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Award Number:
W81XWH-10-1-1029

TITLE:
Regulation of c-Myc mRNA by L11 in Response to UV and Gamma Irradiation

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REPORT DATE: October 2011

TYPE OF REPORT:
Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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1. REPORT DATE (DD-MM-YYYY) 1 October 2011		2. REPORT TYPE Annual		3. DATES COVERED (From - To) 20 Sept 2010-19 Sept 2011	
4. TITLE AND SUBTITLE Regulation of c-Myc mRNA by L11 in Response to UV and Gamma irradiation				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-10-1-1029	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Mu-Shui Dai, M.D., Ph.D. email: daim@ohsu.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Oregon Health & Science University Portland, OR 97239				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research And Material Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT In this funding period, we have discovered that L11 downregulates c-myc mRNA through a microRNA-mediated pathway. L11 binds to c-myc mRNA at its 3'-untranslated region (3'-UTR), the core component of microRNA-induced silencing complex (miRISC) Ago2, as well as miR-24, leading to c-myc mRNA reduction. Knockdown of L11 drastically increases the levels and stability of c-myc mRNA. Ablation of Ago2 abrogated the L11-mediated reduction of c-myc mRNA, whereas knockdown of L11 rescued the miR-24-mediated c-myc mRNA decay. We further show that ribosome-free L11 binds to c-myc mRNA in the cytoplasm and this binding is enhanced in response to ribosomal stress. Meanwhile, we found that c-myc is also down-regulated in response to DNA damage including UV and γ -irradiation (γ -IR) in an L11-dependent manner. Altogether, our results identify a novel regulatory paradigm wherein L11 plays a critical role in controlling c-myc mRNA turnover via recruiting miRISC in response to stress.					
15. SUBJECT TERMS Ribosomal protein, L11, c-Myc, DNA damage, microRNA					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	21	19b. TELEPHONE NUMBER (include area code)

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A. INTRODUCTION

The c-Myc oncoprotein is deregulated in many human cancers. Thus, proper control of c-Myc level and activity is essential for normal cell growth and proliferation. We have previously identified that ribosomal protein L11 suppresses c-Myc transactivation activity^(1,2) and reduces *c-myc* mRNA levels⁽³⁾. Interestingly, *c-myc* mRNA is markedly reduced by treatment of ribosomal stress-inducing agents actinomycin D and 5-fluorouracil in an L11-dependent manner, suggesting that L11 regulates *c-myc* mRNA in response to ribosomal stress. The purpose of this proposal is to examine whether and how L11 is involved in the regulation of c-Myc in response to military-related DNA damage, such as those induced by ultraviolet (UV) and γ -irradiation (IR). Specifically, we will determine whether L11 regulates *c-myc* mRNA levels and stability by recruiting miRNAs in response to UV and γ -IR as well as the mechanism underlying the L11 regulation of *c-myc* mRNA. Results from these experiments would demonstrate an important function of L11 in regulating *c-myc* mRNA in response to DNA damage and offer useful information for developing anti-tumor drugs that target *c-myc* mRNA in cancers and thus have a significant impact on the understanding of c-Myc-induced tumorigenesis.

B. BODY

During the funding period, we have characterized a **general** mechanism underlying L11 regulation of *c-myc* mRNA stability in unstressed conditions or following ribosomal stress. We have found that L11 regulates the stability, but not the translation, of the *c-myc* mRNA. Mechanistically, we have found that L11 recruits microRNA (miRNA)-24 (miR-24) loaded RNA interference silencing complex (miRISC) to suppress *c-myc* mRNA expression. L11 binds to the *c-myc* mRNA at the 3'-end sequence of *c-myc* 3'-UTR (nt 361-470). Overexpression of L11 suppresses the expression of luciferase mRNA and activity, whereas knockdown of L11 increases these levels and activity, in cells transfected with luciferase reporter containing the *c-myc* 3'-UTR (pGL3-myc 3'UTR), but not the control pGL3 vector. We further confirmed that L11 binds to the miRISC component Ago2 and miR-24. Knockdown of L11 rescued the *c-myc* mRNA reduction mediated by either overexpression of miR-24 or knockdown of Ago2, suggesting that L11 recruits miR24/miRISC to repress c-Myc. Interestingly, ribosomal stress induced by actinomycin D (Atc D) or 5-fluorouracil (5-FU)-mediated perturbation of ribosomal biogenesis results in a significant *c-myc* mRNA reduction in cells, and this reduction requires L11. Also, L11 binding to *c-myc* mRNA, miR-24, and Ago2 was significantly increased in response to ribosomal stress, suggesting that L11 play a key role in down-regulating *c-myc* mRNA in response to ribosomal stress, ensuing a tight coordination between the levels and activity c-Myc and ribosomal biogenesis.

Interestingly, we found that it is the ribosome-free L11 that binds to the *c-myc* mRNA in the cytoplasm. Ribosomal stress triggers the release of L11 from the nucleolus to the nucleoplasm, where it binds to c-Myc protein, and to the cytoplasm, where it binds to *c-myc* mRNA. We also found that L11-*c-myc* mRNA binding is independent of the L11-c-Myc protein binding. Furthermore, we show that L11 regulation of *c-myc* mRNA is specific as several tested other ribosomal proteins including L23, L26, L29 and S12 does not regulates *c-myc* mRNA levels and knockdown of L11 does not change the levels of other tested genes including *p53*, *mdm2*, *mdmx*, *c-jun*, *c-fos* and *egr1*.

