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Las1L Is a Nucleolar Protein Required for Cell Proliferation and Ribosome Biogenesis[▽]

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Ribosome biogenesis is a highly regulated process ensuring that cell growth (increase in biomass) is coordinated with cell proliferation. The formation of eukaryotic ribosomes is a multistep process initiated by the transcription and processing of rRNA in the nucleolus. Concomitant with this, several preribosomal particles, which transiently associate with numerous nonribosomal factors before mature 60S and 40S subunits are formed and exported in the cytoplasm, are generated. Here we identify Las1L as a previously uncharacterized nucleolar protein required for ribosome biogenesis. Depletion of Las1L causes inhibition of cell proliferation characterized by a G_1 arrest dependent on the tumor suppressor p53. Moreover, we demonstrate that Las1L is crucial for ribosome biogenesis and that depletion of Las1L leads to inhibition of rRNA processing and failure to synthesize the mature 28S rRNA. Taken together, our data demonstrate that Las1L is essential for cell proliferation and biogenesis of the 60S ribosomal subunit.

Cell growth (increase in cell mass) and cell division must be coordinated during proliferation for cells to remain at a constant size (13). Biogenesis of eukaryotic ribosomes is a finely tuned process ensuring that the protein synthesis capacity of a cell meets the demands of growth associated with proliferation (31). Ribosome biogenesis is initiated by the transcription of rRNA by RNA polymerase I (Pol I) (28S, 18S, and 5.8S rRNA) in the nucleolus and by RNA polymerase III (5S rRNA) in the nucleoplasm. After undergoing cotranscriptional site-specific modification, the 47S pre-rRNA transcript is submitted to a series of endonucleolytic cleavages and exonucleolytic digestion steps to remove internal and external transcribed spacer (ITS and ETS) regions and generate the mature 18S, 28S, and 5.8S rRNAs (21, 26, 27, 36). Mature rRNAs are then assembled with ribosomal proteins to form the 40S (18S rRNA) and 60S (28S, 5.8S, and 5S rRNA) ribosomal particles. These steps involve a large assortment of factors in addition to the RNA and protein components of the ribosome itself. More than 200 additional accessory proteins and noncoding small RNAs engage in complex reactions of processing, assembly, and nuclear export to generate functional cytoplasmic 40S and 60S subunits (36). Although a large collection of evolutionarily conserved proteins has been implicated in processing and assembly of ribonucleoproteins, many details of these pathways and the precise function of several of these factors remain unresolved (28, 54).

Perturbation of rRNA transcription or rRNA processing or deletion of ribosomal proteins has been shown to induce nucleolar stress and stabilization of the tumor suppressor p53 (4, 9–11, 16, 23, 25, 32, 58, 61, 62). It was previously demonstrated that, following nucleolar stress, several ribosomal proteins can bind to HDM2 (an E3 ubiquitin ligase) and inhibit its ability to ubiquitinate and target p53 for degradation, leading to accumulation of p53 and subsequent G_1 cell cycle arrest. This suggests that the tightly concerted process of ribosome biogenesis is also closely linked to regulation of cell cycle progression. Indeed, proteins that are often found deregulated in cancer have been linked to regulation of ribosome biogenesis. For example, c-Myc has been shown to increase transcription and processing of rRNA as well as transcription of ribosomal proteins (2, 18, 19, 40, 48, 56). Moreover, tumor suppressors such as pRb, p53, and PTEN negatively regulate cell proliferation in part by repressing the activity of RNA polymerase I (7, 8, 60). Thus, identification of key players involved in ribosome biogenesis is of high importance to fully understand how deregulation of cell growth and proliferation leads to cancer.

More than a decade ago, Las1 was characterized as a protein essential for cell viability and cell proliferation in *Saccharomyces cerevisiae* (14). Genetic deletion of Las1 induces cells to arrest in G_1, where 80% of the cells are small and unbudded, while overexpression of Las1 results in large cells with multiple surface projections. Together, these results suggest that Las1 could be involved in regulation of cell growth and G_1- to S-phase cell cycle progression. Although Las1 contains a domain that is conserved throughout evolution, the precise mechanism by which Las1 regulates cell proliferation in both budding yeast and higher eukaryotes remains unknown. Here, we demonstrate that human Las1 (Las1L) is a nucleolar protein essential for cell proliferation and ribosome synthesis. Depletion of Las1L by RNA interference (RNAi) causes nucleolar disorganization accompanied by a p53-dependent G_1 cell cycle arrest. Importantly, we found that loss of Las1L abrogates the production of the 60S ribosomal subunit by interfering with 28S rRNA processing. These results indicate that Las1L affects cell proliferation through its fundamental role in ribosome biogenesis.

MATERIALS AND METHODS

Cell culture and cell synchronization. HCT116 and U2OS cells were maintained in Dulbecco's modified Eagle's medium (DMEM) high glucose (HyClone) with 5% fetal bovine serum (FBS) (HyClone) and penicillin-streptomy-

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cin. All cells were grown at 37°C with 5% CO_2. For G_0/G_1 synchronization, cells were washed 3 times in phosphate-buffered saline (PBS) and serum deprived for 72 h. Cells were restimulated by adding culture medium supplemented with 5% FBS.

RNA interference. Synthetic short interfering RNA (siRNA) oligonucleotides (Sigma) were delivered into cells with Lipofectamine RNAi Max (Invitrogen), using the reverse transfection protocol according to the manufacturer's instructions. Briefly, 20 nM siRNA oligonucleotide was diluted in 500 μl of Opti-MEM (Invitrogen) and incubated with 5 μl of Lipofectamine RNAi Max (prediluted in 500 μl of Opti-MEM) in a 6-well plate. Cell suspension (150,000 cells/well) was then added to the well containing the transfection mixture. The following siRNA sequences were used: nontargeting scramble, 5′-GAUCAUACGUGCGAUCA GATT-3′; human Las1L#1, 5′-CUGAUACGCUGUAAGCUCUdTdT-3′ (where dT is deoxyribosylthymine); and human Las1L#2,5′-CAUUUAUACCC AGAGUGGAdTdT-3′. For small hairpin RNA (shRNA), nontargeting scramble (5′-CCTAAGGTTAAGTCGCCCTCGCTCTAGCGAGGGCGACTTAAC CTTAAG-3′) and human Las1L (5′- CCGGGCTGCTACTTTGTCCTGGATT CTCGAGAATCCAGGACAAAGTAGCAGCTTTTTTG-3′) sequences were cloned in pLKO.1-puro lentiviral vector. For virus production, HEK293T cells were transfected with pLKO.1-puro, pVSVG, pMDL, and pREV plasmids in a 3:1:1:1 ratio (53). Medium-containing virus was collected 48 and 72 h after transfection and used to infect cells for shRNA expression.

Cell proliferation assay. HCT116 cells were transfected with the nontargeting scramble or Las1L siRNA oligonucleotide. Twenty-four hours after transfection, cells were replated in a 12-well dish at 25,000 cells per well. Cells were harvested by trypsinization at different time points and counted in the presence of trypan blue on a hemacytometer.

