1 µg) plasmids as indicated. Twenty-four hours post-transfection, the cells were treated with 10 µg/ml of 5-FU for 12 h (lane 5). All transfected cells were treated with MG132 (20 µM) for 6 h before harvesting. Ubiquitylated proteins were detected by immunoblot with the anti-p53 (DO-1) antibody (upper panel). Ubiquitylated p53 (p53-(Ub)n) is indicated. The expression of total p53 and MDM2 is shown in the lower panels. * indicates nonspecific anti-HA antibody-reacting bands.
treated cells. U2OS cells were treated with 10 μg/ml of 5-FU for different time courses as indicated. The clear cell lysates were immunoprecipitated with anti-MDM2 (4B11) antibodies followed by immunoblot using anti-L5, L11, L23, or MDM2 (2A10) antibodies (left panels). The lysates were also directly loaded onto a SDS gel for immunoblot analysis with the above antibodies (left panels). B, 5-FU treatment enhances the interaction between MDM2 and L5. Cell lysates prepared as in A were immunoprecipitated with anti-L5 antibodies followed by immunoblot using anti-L5 or MDM2 (2A10) antibodies (right panels). The lysates were also directly loaded onto a SDS gel for immunoblot analysis with the above antibodies (left panels). C, 5-FU treatment enhances the MDM2-L23 interaction. Cell lysates prepared as in A were immunoprecipitated with anti-L23 antibodies followed by immunoblot using anti-L23 or MDM2 (2A10) antibodies (right panels). The lysates were also directly loaded onto a SDS gel for immunoblot analysis with the above antibodies (left panels). D, MDM2 specifically co-immunoprecipitated with L23 in 5-FU-treated cells. U2OS cells were treated with 10 μg/ml of 5-FU for 12 h and the clear cell lysates were immunoprecipitated with anti-L23 antibody or preimmune serum followed by immunoblot using anti-L23 or MDM2 (2A10) antibodies. E, the enhancement of the MDM2-L23 interaction is not due to the increased levels of MDM2 induced by 5-FU. U2OS cells were treated with 10 μg/ml of 5-FU for 12 h. Six hours before harvest the cells were incubated with or without MG132 (20 μM) as indicated. The clear cell lysates were immunoprecipitated with anti-L23 antibodies followed by immunoblot using anti-L23 or MDM2 (2A10) antibodies (right panels). The lysates were also directly loaded onto a SDS gel for immunoblot analysis with the above antibodies (left panels). F, interaction between MDM2 and L11 in cells treated with 5-FU and MG132. U2OS cells were treated with or without 10 μg/ml of 5-FU for 12 h together with 20 μM MG132 for 6 h as indicated. The clear cell lysates were immunoprecipitated with anti-MDM2 (4B11) antibodies followed by immunoblot using anti-L11 or MDM2 (2A10) antibodies (right panels). The lysates were also directly loaded onto a SDS gel for immunoblot analysis with the above antibodies (left panels).

FIGURE 3. 5-FU treatment increases the ribosome-free form of L5, L11, and L23. A, immunofluorescence staining of the nucleolar marker B23. U2OS cells were treated with or without 10 μg/ml of 5-FU for 12 h. The cells were stained with anti-B23 monoclonal antibody, followed by staining with goat anti-mouse secondary antibody (red), as well as 4′-6-diamidino-2-phenylindole (DAPI) for DNA. B, polysome profile assays. Cytoplasmic extracts containing polysomes from U2OS cells treated with or without 10 μg/ml of 5-FU for 12 h were subjected to a 15–47% linear sucrose gradient sedimentation centrifugation. Fourteen fractions were collected and 30 μl of each fraction was used for immunoblot with anti-L11, anti-L5, anti-L23, or anti-MDM2 antibodies. The distribution of polysome, monosome, and mRNPs are indicated. C, the fold-increase of the ratio of free versus ribosome-associated forms of L11, L5, and L23. The density of each band in B above was determined. The relative ratio was calculated by dividing the total density of L11, L5, or L23 in fractions 11–14 by that of fractions 1–10.

ment. As shown in Fig. 1E, MDM2 drastically reduced the level of p53 (compare lane 3 to lane 2). This reduction of p53 was impaired in the presence of 5-FU treatment (compare lane 4 to lane 3). Furthermore, 5-FU significantly reduced MDM2-mediated p53 ubiquitylation, particularly the polyubiquitylation of p53 (Fig. 1F, compare lane 5 to lane 4). These results suggest that 5-FU treatment stabilizes p53 by inhibiting MDM2-mediated ubiquitylation and degradation of p53.