Together, these data identify a novel regulatory paradigm wherein L11 plays a critical role in controlling *c-myc* mRNA turnover via recruiting miRISC in response to ribosomal stress. The manuscript summarizing these data was recently published in the *Mol Cell Biol* (**Challagundla KB, et al, 2011**)⁽⁴⁾. Please refer this paper (**attached as appendices**) for detail methodology, experimental results, as well as discussion.

In addition, we have performed a purification of L11-associated-miRNAs and mRNAs using deep sequencing. Our initial results identified that L11 associates with a number of novel miRNAs (including miR-16, miR-191, miR-130a, miR-1244, miR-1248, etc.) in addition to miR-24 and mRNAs (including L11 itself, ctBP, Bcl-2, etc.) We will further verify which of these miRNAs are authentic *c-myc* targeting miRNAs and their role in c-Myc regulation.

Specifically relating to the statement of Work (SOW) of this award, following points are either addressed or under planning:

Aim1. To determine if L11 regulates *c-myc* mRNA in response to UV and γ -IR.

We have found that c-myc mRNA is reduced in cells treated with UV and the γ -IR mimicking agent NCS (neocarzinostatin) (**Fig. 1**). This reduction requires L11 as knockdown of L11 completely abolished UV- or IR-mediated reduction of the c-myc mRNA (**Fig. 2**), suggesting that L11 plays an important role in regulating c-myc levels in response to DNA damage.

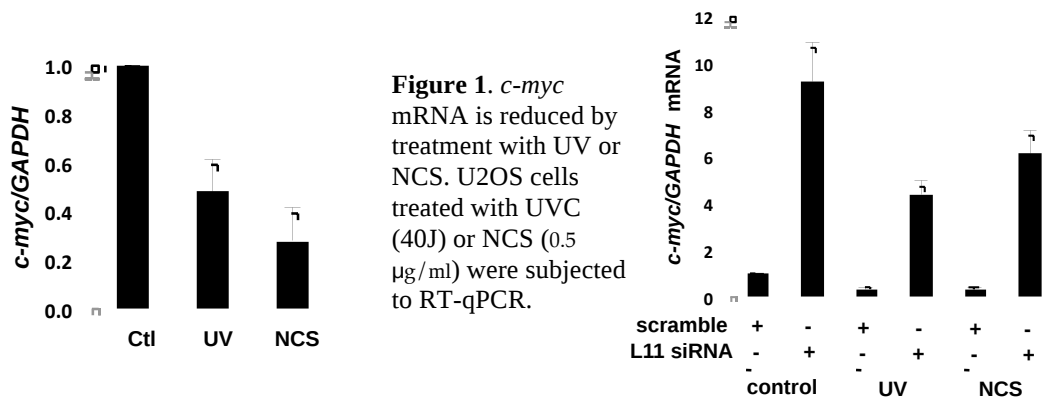


Figure 1. c-myc mRNA is reduced by treatment with UV or NCS. U2OS cells treated with UVC (40J) or NCS (0.5 μ g/ml) were subjected to RT-qPCR.

Figure 2. Knockdown of L11 rescues the downregulation of c-myc mRNA by treatment with UV or NCS. U2OS cells transfected with scrambled or L11 siRNA were treated with UVC (40J) or NCS (0.5 μ g/ml) followed by RT-qPCR detection of c-myc mRNA normalized to GAPDH.

Aim2. To examine if L11 recruits miRNA(s) to the 3'UTR of c-myc mRNA in response to UV and γ -IR.

As expected, we have found that UV damage enhances L11 association with c-myc mRNA (**Fig. 3**). We further showed that L11 binding to the 3'-UTR of c-myc mRNA was induced by UV treatment (**Fig. 4**), suggesting that L11 regulates c-myc mRNA levels by acting on c-myc 3'-UTR. We will continue to examine the questions listed in aim 2(2) through 2(5) by testing whether L11 downregulates c-myc mRNA in response to DNA damage is due to its role in recruiting miR-24 or other above-identified miRNAs (e.g miR-130a). As L11 does not bind to miR-145 and let-7 (4), we will not pursue this direction further.

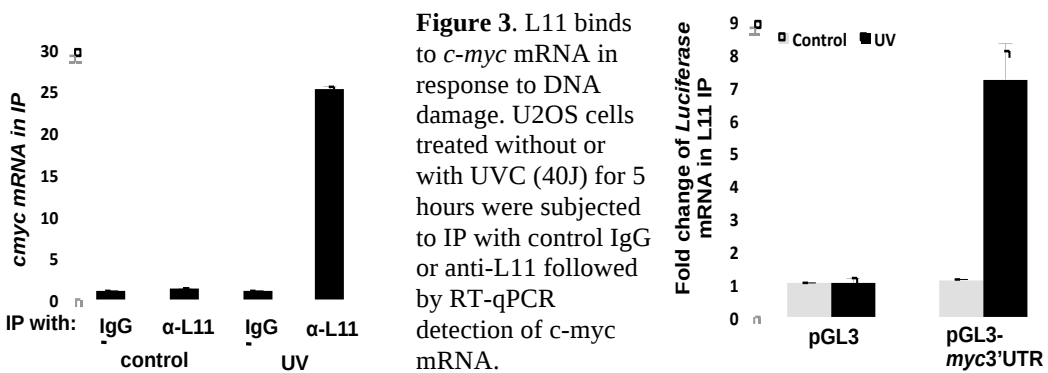


Figure 3. L11 binds to c-myc mRNA in response to DNA damage. U2OS cells treated without or with UVC (40J) for 5 hours were subjected to IP with control IgG or anti-L11 followed by RT-qPCR detection of c-myc mRNA.