Cell cycle analysis. Cell cycle progression was assayed by determination of DNA content using propidium iodide (PI) and bromodeoxyuridine (BrdU) labeling followed by flow cytometry analysis. BrdU was added for 30 min, and cells were harvested and fixed with 70% ethanol overnight. Cells were centrifuged at 10,000 × *g* for 1 min and washed in BrdU wash solution (0.5% Tween 20, 0.5% bovine serum albumin [BSA] in PBS). Cells were resuspended in 2 N HCl for 20 min at room temperature, neutralized with 0.1 M sodium borate, and washed twice with BrdU wash solution. Cells were incubated with anti-BrdU antibody (BD 347580 mouse IgG) at room temperature for 30 min. Cells were washed 3 times in BrdU wash solution and incubated with Alexa Fluor 488 goat anti-mouse IgG (Invitrogen) for 30 min at room temperature. Cells were washed 3 times in BrdU wash solution and then treated with 100 μg/ml RNase and 25 μg/ml propidium iodide. Analyses were performed with a BD FACSCantoII instrument.

Immunoblotting. For immunoblotting, cells were harvested by trypsinization, washed with PBS, and lysed in RIPA buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS) plus protease inhibitors [aprotinin, leupeptin, and 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF)] and phosphatase inhibitor cocktail (ThermoFisher) for 15 min on ice. Lysates were cleared by centrifugation at 22,000 × *g* for 10 min at 4°C. Protein concentrations were evaluated with a BCA kit (Pierce). Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane (Bio-Rad). The Las1L (AV34629) antibody was purchased from Sigma-Aldrich. The p53 (SC-126), β-actin (SC-69879), and CDK2 (SC-163) antibodies were purchased from Santa Cruz Biotechnology. The p21 (556430) antibody was purchased from BD Pharmingen.

Immunofluorescence microscopy. U2OS cells were reverse transfected onto a no. 1 coverglass (Thermo Fisher) with control or Las1L siRNA and incubated for 72 h. For fibrillarin and UBF1 colocalization, cells were fixed with 4% paraformaldehyde at room temperature for 15 min and permeabilized with 0.1% Triton X-100 for 10 min at room temperature. For nucleophosmin (B23) colocalization, cells were fixed for 10 min at −20°C with a 1:1 mixture of ice-cold acetone and methanol or transfected with green fluorescent protein (GFP)-B23 (obtained from Xin Wang [57]) using Lipofectamine LTX (Invitrogen), fixed with 4% paraformaldehyde at room temperature for 15 min, and permeabilized with 0.1% Triton X-100 for 10 min at room temperature. All samples were blocked with 1% BSA for 30 min, washed with PBS, and incubated with the appropriate primary antibody for 1 h. Cells were washed with PBS containing 0.05% Tween 20 and incubated with secondary antibody for 1 h (Alexa Fluor 488 goat anti-rabbit antibody and Alexa Fluor 647 goat anti-mouse antibody; Invitrogen). Cells were washed again with PBS containing 0.05% Tween 20, stained with DAPI (4′,6-diamidino-2-phenylindole) (Molecular Probes), and mounted on slides with 90% glycerol, 4% *N*-propyl gallate. The Las1L (AV34629) antibody was from Sigma-Aldrich. Upstream binding factor (UBF) (SC-13125), fibrillarin (SC-56676), and nucleophosmin (SC-56622) antibodies were purchased from Santa Cruz Biotechnology. Confocal microscopy was performed on a Nikon A1 confocal laser microscope using the Argon multiline laser 458/477/488/514-nm and 638-nm diode at 10 mW with a Plan-Apochromat 100×, 1.4-numerical-aperture (NA) oil objective. Images were acquired using NIS Elements AR 64bit 3.10, SP7, Hotfix9 (Build 550) software. Fluorescence microscopy was performed on a Zeiss Axioskop 40 fluorescence microscope with a Plan-Apochromat 63×, 1.4-NA oil differential interference contrast (DIC) objective. Images were acquired with an Axiocam MRm camera using Axiovision Release 4.6 software. All microscopy was performed at room temperature, and all images were prepared in Adobe Photoshop and Adobe Illustrator.

Metabolic labeling of RNA, RNA extraction, and Northern blot analysis. Metabolic labeling of rRNA was performed as previously described (38). Briefly, cells were starved of phosphate for 1 h by incubation in DMEM without phosphate plus 5% dialyzed fetal bovine serum (Invitrogen). Medium was removed and replaced with DMEM without phosphate plus 5% dialyzed fetal bovine serum with phosphorus-^{32}P (15 μCi per ml), and cells were incubated for 1.5 h. The labeling medium was then replaced with normal growth medium for 2, 4, and 6 h or 0, 0.5, 1, and 2 h. Total RNA was extracted with Trizol reagent (Invitrogen) at 2, 4, and 6 h or 0, 0.5, 1, and 2 h of chase time. RNA was separated on a 1.2% agarose-formaldehyde gel. The gel was rinsed in water and incubated for 20 min in 0.01 M NaOH and 3 M NaCl. RNA was transferred to a Hybond N+ membrane (GE Healthcare) overnight. The membrane was then exposed to film for autoradiography analysis. For Northern blot analysis of rRNA intermediates, total RNA was extracted with Trizol, separated on a 1.2% agarose-formaldehyde gel, and transferred to a Hybond N+ membrane as described above. The DNA oligonucleotide probes were 5′ end labeled using T4 polynucleotide kinase (New England Biolabs) in the presence of [γ-^{32}P]ATP (MP Biomedical). The following probes were used: for ITS-1, 5′- CCTCCGCGCCGGAACGCGCRAGGTACC TGGACGGCGGGGGGGGACG- 3′; for ITS-2, 5′-GCGGCGGCAAGAGGA GGGCGGACGCCGCCGGGTCTGCGCTTAGGGGGA-3′; for 28S rRNA,5′-CCAGCTATCCTGAGGGAAACTTCGGAGGGAACCAGCTACTAGATG GTTCG-3′; and for 18S rRNA, 5′- CACCCGTGGTCACCATGGTAGGCAC GGCGACTACCATCGAAAGTTGATAG-3′. Membranes were prehybridized in Church buffer (1% [wt/vol] bovine serum albumin, 1 mM EDTA, 0.25 M $NaPO_4$ [pH 7.2], 7% [wt/vol] SDS) containing 100 μg/ml of single-stranded salmon sperm DNA (Invitrogen) for 1 h at 65°C. Labeled probes were incubated with the membrane in fresh Church buffer containing 100 μg/ml of single-stranded salmon sperm DNA for 16 h at 65°C. Membranes were washed with 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.1% SDS and exposed on film for autoradiography analysis.