5-FU Treatment Enhances the Interaction of MDM2 with Ribosomal Proteins L5, L11, and L23—Previous studies by others and our lab showed that three ribosomal proteins including L5, L11, and L23 directly bound to MDM2 and inhibited MDM2-mediated p53 ubiquitylation and degradation, leading to p53 stabilization and activation, in response to ribosomal stress (23, 24, 26–28, 37). Because 5-FU metabolites could incorporate into RNAs and inhibit tRNA processing (3–5) and meddling with rRNA processing causes ribosomal stress and often leads to the disruption of the nucleolus (32–34), it is likely...
that 5-FU might also activate p53 by employing these ribosomal proteins to block the MDM2-p53 feedback loop. To test this idea, we first tested whether 5-FU treatment enhances the interaction between MDM2 and these L proteins by treating U2OS cells with 5-FU followed by IP with anti-MDM2 antibodies and immunoblot analysis. As shown in Fig. 2A, 5-FU treatment indeed enhanced the interaction between MDM2 and L5, L11, or L23 when anti-MDM2 antibodies were used for co-IP. Consistently, the enhancement of the MDM2-L5 interaction by 5-FU was also evident when a reciprocal IP was conducted with anti-L5 antibodies (Fig. 2B). Of note, this reciprocal IP could not be done with anti-L11 antibodies, as all the L11 antibodies tested were not suitable for co-IP with endogenous MDM2 (data not shown). When anti-L23 antibodies were used for similar co-IP assays, we found that 5-FU also markedly induced the interaction between MDM2 and L23 (Fig. 2C). The enhanced co-IP of MDM2 by the anti-L23 antibody was specific for L23 because no MDM2 was detected when the control preimmune serum was used (Fig. 2D). Although the overall levels of L5, L11, and L23 were not altered by the treatment of 5-FU (left panels of Fig. 2A), MDM2 levels were significantly induced (Fig. 1B and the left top panels of Fig. 2, A and C). To exclude the possibility that the enhanced interaction between MDM2 and the L proteins might be due to the increased levels of MDM2, we treated the cells with MG132, a proteasome inhibitor, to normalize the levels of MDM2 in the cell, treated with or without 5-FU, and then performed similar co-IP assays. We found that the enhanced interaction between MDM2 and each of the L proteins by 5-FU treatment was still noted when MG132 was used, as shown in the representative results for L23 (Fig. 2E) and L11 (Fig. 2F). Taken together, these results demonstrate that 5-FU treatment can elevate the interactions between MDM2 and the three L proteins.

5-FU Treatment Results in an Increase in the Free Form of Ribosomal Proteins—To illustrate how 5-FU may enhance the MDM2-L protein interactions, we first tested whether 5-FU treatment could alter the nucleolar structure by using the B23 protein as a nucleolar marker (34). As shown in Fig. 3A, 5-FU treatment resulted in diffusion of B23 into the nucleoplasm, suggesting that 5-FU might have disrupted the normal nucleolus structure and caused nucleolar stress (34). Because none of our polyclonal antibodies against these L proteins is suitable for immunofluorescence staining (data not shown), we were unable to assess the distribution of these L proteins in response to 5-FU treatment using this method. To overcome this obstacle, we next conducted polysome profile assays as described previously (22, 23), which allowed us to examine the distribution of ribosome-associated and ribosome-free forms of these proteins. Cytoplasmic extracts prepared from 5-FU or mock Me2SO-treated U2OS cells were subjected to linear sucrose gradient sedimentation centrifugation. Fourteen fractions were collected and subjected to Western blot assays for detection of MDM2, L5, L11, and L23. Consistent with our previous study (23), MDM2 was not co-eluted with either the polysomes (fractions 1–7) or the monosomes (fractions 8–10), both of which contain L11, L5, and L23 (Fig. 3B). Instead, MDM2 stayed near the top of the gradient where the ribosome-free fractions of L11, L5 and L23 were also detected (Fig. 3B). The profile of polysome, monosome, and free mRNPs (small ribonuclear protein) was verified by determining the distribution of rRNAs (data not shown). Interestingly, treatment of 5-FU resulted in a marked increase of the ribosome-free L11, L5, and L23, as compared with the cells treated with Me2SO.