Figure 4. L11 binds to c-myc 3'-UTR response to DNA damage. U2OS cells transfected with control pGL3 or pGL3-myc3'-UTR luciferase vector were treated without or with UVC (40J) for 5 hours. Cell lysates were then subjected to IP with control IgG or anti-L11 followed by RT-qPCR detection of c-myc mRNA. The data were normalized to the c-myc mRNA in IP with control IgG.

Aim3. To elucidate the mechanism underlying L11 regulation of c-myc mRNA in response to UV and γ -IR.

We will examine whether UV or γ -IR enhances the L11-miRISC formation and whether other RNA-binding proteins or other miRNAs could participate in the L11 regulation of c-myc mRNA stability in response to DNA damage as proposed.

C. KEY RESEARCH ACCOMPLISHMENTS:

- (1). L11 destabilizes c-myc mRNA via a miRNA-mediated pathway.
- (2). c-myc mRNA is reduced in response to DNA damage (UV or IR) and ribosomal stress.
- (3). DNA damage or ribosomal stress-induced c-myc mRNA down regulation requires L11.

D. Reportable Outcomes.

(1) Manuscript: This award supports the manuscript by Challagundla KB, Sun XX, Zhang X, DeVine T, Zhang Q, Sears R, **Dai MS.** (2011) Ribosomal protein L11 recruits miR-24/miRISC to repress c-Myc in response to ribosomal stress. *Mol Cell Biol*, 31(19): 4007-4021

(2) Employment/training. This award supports one postdoctoral in the lab for his employment and training.

E. CONCLUSIONS

L11 plays an important role in *c-myc* down-regulation in response to DNA damage, suggesting that microRNA-mediated *c-myc* mRNA decay is an important mechanism that coordinates ribosomal biogenesis and c-Myc activity during stress conditions.

F. REFERENCE.

1. **Dai MS**, Arnold H, Sun XX, Sears R, Lu H. (2007) Inhibition of c-Myc activity by ribosomal protein L11. *EMBO J*, 26: 3332-3345
2. **Dai MS**, Sun XX, Lu H. (2010) Ribosomal protein L11 associates with c-Myc at the 5S rRNA and tRNA genes and regulates their expression. *J Biol Chem*, 285(17): 12578-12594
3. **Dai MS**, Sears R, Lu H. (2007) Feedback inhibition of c-Myc by ribosomal protein L11. *Cell Cycle*, 6: 2735-2741
4. Challagundla KB, Sun XX, Zhang X, DeVine T, Zhang Q, Sears R, **Dai MS**. (2011) Ribosomal protein L11 recruits miR-24/miRSIC to repress c-Myc in response to ribosomal stress. *Mol Cell Biol*, 31(19): 4007-4021

G. APPENDICES

Please find the attached article by Challagundla KB, et al. *Mol Cell Biol*, 31(19): 4007-4021 (2011).

H. SUPPORTING DATA

N/A

Ribosomal Protein L11 Recruits miR-24/miRISC To Repress c-Myc Expression in Response to Ribosomal Stress[▽]

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Received 14 June 2011/Returned for modification 18 July 2011/Accepted 19 July 2011

c-Myc promotes cell growth by enhancing ribosomal biogenesis and translation. Deregulated expression of c-Myc and aberrant ribosomal biogenesis and translation contribute to tumorigenesis. Thus, a fine coordination between c-Myc and ribosomal biogenesis is vital for normal cell homeostasis. Here, we show that ribosomal protein L11 regulates c-myc mRNA turnover. L11 binds to c-myc mRNA at its 3' untranslated region (3'-UTR), the core component of microRNA-induced silencing complex (miRISC) argonaute 2 (Ago2), as well as miR-24, leading to c-myc mRNA reduction. Knockdown of L11 drastically increases the levels and stability of c-myc mRNA. Ablation of Ago2 abrogated the L11-mediated reduction of c-myc mRNA, whereas knockdown of L11 rescued miR-24-mediated c-myc mRNA decay. Interestingly, treatment of cells with the ribosomal stress-inducing agent actinomycin D or 5-fluorouracil significantly decreased the c-myc mRNA levels in an L11- and Ago2-dependent manner. Both treatments enhanced the association of L11 with Ago2, miR-24, and c-myc mRNA. We further show that ribosome-free L11 binds to c-myc mRNA in the cytoplasm and that this binding is enhanced by actinomycin D treatment. Together, our results identify a novel regulatory paradigm wherein L11 plays a critical role in controlling c-myc mRNA turnover via recruiting miRISC in response to ribosomal stress.

The c-myc proto-oncogene product c-Myc regulates expression of a large number of genes involved in the control of cell growth, proliferation, apoptosis, differentiation, angiogenesis, stem cell renewal, and metabolism, as well as ribosomal biogenesis and translation (1, 62). However, deregulated overexpression of c-Myc occurs in a wide range of human cancers (43). Transgenic animal studies have clearly shown the oncogenic potential of c-Myc (45). Thus, the levels and activity of c-Myc must be precisely regulated in normal cells.

The c-Myc activity seen in promoting cell proliferation and tumorigenesis is thoroughly integrated with its role in enhancing ribosomal biogenesis (13, 62). Ribosomal biogenesis is a multistep cellular process, which includes synthesis of rRNA and ribosomal proteins (RPs), rRNA processing, and the assembly of the mature ribosome subunits in the nucleolus followed by their transport into the cytoplasm (50). This process requires coordinated transcription catalyzed by all three RNA polymerases (RNA Pol I, II, and III). c-Myc enhances RNA Pol I-catalyzed synthesis of rRNA (3, 24, 25) and Pol III-catalyzed synthesis of 5S rRNA and tRNAs (23). c-Myc also promotes Pol II-catalyzed transcription of ribosomal proteins and translation initiation and elongation factors, as well as many nucleolar proteins required for rRNA processing and ribosome subunit assembly and transport (62). Collectively, c-Myc potentiates ribosomal biogenesis and translation activity.