Polysome preparation. HCT116 cells were infected with control shRNA and Las1 shRNA through lentiviral infection. Five days after selection in 1 μg/ml of puromycin, cells were seeded into four 150-mm cell culture plates and treated with 100 μg/ml of cycloheximide (Fisher Scientific) for 10 min. Cells were then washed in ice-cold PBS containing 100 μg/ml of cycloheximide and harvested. The pellet was resuspended in 500 μl of lysis buffer (20 mM Tris-Cl, pH 7.5, 5 mM $MgCl_2$, 100 mM KCl, 2.5 mM dithiothreitol, 0.5% NP-40) containing 200 U of Optizyme RNase inhibitor (Fisher Scientific), 100 μg/ml of cycloheximide, and 100 μg/ml of heparin (Fisher Scientific). Cells were lysed on ice for 10 min. Nuclear and cell debris was removed by two rounds of centrifugation at 4°C for 5 min at 5,000 × *g* and 15,000 × *g*. Cleared lysates were normalized based on protein concentrations and then layered onto a 12-ml 10% to 50% (wt/vol) sucrose gradient made in 20 mM Tris-Cl (pH 7.5), 5 mM $MgCl_2$, 100 mM KCl, 0.5 mM dithiothreitol, 100 μg/ml cycloheximide, and 100 μg/ml heparin. Each gradient was centrifuged at 38,000 rpm in an SW41 Beckman rotor for 3 h at 4°C. Fractions were collected, and the optical density of individual fractions was measured at 260 nm. To purify RNA for electrophoresis analysis, fractions were treated with proteinase K for 30 min at 42°C, followed by 1 extraction with phenol-chloroform-isoamyl alcohol (25:24:1) and 1 extraction with chloroform-isoamyl alcohol (24:1). RNA was precipitated with ethanol and 3 M sodium acetate, pH 5.2.

RESULTS

Las1L is required for cell proliferation. It was previously demonstrated that Las1 is essential for cell proliferation in *S. cerevisiae* (14). Las1 (herein referred to as Las1L) is conserved across species. An evolutionarily conserved domain in Las1 and its homologs was used to define a Las1 motif that is located on the N terminus of the protein (Fig. 1A and B). In addition, Las1L contains a glutamic acid-rich region on its C terminus

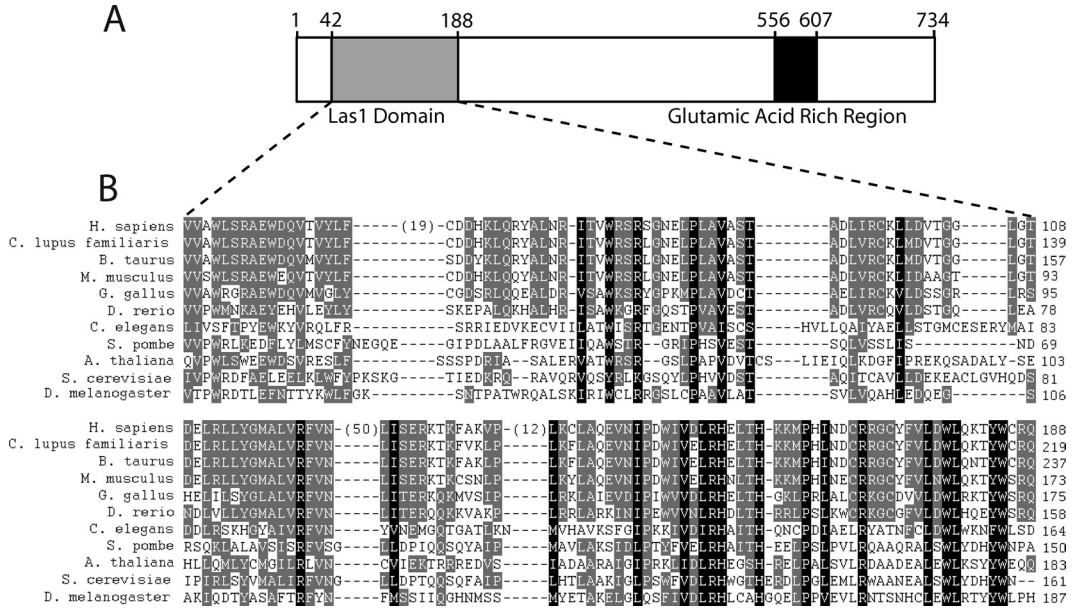

FIG. 1. Las1L protein is conserved throughout evolution. (A) Diagram of human Las1L protein, with the conserved Las1L domain and a C-terminal glutamic acid-rich region indicated. (B) Sequence alignment of human Las1L domain (residues 42 to 188) across 11 model organisms. Black shading denotes identical residues and conserved substitutions in 100% of the sequences, while gray shading indicates conservation in more than 50% of the sequences. Numbers in parentheses represent the gap lengths between residues. Organisms and GenBank sequence accession numbers are as follows: *Homo sapiens*, Q9Y4W2.2; *Canis lupus familiaris*, XP_538062.2; *Bos taurus*, NP_001070283.2; *Mus musculus*, A2BE28.1; *Gallus gallus*, Q5ZIQ0; *Danio rerio*, NP_001119872.1; *Caenorhabditis elegans*, Q9U1T1; *Schizosaccharomyces pombe*, O42936.1; *Arabidopsis thaliana*, Q8RXE3; *Saccharomyces cerevisiae*, P36146.1; and *Drosophila melanogaster*, Q8SZY9. Alignment was performed using ClustalX 2.0.12.

(Fig. 1A). The exact functions of Las1 or Las1L in both *S. cerevisiae* and higher eukaryotes are unknown.

To evaluate whether Las1L is required for cell proliferation, we depleted HCT116 colon carcinoma cells of Las1L by RNAi treatment and compared proliferation rates with those of cells treated with control short interfering RNA (siRNA). Knockdown of Las1L with two separate siRNAs was confirmed by Western blotting (Fig. 2A). Cell counts revealed that Las1L siRNA-treated cells proliferated at a much lower rate than those treated with control siRNA (Fig. 2B). To determine if this decrease in proliferation was due to a specific arrest in the cell cycle, we then analyzed DNA profiles of cells treated with

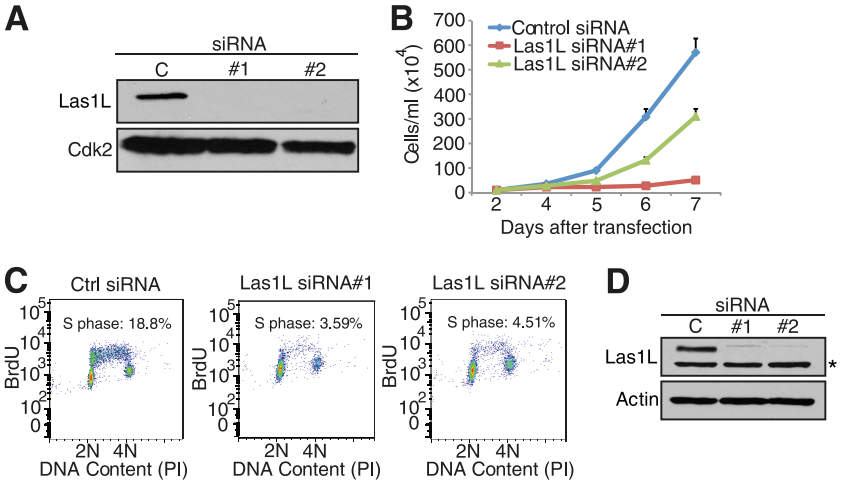

FIG. 2. Las1L is required for cell proliferation. (A) HCT116 cells were transfected with nontargeting control (represented by the letter C), Las1L#1, or Las1L#2 siRNA. Depletion of Las1L at day 7 posttransfection was confirmed by Western blotting with a Las1-specific antibody. A Cdk2 antibody was used as a loading control. (B) Growth curve of HCT116 cells transfected with control siRNA or Las1L#1 and Las1L#2 siRNA as indicated. Twenty-four hours after transfection, cells were replated and counted at days 2, 4, 5, 6, and 7 posttransfection. Error bars indicate standard deviations. (C) BrdU and PI labeling of HCT116 cells transfected with control (Ctrl) or Las1L siRNAs. Seventy-two hours after transfection, the cells were analyzed by flow cytometry for DNA content. The percentage of cells in S phase is indicated on each graph. (D) Western blot analysis from protein lysates of cells used for the experiment with results shown in panel C, using anti-Las1L and antiactin antibodies to confirm knockdown. The asterisk indicates the presence of an unspecific band.