5-FU Enhances MDM2-Ribosomal Protein Interaction

FIGURE 4. Knockdown of endogenous L5, L11, or L23 by siRNA inhibits the effect of 5-FU to induce p53 levels and activation. A, ablation of endogenous L5 by siRNA inhibits 5-FU-induced p53. U2OS cells were transfected with L5 siRNA (0.2 μg, lanes 3 and 4) or with scrambled RNA duplex (0.2 μg, lanes 1 and 2) for 48 h. Twelve hours before harvest the cells were treated with (lanes 2 and 4) or without (lanes 1 and 3) 10 μg/ml of 5-FU. The clear cell lysates were assayed for protein expression of p53, p21, and MDM2 by immunoblot with specific antibodies. B, ablation of endogenous L11 by siRNA inhibits 5-FU-induced p53 induction. U2OS cells were transfected with L11 siRNA (0.2 μg, lanes 3 and 4) or with scrambled RNA duplex (0.2 μg, lanes 1 and 2) for 48 h. Twelve hours before harvesting the cells were treated with (lanes 2 and 4) or without (lanes 1 and 3) 10 μg/ml of 5-FU. The clear cell lysates were assayed for protein expression of p53, p21, and MDM2 by immunoblot with specific antibodies. C, ablation of endogenous L23 by siRNA inhibits 5-FU-induced p53 induction. U2OS cells were transfected with L23 siRNA (0.2 μg, lanes 3 and 4) or with scrambled RNA duplex (0.2 μg, lanes 1 and 2) for 48 h. Twelve hours before harvesting the cells were treated with (lanes 2 and 4) or without (lanes 1 and 3) 10 μg/ml of 5-FU. The clear cell lysates were assayed for protein expression of p53, p21, and MDM2 by immunoblot with specific antibodies. D and E, ablation of endogenous L5, L11, or L23 by siRNA inhibits the p21 (D) and mdm2 (E) mRNA levels induced by 5-FU. Total RNAs was prepared from cells transfected with L5, L11, or L23 siRNA, or scrambled RNA followed by treatment with or without 5-FU as shown above in A–C and retrotranscribed. Real-time PCR analysis was then conducted to determine the expression of the p21 (D) and mdm2 (E) mRNA levels. The expression of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA was used as control.
Estimation of the ratio of the free form to ribosome-associated L proteins revealed an ∼2–8-fold increase of the free ribosomal proteins in 5-FU-treated cells (Fig. 3C).

Together with results in Fig. 2, these results demonstrate that 5-FU treatment causes ribosomal stress and subsequently induces the release of ribosome-free L proteins that interact with MDM2.

Knockdown of L5, L11, or L23 by siRNA Inhibits 5-FU-induced p53 Activation—The enhancement of the ribosomal protein-MDM2 interactions has been shown to suppress MDM2 inhibitory activity toward p53 (23, 24, 26–28). Thus, we predicted that this effect would also happen in response to 5-FU treatment. To demonstrate that L11, L5, and L23 indeed play roles in 5-FU-induced p53 activation, we examined if knocking down their expression by siRNAs would influence the 5-FU-induced p53 level and activity in U2OS cells. As shown in Fig. 4, reduction of L5 (Fig. 4A) or L11 (Fig. 4B) levels by siRNA markedly inhibited the 5-FU-induced levels of p53, as compared with that in scrambled RNA duplex transfected cells. Consistently, 5-FU induction of p21 and MDM2 protein levels was also drastically impaired by L5 and L11, but not scrambled, siRNA, respectively (Fig. 4, A and B). This trend was also true to the mRNA level of p21 and mdm2, as measured by real time reverse transcriptase-PCR assays (Fig. 4, D and E). These results demonstrate that L5 and L11 are required for 5-FU-mediated induction of p53 level and activity.

Consistent with our previous observation (23), L23 siRNA induced p53 (Fig. 4C) as well as the protein (Fig. 4C) and mRNA (Fig. 4, D and E) levels of MDM2 and p21. Interestingly, when L23 siRNA-transfected cells were treated with 5-FU, the induction of p53, p21, and MDM2 levels were not further enhanced in comparison with the scrambled RNA transfected to 5-FU-treated cells (Fig. 4, C–E). Instead, the levels of mdm2 mRNA and p21 and MDM2 proteins (Fig. 4C) were reduced by L23 siRNA in the presence of 5-FU, as compared with the cells treated with 5-FU alone. This lack of synergy between L23 siRNA and 5-FU treatment indicates that L23 also plays a role in 5-FU-induced p53 activation.

As shown in previous reports (22, 24), siRNAs against these ribosomal proteins did not negate protein synthesis within a few days after transiently depleting these proteins. Thus, our results demonstrate that L5, L11, and L23 are crucial for 5-FU-triggered p53 activation.