Accumulating evidence has suggested that enhanced ribosomal biogenesis and translation contribute to cell transformation (13,

54). For example, overexpression of RNA Pol III-specific transcription factor Brf1 or RNA Pol III-transcribed tRNA^{Met} alone is sufficient to induce cell transformation and tumorigenicity in mice (40). Likewise, overexpression of translation initiation factors such as eukaryotic translation initiation factors 4E (eIF4E) (53), eIF4G (20), and eIF3s (66) promotes transformation in cells. Thus, c-Myc-enhanced ribosomal biogenesis and translation conceivably contribute to its oncogenic activity and should be tightly regulated during normal cell homeostasis.

We have previously demonstrated that RP L11 binds to c-Myc and inhibits the recruitment of the key coactivator TRRAP at c-Myc target gene promoters, leading to direct inhibition of c-Myc-driven gene transcription by all the three RNA Pols (12, 17). We have also found that L11 regulates c-Myc levels (12, 15). However, the underlying mechanism is not clear. Interestingly, L11, together with several other RPs, also plays a key role in transmitting ribosomal stress signals to p53-dependent cell cycle checkpoints via suppressing MDM2, a major p53 negative regulator (10, 14, 18, 28, 36, 44, 69, 70). Ribosomal stress, also called nucleolar stress because it is often accompanied by nucleolar disruption (52), is triggered by perturbation of any of the steps involved in ribosomal biogenesis (68), such as treatment of cells with a low dose of actinomycin D (Act D) (4, 14, 18), 5-fluorouracil (5-FU) (57), or mycophenolic acid (58), expression of the dominant-negative mutant of the Bop1 rRNA processing factor (56), serum starvation or contact inhibition (4), genetic disruption of the TIF-IA Pol I transcription initiation factor (65), or knockdown of certain ribosomal proteins (21, 59). In response to ribosomal stress, these RPs, including L11, are released from the nucleolus or from intact ribosomes to suppress MDM2 (68). However, whether L11 suppresses c-Myc in response to ribosomal stress is not known.

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[▽] Published ahead of print on 1 August 2011.

MicroRNAs (miRNAs) are evolutionarily conserved small noncoding RNAs that are ~22 nucleotides (nt) in length and have emerged as key posttranscriptional regulators of gene expression (33). The mature miRNA is incorporated into an RNA-induced silencing complex (RISC) called miRNA-induced silencing complex (miRISC) and pairs with the 3' untranslated region (3'-UTR) of target mRNAs, leading to their translational inhibition and/or mRNA degradation (33). In this study, we found that L11 regulates *c-myc* mRNA stability via a miRNA-mediated pathway. L11 binds to *c-myc* mRNA at its 3'-UTR, recruits a miR-24-loaded miRISC to the *c-myc* mRNA, and subsequently promotes *c-myc* mRNA degradation. Interestingly, ribosomal stress induced by treatment of cells with a low dose of Act D or 5-FU drastically reduced the levels of *c-myc* mRNA in an L11- and argonaute 2 (Ago2)-dependent manner. Both treatments enhance the association of L11 with Ago2, miR-24, and *c-myc* mRNA. These results reveal a novel regulatory paradigm wherein L11 plays a critical role in controlling *c-myc* mRNA stability in response to ribosomal stress.

MATERIALS AND METHODS

Cell culture, antibodies, plasmids, and reagents. Human lung non-small cell carcinoma H1299 cells, human osteosarcoma U2OS cells, and human embryonic kidney epithelial 293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 U of penicillin/ml, and 0.1 mg of streptomycin/ml at 37°C in a 5% CO₂ humidified atmosphere (18). Human diploid lung fibroblast WI38 cells were cultured in DMEM supplemented with 15% FBS and MEM nonessential amino acids (Gibco). To observe the c-Myc regulation by overexpression of L11, cells were transfected and cultured in DMEM containing 0.2% FBS. Anti-Flag (M2; Sigma), rabbit polyclonal anti-Ago2 (Millipore), mouse monoclonal anti-Ago2 (Abcam), mouse monoclonal anti-Myc (9E10; Zymed), and mouse polyclonal anti-Myc (Y69; Abcam) antibodies were purchased. Rabbit polyclonal anti-L11 (12) and anti-L23 (18) antibodies were previously described. Act D and 5-FU were purchased from Sigma. The Flag-tagged L11 (Flag-L11) plasmid was previously described (12). To construct a pGL3-*myc*3'UTR luciferase reporter, the *c-myc* 3'-UTR was amplified from U2OS cell mRNA by reverse transcriptase PCR (RT-PCR) with primers 5'-CGCTCTAGAGGAAAAGTAAGGAAAACGATTCCTTC-3' and 5'-CGCTCTAGATTGGCTCAATGATATATTTGCCAG-3', where the underlined sequences represent the XbaI site. The PCR product was then cloned into pGL3-promoter plasmid (Promega) at the XbaI site and sequenced. The luciferase reporters containing different fragments of *c-myc* 3'-UTR were cloned by inserting PCR products into the pGL3-promoter plasmid at the XbaI site.