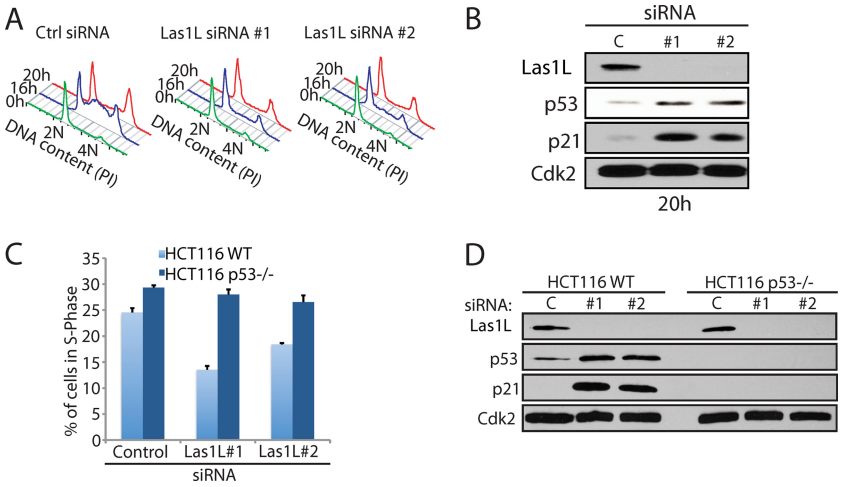

FIG. 3. Depletion of Las1L induces a p53-dependent G_1 cell cycle arrest. (A) Cell cycle profiles of HCT116 cells transfected with control (Ctrl), Las1L#1, or Las1L#2 siRNA. Twenty-four hours after transfection, cells were incubated in serum-free medium for an additional 72 h to synchronize the cells in G_1. Serum was reintroduced, and the cell cycle phase was monitored at the indicated time points by staining with propidium iodide (PI) followed by analysis by flow cytometry. (B) Western blot analysis of cells from the 20-h time point release described for panel A, with specific antibodies against Las1L, p53, p21, and Cdk2. (C) Flow cytometry analysis of HCT116 WT and p53-null cells transfected with control, Las1L#1, or Las1L#2 siRNA. Seventy-two hours after transfection, cells were analyzed for BrdU incorporation, and the results are graphed as percentages of cells in S phase. Data represent the means from 3 independent experiments. Error bars indicate standard deviations. (D) Western blot analysis from protein lysates of the cells used for the experiments with results shown in panel C, with anti-Las1L, anti-p53, anti-p21, and anti-Cdk2 antibodies.

control and Las1L siRNAs, and Las1L knockdown was confirmed by Western blot analysis (Fig. 2D). BrdU and propidium iodide labeling revealed an increase in the G_1 population and a considerable decrease in the number of cells in S phase for the Las1L-depleted cells compared to the control siRNA-treated cells, suggesting an arrest in G_1 (Fig. 2C). To assess whether Las1L is required for the progression from G_1 to S phase, cells were treated with control or Las1L siRNA, synchronized in G_1 by serum starvation, released into the cell cycle by addition of serum, and then analyzed at 0, 16, and 20 h for DNA content. Flow cytometry profiles after release showed that control siRNA-treated cells progressed into S phase at 16 h as expected, while cells lacking Las1L failed to advance out of G_1 (Fig. 3A). Similar results were obtained using the U2OS osteosarcoma cell line (data not shown).

To further investigate the mechanism by which Las1L causes cells to arrest in G_1, we next analyzed whether the levels of cyclin/CDK inhibitors ($p21^{CIP1}$ and $p27^{kip1}$) and the tumor suppressor p53 were changed upon depletion of Las1L. Western blot analysis of cells released from starvation revealed stabilization of p53 and accumulation of $p21^{CIP1}$ in the cells depleted of Las1L (Fig. 3B), while levels of $p27^{kip1}$ remained unchanged (data not shown).

To examine if stabilization of p53 was necessary to mediate the observed G_1 arrest, we transfected asynchronous HCT116 wild-type (WT) and HCT116 $p53^{-/-}$ cells with control or Las1L siRNA and analyzed their cell cycle profiles by flow cytometry. Wild-type cells treated with Las1L siRNA arrested in G_1, with substantially fewer cells in S phase and increased p53 and p21 levels (Fig. 3C and D). However, $p53^{-/-}$ cells continued to proliferate, as they showed similar percentages of cells in S phase despite the absence of Las1L (Fig. 3C and D). Taken together, these findings indicate that depletion of Las1L affects cell proliferation and specifically the G_1- to S-phase progression of the cell cycle. Moreover, our data suggest that depletion of Las1L induces cellular stress signaling through the $p53\text{-}p21^{CIP1}$ axis to prevent S-phase entry.

Las1L is localized to the nucleolus. The subcellular localization of endogenous Las1L was evaluated by immunofluorescence. Using a Las1L-specific antibody, we observed that Las1L was found to localize predominantly in the nucleolus of U2OS cells (Fig. 4A). The same localization was observed in MCF7, HEK293T, HCT116, and HeLa cells, suggesting that nucleolar localization of Las1L is not cell type specific (data not shown). The nucleolar localization was lost upon Las1L RNAi treatment, confirming the specificity of the antibody used (Fig. 4A). In support of this observation, Las1L was also found to be localized in the nucleolus by mass spectrometry as part of a large-scale proteomic analysis of the human nucleolus (47).

The nucleolus can generally be divided into 3 specific regions: the fibrillar component, where rRNA transcription machinery is localized; the dense fibrillar component, where early steps of rRNA processing take place; and the granular component, where later steps in rRNA processing and nascent ribosomal assembly occur (5, 46, 49). To better define the nucleolar sublocalization of Las1L, we performed confocal microscopy on U2OS cells using upstream binding factor (UBF), fibrillarin, and nucleophosmin (B23) as markers for the fibrillar component, dense fibrillar component, and granular component, respectively. Las1L did not colocalize with UBF and colocalized only minimally with fibrillarin (Fig. 4B). Of the three markers, Las1L colocalized the most with nucleophosmin (B23), although not entirely (Fig. 4B). Because a methanol-acetone fixation method was used for the nucleophosmin (B23) antibody, we confirmed colocalization using a GFP-nu-