Reduction of Endogenous L5, L11, or L23 by siRNA Alleviates 5-FU-induced Cell Cycle Arrest—It has been shown that 5-FU treatment results in G1/S phase cell cycle arrest (38–43). To determine whether the above L proteins are also required for this 5-FU effect, we conducted cell cycle analysis after introducing L5, L11, L23, or scramble siRNAs into U2OS cells. As shown in Fig. 5, A and B, 5-FU treatment resulted in the accumulation of cells at later G1 or the concurrent loss of G2 cells, consistent with previously reported results (7, 38–43). Consistent with the above results in Fig. 4, knockdown of either
endogenous L5 or L11 by siRNA alleviated this 5-FU effect on cell cycle progression. Without 5-FU treatment, L5 or L11 siRNA alone would not significantly affect the cell cycle profile (Fig. 5, A and B). Consistent with our previous observation (23) and the result in Fig. 4, knockdown of L23 by siRNA induced G₁ cell cycle arrest (Fig. 5, A and B). Again, L23 siRNA did not synergize the effect of 5-FU on the cell cycle, suggesting that L23 was also required for 5-FU-induced G₁/S arrest. These results demonstrate that L5, L11, and L23 are each required for the cell cycle arrest induced by 5-FU.

**DISCUSSION**

It has been shown that 5-FU activates p53 to induce cell cycle arrest and/or apoptosis (6, 7). However, the mechanism underlying this 5-FU–p53 signaling pathway has remained unknown (1). Here we provide the first evidence demonstrating that ribosomal proteins L5, L11, and L23 play crucial roles in mediating 5-FU-induced p53 activation. We showed that 5-FU treatment enhanced the interaction between MDM2 and L5, L11, and L23 in cells (Fig. 2). These enhanced interactions are not due to the induced expression of MDM2 by 5-FU treatment, as the enhanced interactions were still noted when MDM2 levels were normalized by MG132 (Fig. 2, E and F). Further supporting the role of these L proteins in 5-FU-induced p53 activation is the evidence showing that knockdown of either of the three L proteins reduced the 5-FU-induced levels of p53 and its targets, p21 and MDM2 (Fig. 4), and consequently alleviated 5-FU-induced G₁/S phase arrest (Fig. 5). Taken together, our results as described above link L5, L11, and L23 with the 5-FU–p53 signaling pathway.

The ribosomal proteins L5, L11, and L23 have been shown to bind to MDM2 and inhibit MDM2 suppression of p53 activity in response to nucleolar/ribosomal biogenesis stress (22–28). This stress is induced by perturbation of ribosomal biogenesis through inhibition of rRNA synthesis, processing, and assembly (32–34). In response to this type of stress it is believed that the nucleolus is disrupted and ribosomal proteins are consequently released from the nucleolus to interact with MDM2 in either the nucleoplasmic or the cytoplasmic compartments (22, 23). Because the incorporation of the 5-FU metabolite 5-fluorouridine triphosphate into RNAs, particularly rRNA, has been suggested to represent a major mechanism underlying the cytotoxicity of 5-FU to cells due to interference with rRNA processing (3–5), we predicted that 5-FU would cause ribosomal stress similar to that by actinomycin D (22–24, 26, 33). Indeed, 5-FU treatment resulted in the inhibition of MDM2-mediated p53 ubiquitylation and degradation (Fig. 1, E and F), the alteration of the nucleolar structure (Fig. 3A), and the increase of ribosome-free forms of L5, L11, and L23 (Fig. 3, B and C). Together with the results of Figs. 2, 4, and 5, these results suggest that 5-FU activates p53 by causing ribosomal stress and releasing these L proteins to block the MDM2–p53 negative feedback regulation (Fig. 5C).

The finding that the above L proteins play critical roles in 5-FU-induced p53 activation has several implications. First, it provides another line of evidence showing the importance of these L proteins in regulating the p53 pathway, in addition to their responses to a low dose of actinomycin D (22–24, 26), further supporting the model that the nucleolus plays a central role in p53 response to cellular stress (34). Also, our results using siRNAs specifically against the L proteins demonstrate a key role for these L proteins in the cell cycle checkpoint control. By doing so, these ribosomal proteins may coordinate ribosomal biogenesis with the cell cycle progression, thus maintaining the integrity of ribosomal biogenesis and genomes in response to ribosomal stress. Finally, these L proteins may have implications in the development of cellular resistance to 5-FU. Emerging evidence suggests that alterations of the ribosomal biogenesis pathway might contribute to tumorigenesis (31). Thus it is possible that the expression of these L proteins may be altered by mutation or deletion in certain tumors, leading to impotence of 5-FU treatment in tumors. Because these L proteins are small proteins, further characterization of their interactions with MDM2 would be informative for designing small molecules or peptides that could restore the sensitivity of cancer cells to 5-FU treatment. These are interesting topics for future investigation.

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