Transfection, immunoblot, and coimmunoprecipitation analyses. Cells were transfected with plasmids by the use of TransIT-LT1 reagents following the manufacturer's protocol (Mirus Bio Corporation). Cells were harvested at 48 h posttransfection and lysed in lysis buffer consisting of 50 mM Tris-HCl (pH 8.0), 0.5% Nonidet P-40, 5 mM EDTA, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), 1 µg of pepstatin A/ml, and 1 mM leupeptin. Equal amounts of cell lysates were used for immunoblot (IB) analysis as described previously (16). Coimmunoprecipitation (co-IP) was conducted as described previously (14, 16). Sequential co-IP of L11 and c-Myc was conducted as described previously (12, 17). RNA was extracted from the immunoprecipitates and subjected to RT-quantitative PCR (RT-qPCR) assays as described below. For analysis of the RNA dependency of L11-Ago2 interaction (see Fig. 3), RNase A (200 µg/ml) was added to cell lysates followed by IP.

[³⁵S]methionine pulse-labeling. To examine c-Myc translation upon L11 knockdown, cells were prestarved in methionine-free medium supplemented with dialyzed 10% FBS for 30 min followed by pulse-labeling with 50 µCi of [³⁵S]methionine/ml for 15 min. Cells were lysed, and the cleared cell lysates were immunoprecipitated using anti-c-Myc (C33; Santa Cruz Biotechnology) monoclonal antibody-conjugated beads. After washing, half of the immunoprecipitate was separated using a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. The gel was incubated in Amplify solution (Amersham Biosciences) for 10 min, dried, and exposed to X-ray film. The other half was subjected to IB using anti-c-Myc (Y69) antibodies. Radiolabeled and total c-Myc were quantified, and the ratio of radiolabeled to total c-Myc represents the

relative translational activity of c-Myc. Equal amounts of total proteins were also loaded onto an SDS-PAGE gel to examine total ³⁵S-labeled cellular proteins.

Immunoprecipitation of protein-associated RNAs (RNA IP). Immunoprecipitation of RNA-protein complexes was performed as previously described, with minor modifications (37, 60). Briefly, cells were lysed in polysome lysis buffer (PLB) (100 mM KCl, 5 mM MgCl₂, 10 mM HEPES [pH 7.0], 0.5% Nonidet P-40, 1 mM DTT, 100 U of RNase inhibitor/ml) supplemented with 20 mM EDTA and protease inhibitors on ice for 20 min followed by centrifugation. The supernatants were precleared with protein A-Sepharose beads. The cleared supernatants were then diluted (1:10 [vol/vol]) in NT2 buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 1 mM MgCl₂, 0.05% Nonidet P-40, 1 mM DTT, 100 U of RNase inhibitor/ml) supplemented with 20 mM EDTA and protease inhibitors and incubated with primary antibodies at 4°C for 4 h, followed by incubation of protein A/G beads for an additional 2 h at 4°C. The beads were washed five times with NT2 buffer supplemented with protease inhibitors. The bead-bound protein-RNA complexes were then treated with DNase I and proteinase K and eluted twice with NT2 buffer containing 0.1% SDS. RNAs were extracted from the elution with phenol-chloroform and ethanol precipitation and subjected to RT-qPCR assays as described below.

Luciferase reporter assays. Cells were transfected with pCMV-β-galactoside (β-gal) and luciferase reporter plasmid pGL3, with pGL3-*myc*3'UTR or its fragments, together with control or Flag-L11 plasmid, or with scrambled or L11 small interfering RNA (siRNA), as indicated in the figures. Luciferase activity was determined and normalized by calculating β-gal activity in the same assay as described previously (12).

RT-qPCR analysis. Total RNA was isolated from cells by the use of TRIzol reagent (Invitrogen). Reverse transcriptions were performed as described previously (59). qPCR was performed using an ABI StepOne real-time PCR system (Applied Biosystems) and SYBR green mix (Bio-Rad) for mRNA expression determinations as described previously (59). Analysis of expression of mature miRNAs was performed using a TaqMan miRNA assay kit (Applied Biosystems) following the manufacturer's protocol. All reactions were carried out in triplicate. Relative gene expression levels were calculated using the ΔCt method following the manufacturer's instructions. The primers for *c-myc* and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) were previously described (12). The primers for other genes were 5'-CATCGTTGACCGCCTGAAGT-3' and 5'-GGAGCAAGATGGATTCCAATTC-3' (for luciferase); 5'-TCAACGCGCAGGACTTCTG-3' and 5'-CAGTGACCGTGGGAATGAAGT-3' (*c-fos*); 5'-GCAAAGATGGAAACGACCTTCT-3' and 5'-GCTCTCGGACGGGAGGA A-3' (*c-jun*); 5'-GCCTGCGACATCTGTGGAA-3' and 5'-CGCAAGTGGATC TTGGTATGC-3' (*egr1*); 5'-AGGCCTTGGAACTCAAGGAT-3' and 5'-TGA GTCAGGCCCTTCTGTCT-3' (*p53*); 5'-ATGAATCCCCCTCCATTCAT-3' and 5'-CAGGAAGCCAATTCTCAGAA-3' (*mdm2*); and 5'-GCCTTGAGGAAG GATTGTA-3' and 5'-TCGACAATCAGGGACATCAT-3' (*mdmx*).