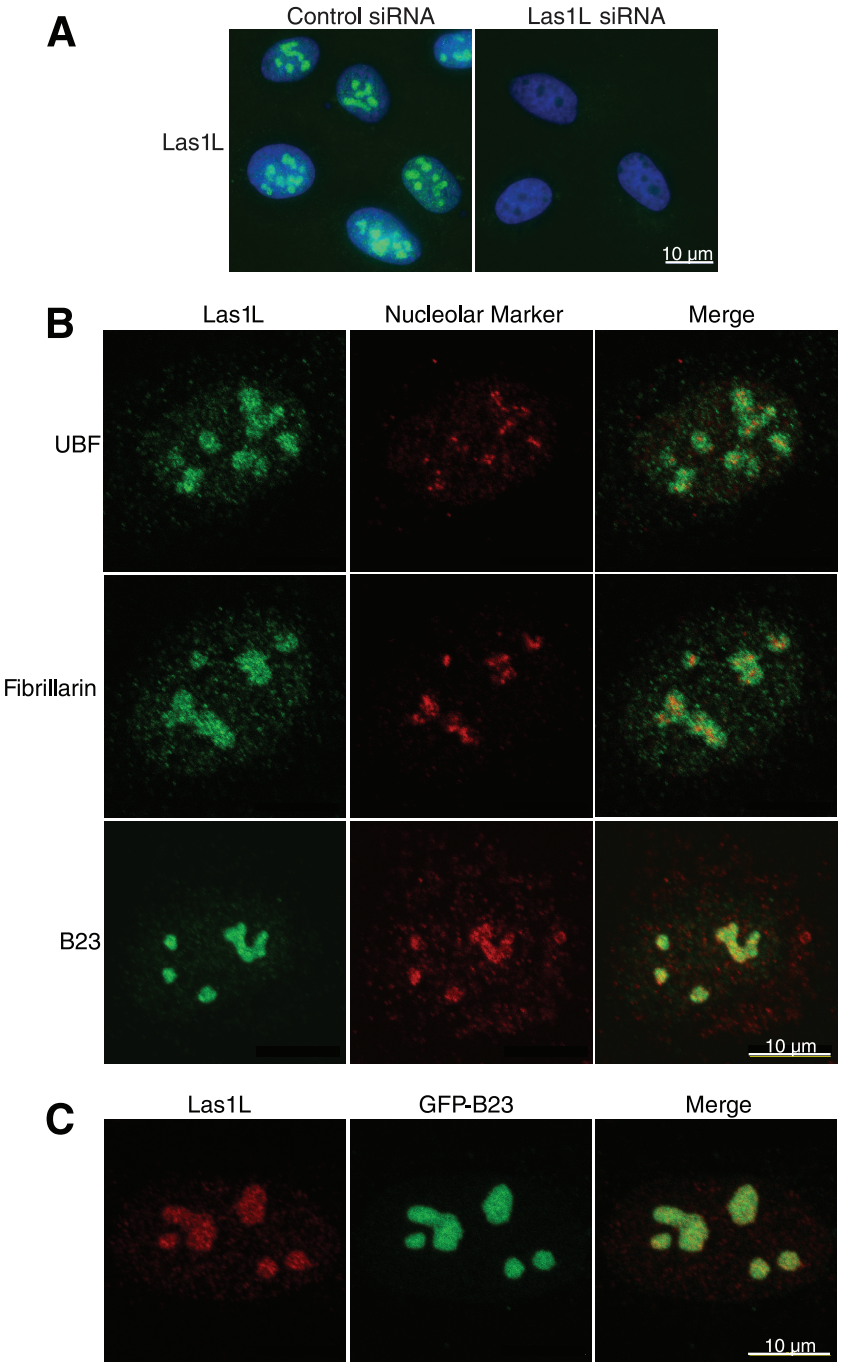

FIG. 4. Las1L localizes to the nucleolus. (A) Immunofluorescence analysis of U2OS cells transfected with control or Las1L#1 siRNA. Seventy-two hours after transfection, cells were fixed and immunostained with anti-Las1L (green). DNA was visualized by staining with Hoechst 33342 (blue). The scale bar applies to both panels. (B) Confocal microscopy representing colocalization of Las1L (green) with nucleolar compartmental markers UBF, fibrillarin, and B23 (red) by immunostaining. Before staining, cells were fixed with 4% formaldehyde, except for B23, where methanol-acetone was used. The scale bar applies to all panels. (C) Colocalization of Las1L with B23 was confirmed using a GFP-B23 construct (green) and an anti-Las1L antibody (red). Cells were fixed with 4% formaldehyde. The scale bar applies to all panels.

cleophosmin (GFP-B23) expression construction and formaldehyde fixation (Fig. 4C). We conclude that Las1L is localized mainly to the granular component, the region implicated in the later steps of rRNA processing and subunit assembly and export.

Using Las1L siRNA, we observed that depletion of Las1L often resulted in a reorganization of the nucleolar architecture, as shown by abnormal staining of fibrillarin (Fig. 5A). Cells treated with control siRNA showed multiple lobulated nucleoli, whereas Las1L-depleted cells had a large collapsed nucleolus. Similar observations were made using UBF and nucleophosmin as nucleolar markers (data not shown).

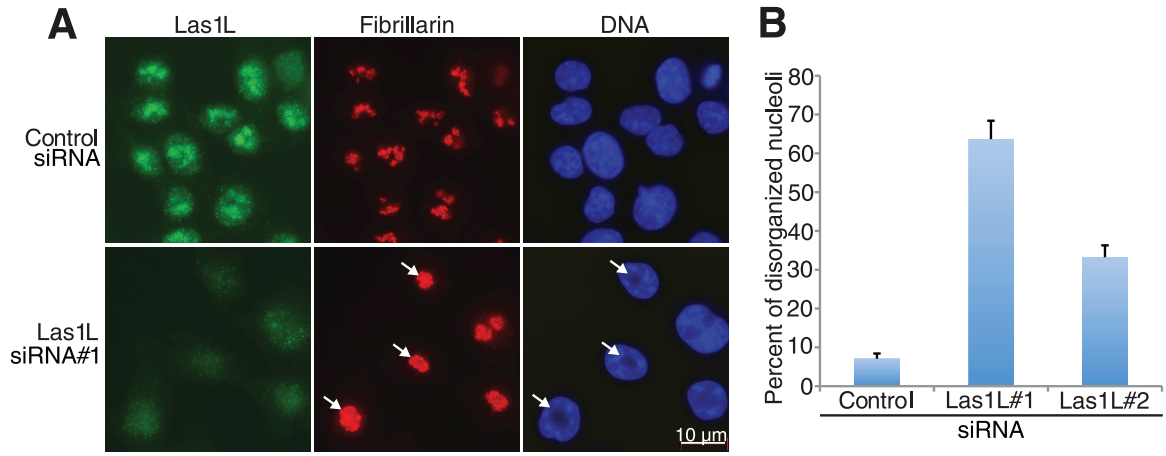

FIG. 5. Las1L depletion causes nucleolar disorganization. (A) Nucleolar disorganization in the absence of Las1L was analyzed by immunofluorescence. HCT116 cells were transfected with control or Las1L#1 siRNA for 72 h. Cells were fixed and immunostained with anti-Las1L antibody (green) or antifibrillarin antibody (red), and DNA was visualized with Hoechst 33342 (blue). The scale bar applies to all panels. Arrows indicate perturbed nucleoli. (B) The numbers of cells with disorganized nucleoli were counted in 5 different low-power fields and graphed. Error bars indicate standard deviations.

Quantification of the number of disorganized nucleoli demonstrates a dramatic increase in cells depleted of Las1L compared to cells treated with control siRNA (Fig. 5B), suggesting that Las1L is required to maintain the integrity of the nucleolus.

Depletion of Las1L results in loss of the 60S ribosomal subunit. The primary function of the nucleolus in eukaryotic cells is the synthesis and assembly of ribosomal subunits (5). Nucleoli assemble around tandem repeats of ribosomal DNA gene clusters, where Pol I initiates the transcription of the 47S pre-rRNA. The 47S transcript is then processed by nonribosomal factors and assembled with ribosomal proteins to form the 60S and 40S preribosomal subunits. Recent studies in both *S. cerevisiae* and mammalian cells suggest that Pol I transcription and site-specific modification and initial processing of the 47S rRNA occur concomitantly (26, 27, 36). Additional processing occurs as the subunits are exported separately through the nucleus into the cytoplasm, where the final steps of rRNA processing occur. The 40S subunit then initiates translation, and the 60S subunit is incorporated into a translationally competent 80S ribosome (28, 36). To determine if Las1L is required for the formation of functional ribosomes, polysome profiles of cells infected with control or Las1L small hairpin RNA (shRNA) were analyzed. Ablation of Las1L (Fig. 6B) resulted in a dramatic loss of the 60S subunit, whereas levels of the 40S subunit remained unchanged (Fig. 6A). Positions of ribosomal subunits on the polysome profile were confirmed by the appearance of the 18S or 28S rRNA in each fraction (Fig. 6C). Pronounced depletion of the 60S ribosomal subunit in the absence of Las1L suggests a vital function in the synthesis of mature ribosomes.

Las1L is necessary for rRNA processing. In both budding yeast and higher eukaryotes, loss of preribosomal subunits can occur by different mechanisms, including depletion of ribosomal proteins, abrogation of rRNA transcription, or impairment of the rRNA processing pathway (6, 16, 50, 58). Processing of the rRNA transcript from the 47S rRNA to the mature 18S, 5.8S, and 28S rRNAs (Fig. 7A) involves the cooperation of various nonribosomal proteins. During this process, the two internal transcribed spacers (ITS-1 and ITS-2) are cleaved from the rRNA transcript. Accrual of intermediates containing either ITS-1 or ITS-2 denotes impairment in rRNA processing. We therefore examined the accumulation of rRNA processing intermediates by Northern blotting using probes specific for the ITS-1, ITS-2, 28S rRNA, and 18S rRNA regions (Fig. 7A) to determine if Las1L is involved in pre-rRNA processing. Depletion of Las1L was confirmed by Western blotting (Fig. 7B). Northern blot analysis using a probe specific for the 18S region showed that there was little change in the levels of the mature 18S rRNA upon Las1L knockdown (Fig. 7C and D). In contrast, investigation using the ITS-2 probe revealed a substantial accumulation of the 32S pre-rRNA and a distinct loss of the 12S pre-rRNA in cells treated with Las1L siRNA compared to levels in cells treated with control siRNA (Fig. 7C and D), suggesting that Las1L is instrumental in the processing of ITS-2. Accumulation of the 32S pre-RNA upon depletion of Las1L was also apparent in analysis with the probe specific for the 28S region (Fig. 7C). Interestingly, when the ITS-1 probe was used, a decrease of the 30S intermediate was observed in Las1L siRNA-treated cells compared with the level in control siRNA-treated cells (Fig. 7C and D). To confirm that the processing defect observed in the absence of Las1L is not due to cell cycle arrest by upregulation of p53, Northern blot analyses were performed in HCT116 $p53^{-/-}$ cells (which do not arrest upon Las1L depletion). The same accumulation of the 32S pre-RNA was also observed with Las1L siRNA in HCT116 $p53^{-/-}$ cells, indicating that accrual of the 32S rRNA intermediate is due to Las1L depletion and not a secondary effect of the G_1 cell cycle arrest. Collectively, these data indicate that Las1L plays a fundamental role in rRNA processing, principally of the ITS-2 segment during the processing from the 32S strand to the 28S rRNA.

Las1L is required for maturation of the 28S rRNA. Thus far, we have shown that Las1L is required for the formation of the 60S ribosomal subunit, which contains the processed 28S rRNA (Fig. 6A). We also demonstrated that the depletion of

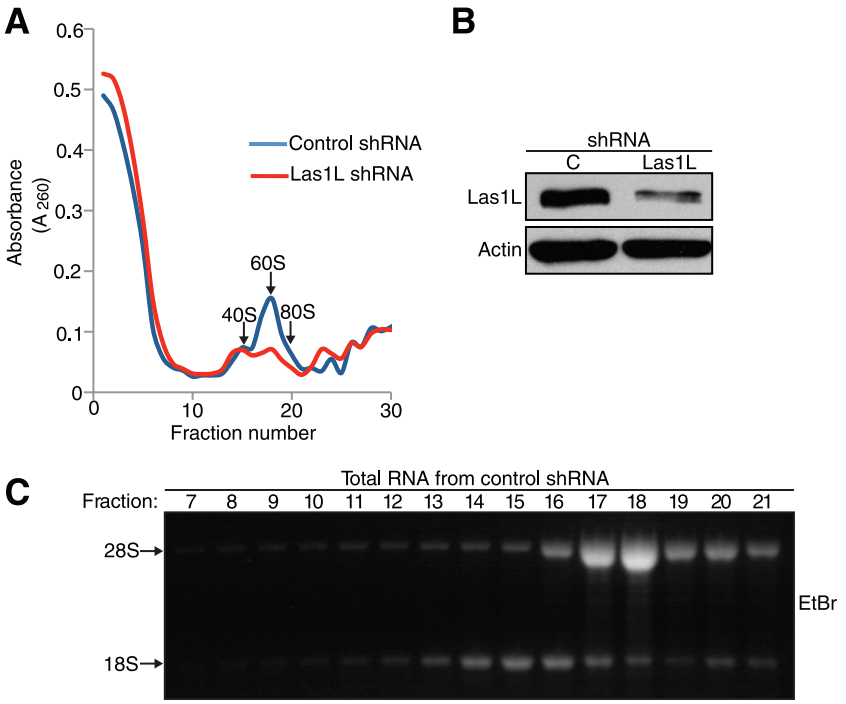

FIG. 6. Depletion of Las1L causes defects in ribosome biogenesis. (A) Polysome profile of HCT116 cells infected with lentivirus-expressing control shRNA or Las1L shRNA 5 days after puromycin selection. Polysome profiles were performed by fractionation on a 10 to 50% sucrose gradient. Fractions were collected, and the optical density at 260 nm (A_{260}) was measured. The positions of the 40S and 60S native subunits and the 80S monosomes are indicated. (B) Western blot analysis showing the levels of Las1L in control shRNA- or Las1L shRNA-infected cells used for the experiments with results shown in panel A. Actin levels were measured to the control level for protein loading. (C) RNA (stained with ethidium bromide [EtBr]) from fractions 7 to 21 was extracted and separated on a 1.2% formaldehyde gel to confirm the presence of the 40S or 60S subunit by monitoring the appearance of the 18S or 28S rRNA, respectively.