RNA interference (RNAi) and miRNA overexpression. The 21-nt siRNA duplexes with a 3' dTdT overhang were synthesized by Dharmacon Inc. (Lafayette, CO). The target sequences for L11 and control scramble II RNA were previously described (12). L11 siRNA-1 was used in all the experiments except where indicated. The target sequence for Ago2 was 5'-GCACGGAAGUCCAUCUGAA-3'. The miR-24 mimics and control cel-miR-67 were purchased from Dharmacon Inc. These siRNA duplexes (100 nM) and miRNA mimics (25 to 50 nM) were introduced into cells by the use of SilentFect lipid reagent (Bio-Rad) following the manufacturer's protocol. The cells were analyzed 48 h after transfection.

Cell fractionations. To isolate the cytoplasmic and nuclear fractions, cells were resuspended in hypotonic buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM DTT) in the presence of protease inhibitors. Cell membranes were then broken using a Dounce homogenizer (tight pestle) and 10 up-and-down strokes. After centrifugation, the supernatant was collected as the cytoplasmic fraction and supplemented with a 0.1 volume of 10× buffer B (0.3 M Tris-HCl [pH 7.9], 1.4 M KCl, 30 mM MgCl₂). The nuclear pellets were washed with buffer A and then resuspended in buffer C (20 mM HEPES [pH 7.9], 420 mM NaCl, 0.2 mM EDTA, 1.5 mM MgCl₂, 0.5 mM DTT, 25% glycerol) in the presence of protease inhibitors and sonicated. The nuclear fraction (supernatant) was collected by centrifugation.

For isolation of the nucleolus fraction, the nuclear pellets were resuspended in buffer S1 containing 0.25 M sucrose and 10 mM MgCl₂, layered over buffer S2 containing 0.35 M sucrose and 0.5 mM MgCl₂, and centrifuged at 1,430 × g for 10 min at 4°C. The pelleted nuclei were resuspended in buffer S2 followed by sonication. The sonicated nuclei were then layered over buffer S3 containing 0.88 M sucrose and 0.5 mM MgCl₂ and centrifuged at 3,000 × g for 10 min at 4°C. The pellet contained purified nucleoli, and the supernatant represented the nucleoplasm (2, 4).