Las1L leads to a substantial accumulation of the 32S rRNA intermediate (Fig. 7B). We therefore predicted that loss of Las1L would diminish the production of the mature 28S rRNA. To test this hypothesis, we examined rRNA synthesis and processing using pulse-chase analysis by ^{32}P-orthophosphate labeling. Compared to results for cells treated with control siRNA, a minimal reduction in the 18S rRNA was seen with Las1L RNAi. However, cells depleted of Las1L displayed a major decrease in the synthesis of the 28S rRNA from the 32S precursor (Fig. 8A). An increase in the 32S/28S ratio compared to that for cells treated with the control signifies an accumulation of 32S rRNA accompanied by little to no change in the 28S rRNA (Fig. 8B).

Previous evidence has demonstrated that p53 suppresses Pol I transcription by preventing its interaction with the rRNA transcriptional auxiliary factors UBF and SL1 (7, 59). As we have shown, p53 levels increase with depletion of Las1L (Fig. 3B and D). We investigated by pulse-chase analysis whether the observed reduction of the mature 28S rRNA was a consequence of p53-mediated cell cycle arrest and subsequent repression of the Pol I promoter. 47/45S pre-RNA and 18S rRNA levels were reduced upon Las1L siRNA treatment in WT cells but not in p53$^{-/-}$ cells, suggesting that this could be due to inhibition of Pol I activity by p53 (Fig. 8A and C). However, both cell types failed to accumulate the 28S rRNA upon reduction of Las1L (Fig. 8C), and an increase in the 32S/28S ratio compared to that for control siRNA-treated cells was also observed upon treatment with Las1L siRNA in both cell types (Fig. 8D). Taken together, these data suggest that the dramatic reduction in the 28S rRNA is due to depletion of Las1L and not a reduction in Pol I activity induced by stabilization of p53, corroborating our findings that Las1L is required for production of the 60S ribosomal subunit.

DISCUSSION

Recently, genetic screens combined with proteomic approaches in *S. cerevisiae* have revealed the identity of many evolutionarily conserved proteins involved in ribosome biogenesis (29, 34, 37, 45). The exact mechanism of action and specific interactors of many of these proteins remain to be clarified. Our results reveal Las1L as a novel protein necessary for processing of the 32S to the 28S rRNA and formation of the 60S preribosomal subunit.

Although ribosome biogenesis is a highly conserved process, characterized proteins and complexes involved in 60S maturation in mammals are limited. Known proteins implicated in 60S preribosome synthesis via processing of the 32S to the 28S rRNA include nucleostemin and nucleophosmin (22, 42). The proteins Bop1, Pes1, and WDR12 (PeBoW complex) were shown to function in maturation of the 60S subunit, and removal or loss of function of any of these proteins causes defects in 28S rRNA production through accumulation of the 32S rRNA intermediate (20, 24, 30, 41, 50, 51). Members of the PeBoW complex have also been implicated in the processing of both ITS-1 and the 3′ ETS (30, 50, 51). It remains to be

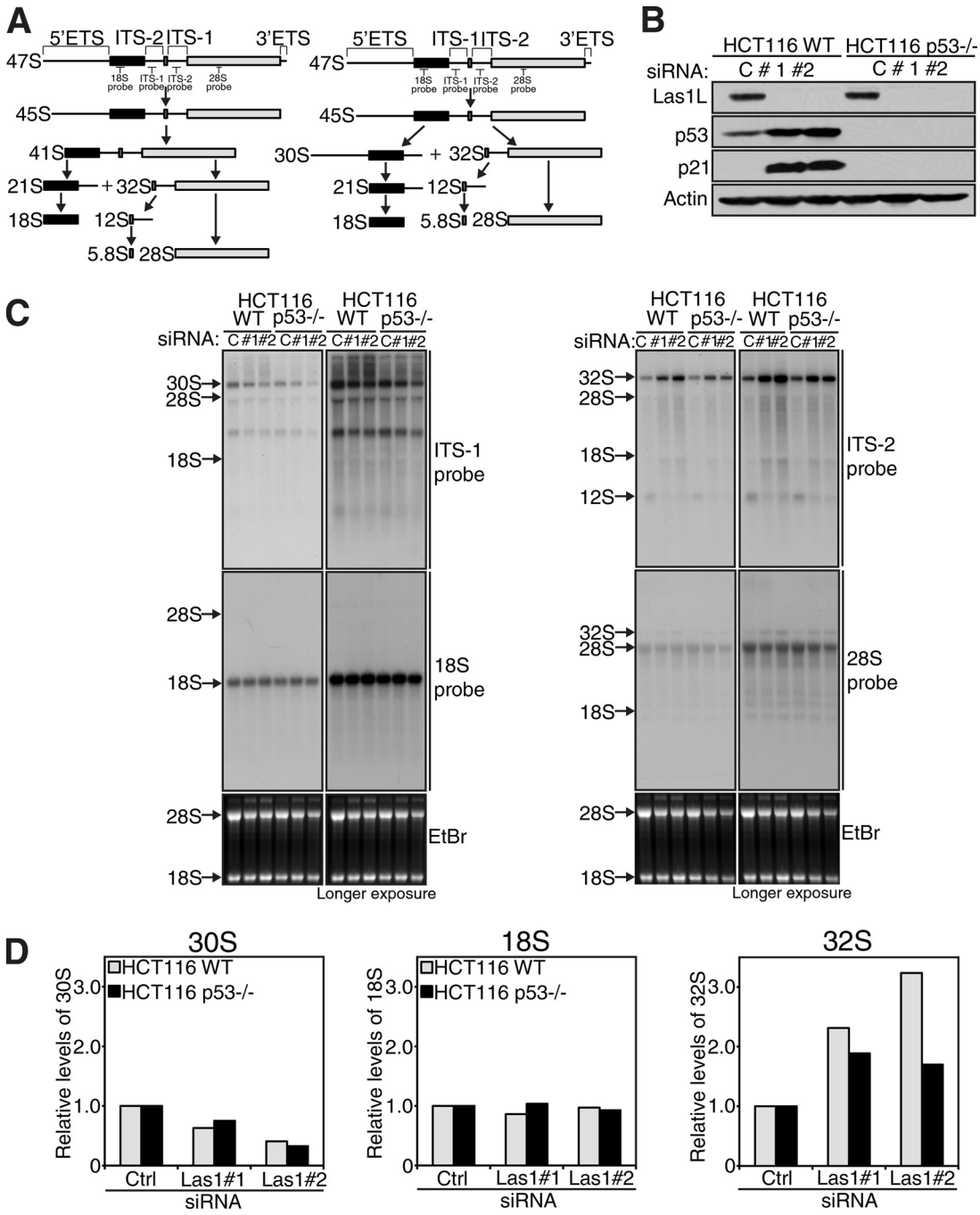

FIG. 7. Las1L is required for proper processing of ITS-2. (A) Schematic representation (21) of the 47S rRNA primary transcript and the two major processing pathways, with indicated processed rRNA intermediates. The positions of the specific probes used for Northern blot analysis are indicated. (B) Western blot analysis with anti-Las1L, anti-p53, anti-p21, and antiactin antibodies to confirm knockdown of Las1L in the cells used for Northern blot analysis with results shown in panel C. (C) Northern blot analysis of total RNA from HCT116 WT or HCT116 p53$^{-/-}$ cells transfected with control (represented by the letter C), Las1L#1, or Las1L#2 siRNA for 72 h. Equal amounts of total RNA were hybridized with probes specific for ITS-1, ITS-2, 28S, and 18S rRNA intermediates, as indicated to the right of each panel. Right panels for each probe represent longer exposure times. (D) Quantification of relative band intensities of 32S, 30S, and 18S rRNAs in the shorter exposures shown in panel C. Intensities were normalized to the control level for each respective cell type for each panel.

determined whether Las1L functions have a link with the PeBoW complex or whether Las1L acts in a similar, parallel pathway.