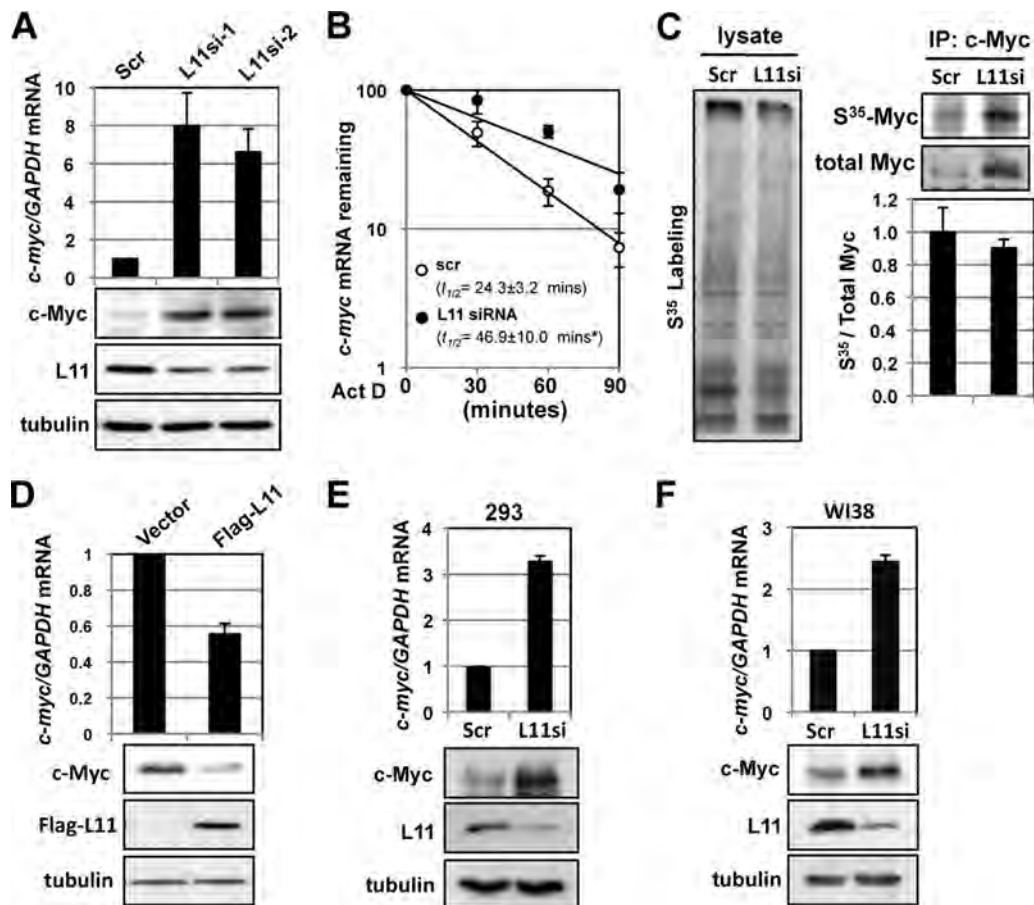


FIG. 1. L11 regulates *c-myc* mRNA levels and stability. (A) Knockdown of L11 increases the levels of *c-myc* mRNA and c-Myc protein. U2OS cells were transfected with scrambled (Scr) siRNA or one of two L11 siRNAs (L11si-1 or L11si-2). The cells were assayed for expression of *c-myc* mRNA by the use of RT-qPCR and of c-Myc protein by the use of IB assays. (B) Knockdown of L11 stabilizes *c-myc* mRNA. U2OS cells transfected with scrambled or L11 siRNA were treated with 2 μ M Act D. The cells were harvested at the indicated time points and assayed for relative levels of *c-myc* mRNA normalized to the expression of *GAPDH* mRNA by the use of RT-qPCR assays. The average *c-myc* mRNA half-life value is shown. *, $P < 0.01$ compared to $t_{1/2}$ of *c-myc* mRNA in scrambled-RNA transfected cells. (C) Knockdown of L11 does not affect *c-myc* mRNA translation. U2OS cells transfected with scrambled or L11 siRNA were pulse-labeled with [35 S]methionine. Equal amounts of cell lysates were immunoprecipitated with anti-c-Myc (C33) antibody-conjugated beads followed by autoradiography (top right panel) and IB with anti-c-Myc antibody (Y69) (middle right panel). The total lysates were also loaded on an SDS-PAGE gel followed by autoradiography (left panel). The relative translation efficiency of *c-myc* mRNA was calculated based on the ratio of radiolabeled to total immunoprecipitated c-Myc protein and is plotted in the bottom right panel. (D) Overexpression of L11 reduces the levels of *c-myc* mRNA and c-Myc protein. U2OS cells transfected with control or Flag-L11 vector were assayed for expression of *c-myc* mRNA by the use of RT-qPCR and of protein by the use of IB assays. (E and F) Knockdown of L11 increases the levels of *c-myc* mRNA and c-Myc protein in 293 and WI38 cells. 293 (E) and WI38 (F) cells were transfected with scrambled or L11 siRNA. The cells were assayed for expression of *c-myc* mRNA by the use of RT-qPCR and of c-Myc protein by the use of IB assays.

Ribosome fractionation. For separation of ribosome-free L11 from the total ribosome volume, cells were lysed in polysome lysis buffer. The extracts were overlaid on a 20% (wt/vol) sucrose cushion and centrifuged at $150,000 \times g$ for 2 h. The polysome-containing pellet and the nonribosomal supernatant were collected separately (42) and immunoblotted with anti-L11 antibody. The non-ribosomal supernatant was also used for RNA IP assays.

RESULTS

L11 regulates the levels and stability of *c-myc* mRNA. We previously showed that L11 directly suppresses c-Myc transactivation activity (12). Interestingly, L11 also regulates c-Myc levels. Knockdown of L11 drastically increased the levels of c-Myc protein (12) (bottom panels of Fig. 1A) and *c-myc* mRNA (15) in U2OS cells. RT-qPCR assays revealed that the levels of *c-myc* mRNA were markedly increased upon knock-

down of L11 (top panel of Fig. 1A). This effect was unlikely to have been an off-target effect, as similar results were observed when two different siRNAs against L11 were used. To examine how *c-myc* mRNA was induced by knockdown of L11, we determined the half-life ($t_{1/2}$) of *c-myc* mRNA. As shown in Fig. 1B, knockdown of L11 significantly prolonged the half-life of *c-myc* mRNA, suggesting that knockdown of L11 stabilizes *c-myc* mRNA. To examine whether knockdown of L11 affects c-Myc translation, we performed [35 S]methionine pulse-labeling assays. Consistent with a previous report (4), transient knockdown of L11 did not significantly affect global translation (left panel of Fig. 1C). It also appears that knockdown of L11 did not affect c-Myc translation, as levels of nascent synthesized c-Myc were increased proportionally to total cellular c-Myc levels (right panels of Fig. 1C). Therefore, we conclude

that transient knockdown of L11 increases *c-Myc* levels by stabilizing *c-myc* mRNA without affecting *c-Myc* translation. Consistently, overexpression of L11 reduced the levels of *c-myc* mRNA and protein (Fig. 1D) in U2OS cells. Similarly, knockdown of L11 also increased the levels of *c-myc* mRNA and protein in 293 cells (Fig. 1E), human normal fibroblast WI38 cells (Fig. 1F), and H1299 cells (data not shown), suggesting that the regulation of *c-myc* mRNA by L11 was not specific to cell type. Taken together, these results demonstrate that L11 regulates *c-myc* mRNA turnover.

L11 binds to *c-myc* at its 3'-UTR. To understand how L11 regulates *c-myc* mRNA stability, we examined whether L11 physically associates with *c-myc* mRNA by the use of RNA immunoprecipitation (IP) assays. Lysates from 293 or U2OS cells transfected with Flag-L11 were immunoprecipitated with control IgG or anti-Flag antibody, followed by detection of *c-myc* mRNA by the use of RT-qPCR assays. As shown in Fig. 2A, *c-myc* mRNA was specifically detected in anti-Flag but not control IgG immunoprecipitates from both cell lines, suggesting that L11 binds to *c-myc* mRNA in cells.

c-myc mRNA turnover is fast and highly regulated in cells, with a normal half-life of about 15 to 30 min (19) (Fig. 1B). The *c-myc* 3'-UTR contains several AU-rich elements (AREs) and plays an important role in the regulation of *c-myc* mRNA turnover (30). Several ARE binding proteins, including AUF1 (67), HuR (32), and tristetraprolin (TTP) (39), have been found to bind to *c-myc* AREs and regulate *c-myc* mRNA stability. To test whether L11 binds to *c-myc* mRNA through its 3'-UTR, we generated a luciferase reporter that contained a *c-myc* 3'-UTR in the 3' portion of the *luciferase* gene (pGL3-*myc3'UTR*; Fig. 2E). U2OS cells were transfected with Flag-L11 together with pGL3-*myc3'UTR* or control pGL3 vector. The cell lysates were immunoprecipitated with anti-Flag antibody or control IgG followed by RT-qPCR detection of the *luciferase* gene. As shown in Fig. 2B, *luciferase* mRNA was specifically coimmunoprecipitated with Flag-L11 in cells transfected with *c-myc* 3'-UTR-containing but not control luciferase plasmid. Similar results were also observed in 293 cells (Fig. 2F; see also Fig. 8). These results demonstrate that L11 binds to the *c-myc* 3'-UTR in cells.