In a recent screen in *S. cerevisiae*, deletion of the *GRC3* gene resulted in comparable defects in processing of the 32S, 28S, and 12S rRNA homologues (37). Several of the proteins that were found to be involved in pre-rRNA processing are also involved in other steps of ribosome biogenesis, such as trans-

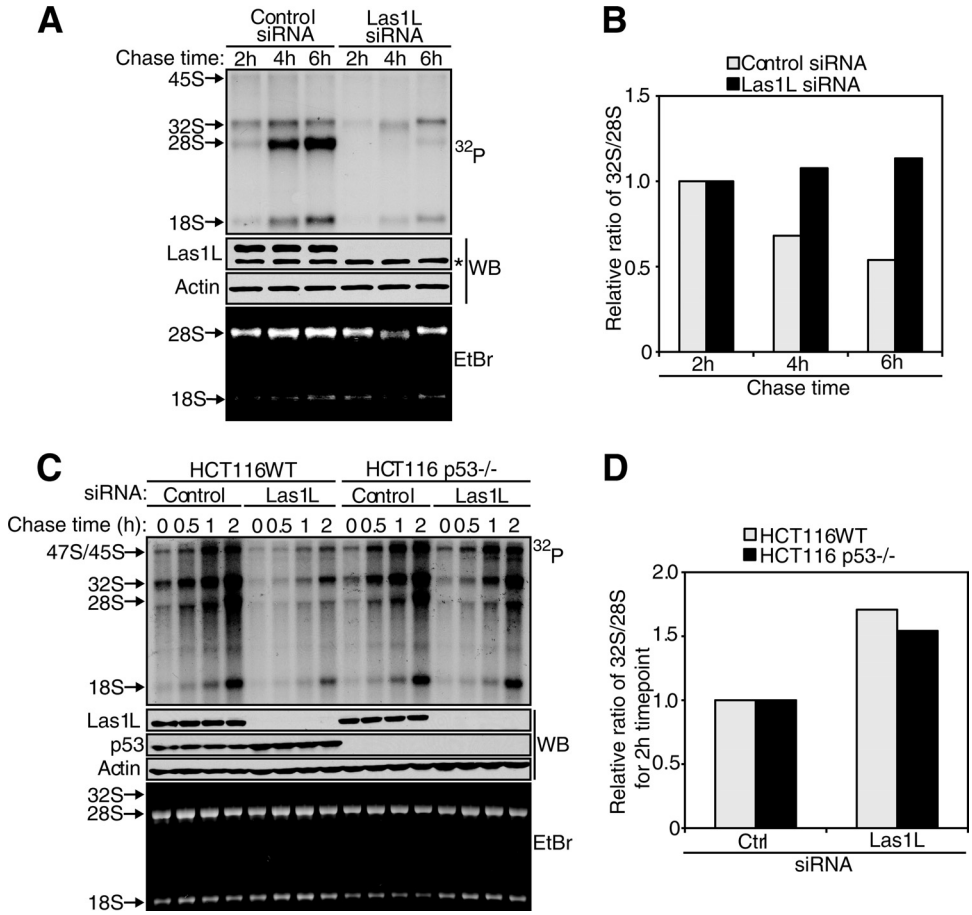

FIG. 8. Las1L is necessary for 28S rRNA synthesis. (A) Pulse-chase analysis of rRNA in HCT116 WT cells transfected with control or Las1L#1 siRNA. Cells were pulse-labeled with ^{32}P-orthophosphate for 2 h and chased in normal growth medium for the indicated amounts of time. Total RNA was harvested and separated on a 1.2% formaldehyde denaturing gel. Knockdown of Las1L was verified by Western blot analysis (WB) with an anti-Las1L antibody. The asterisk indicates the presence of an unspecific band. Equal protein loading was confirmed using an antiactin antibody. The bottom panel represents an ethidium bromide (EtBr) RNA gel before transfer. (B) Quantification of band intensities from the experiment with results shown in panel A. 32S/28S ratios for the indicated chase times are shown. Intensities were normalized to the 32S/28S ratio at the 2-h time point. (C) Pulse-chase analysis of rRNA in HCT116 WT and HCT116 p53$^{-/-}$ cells. Cells were labeled as described for panel A and chased in normal growth medium for the indicated amounts of time. Knockdown of Las1L was verified by Western blot analysis with an anti-Las1 antibody. Levels of p53 were determined using an anti-p53 antibody. Equal protein loading was confirmed using an antiactin antibody. (D) Quantification of band intensities from the experiment with results shown in panel C. The relative 32S/28S ratio at the 2-h time point is shown. Intensities were normalized to the control level for each respective cell type.

port into the nucleoplasm and export of subunits to the cytoplasm in *S. cerevisiae* (12, 15, 17, 33, 35, 43, 52, 55). For example, deletion of the Rix complex proteins and the AAA ATPase Rea1 inhibited both rRNA processing and 60S subunit export (17). Also in budding yeast, depletion of Nop53p results in defects in rRNA processing and export of the 60S ribosomal subunit (52). The possibility that Las1L could be another multifunctional protein involved in multiple steps of 60S maturation requires further clarification. In budding yeast, the function of Las1 is uncharacterized (14), and Las1 is not found in any of the purified pre-60S complexes, nor is it represented in any of the large-scale protein interaction analyses (29, 34, 37, 45). It will be interesting to evaluate whether *S. cerevisiae* Las1 is involved in rRNA processing and formation of the 60S subunit as well.

The cell cycle is intimately coupled to nucleolar function, and disrupted nucleolar integrity is a featured cellular stress that leads to p53-mediated G_1 cell cycle arrest (44). Indeed, inhibition of rRNA transcription, impairment in rRNA processing, and depletion of ribosomal proteins have all been associated with nucleolar stress and induction of p53 (11, 25, 32, 39, 44, 58, 61). Reduction of both 40S and 60S subunit synthesis was shown to induce a p53-dependent cell cycle arrest (23, 24). Once a nucleolar stress is induced, p53 stabilization is achieved via the binding of ribosomal proteins to HDM2 to inhibit its E3 ubiquitin ligase activity, which is responsible for the ubiquitination and consequent degradation of p53 (4, 9–11, 16, 23, 25, 32, 58, 61, 62). Similarly to results found with other proteins involved in rRNA processing, we found that removal of Las1L results in nucleolar stress and stabilization of p53, leading to G_1 arrest, thus intimately linking Las1L to cell cycle progression.

In recent years, the link between deregulated ribosome biogenesis and cancer has become more evident. Zebrafish har-

boring heterozygous mutations in ribosomal proteins incur a high incidence of tumorigenesis (1). Aggressive forms of breast cancers show deregulated ribosome biogenesis through increased rRNA synthesis and alterations in the rRNA processing pathway (3). Future experiments include the elucidation of proteins interacting with Las1L to give further insight into the molecular mechanisms underlying the involvement of Las1L in maturation of the 60S ribosomal subunit and to further evaluate the role of Las1L in human disease and cancer.

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