L11 regulates *c-myc* mRNA levels through the *c-myc* 3'-UTR. Next, we examined whether L11 regulates *c-myc* mRNA levels through the *c-myc* 3'-UTR. 293 cells were transfected with pGL3 or pGL3-*myc3'UTR* together with control or Flag-L11 plasmid. The cells were assayed for relative luciferase activity levels as well as expression of *luciferase* mRNA by RT-qPCR. As shown in Fig. 2C, overexpression of L11 specifically suppressed the luciferase activity (top panel) and reduced *luciferase* mRNA levels (middle panel) in pGL3-*myc3'UTR* but not control pGL3 transfected cells. Similar results were also observed in H1299 cells (data not shown). Conversely, knockdown of endogenous L11 significantly increased the luciferase activity and the *luciferase* mRNA levels in pGL3-*myc3'UTR* (compare lane 4 to lane 3) but not control pGL3 (compare lane 2 to lane 1) transfected U2OS cells (Fig. 2D). Together, these results suggest that L11 regulates *c-myc* mRNA levels by acting on the *c-myc* 3'-UTR.

To demonstrate the specificity of L11 regulation of *c-myc* mRNA through the *c-myc* 3'-UTR, we constructed a panel of luciferase reporter plasmids containing overlapping fragments

of the *c-myc* 3'-UTR (Fig. 2E) and mapped the L11-binding site at the *c-myc* 3'-UTR. 293 cells were transfected with Flag-L11 together with control pGL3 and pGL3-*myc3'UTR* or its deletion fragments followed by RNA IP using control IgG or anti-Flag antibodies. As shown in Fig. 2F, Flag-L11 was associated specifically with the luciferase mRNA containing the full-length (FL) *c-myc* 3'-UTR and the fragment consisting of nt 309 to 470 (F4) but not other fragments encompassing nt 1 to 360. Furthermore, overexpression of L11 significantly suppressed the luciferase activity in cells transfected with pGL3-*myc3'UTR*-F4 but not those transfected with other *c-myc* 3'-UTR fragments containing luciferase reporter plasmids (F1, F2, and F3) (Fig. 2G). These results suggest that L11 regulates *c-myc* mRNA through nt 361 to 470 of the *c-myc* 3'-UTR.

L11-mediated *c-myc* mRNA reduction requires Ago2. miRNAs have emerged as key regulators of gene expression by suppressing translation and/or inducing mRNA decay of target genes through the 3'-UTR (47, 61). Therefore, we asked whether L11 regulates *c-myc* mRNA turnover through a miRNA-mediated pathway. As Ago2 is a core component of miRISC (47, 61) and, consistent with a previous report (32), knockdown of endogenous Ago2 significantly increased the levels of *c-Myc* protein and mRNA (Fig. 3A), we tested whether Ago2 is involved in L11-mediated *c-myc* mRNA reduction. We first asked whether L11 could facilitate the recruitment of Ago2 to the *c-myc* mRNA. To this end, we performed RNA IP followed by RT-qPCR assays and found that overexpression of L11 increased the binding of Ago2 to the *c-myc* mRNA (Fig. 3B), whereas knockdown of L11 significantly reduced the Ago2 binding to the *c-myc* mRNA (Fig. 3C), suggesting that L11 facilitates the recruitment of Ago2 to the *c-myc* mRNA. Furthermore, knockdown of Ago2 extensively rescued the L11-mediated reduction of *c-Myc* protein (compare the ratio of lane 4 to lane 3 with the ratio of lane 2 to lane 1 in Fig. 3D) and *c-myc* mRNA (Fig. 3E) in U2OS cells. Together, these results suggest that L11 decreases *c-myc* mRNA levels by recruiting Ago2 to the *c-myc* mRNA.

L11 associates with Ago2. Given that L11 regulates Ago2 targeting of *c-myc* mRNA (Fig. 3B and C), we tested whether L11 physically associates with Ago2. 293 cells were transfected with control or Flag-L11 followed by co-IP assays using anti-Flag antibodies. As shown in Fig. 3F, endogenous Ago2 was specifically coimmunoprecipitated with Flag-L11 in 293 cells. Similar results were also observed in U2OS cells (Fig. 3G) and H1299 cells (data not shown). Also, endogenous Ago2 and endogenous L11 were specifically coimmunoprecipitated with each other using either anti-Ago2 or anti-L11 antibodies, but not control IgG, in 293 cells (Fig. 3H) and U2OS cells (Fig. 3I and data not shown). RNase treatment abolished the interaction between L11 and Ago2 (Fig. 3I). These results suggest that the interaction between L11 and Ago2 in cells is dependent on the presence of cellular RNA.

L11 recruits miR-24 to target *c-myc* mRNA. We then asked whether L11 regulates *c-myc* mRNA through the activity of miRNAs. At least four miRNAs, including Let-7 (32), miR-145 (55), miR-24 (34), and miR-34c (7), have been previously shown to downregulate *c-Myc* by either repressing *c-Myc* translation (let-7 and miR-34c) or reducing *c-myc* mRNA levels (miR-24 and miR-145). Thus, we examined whether L11 associates with these miRNAs in cells. Lysates from 293 or

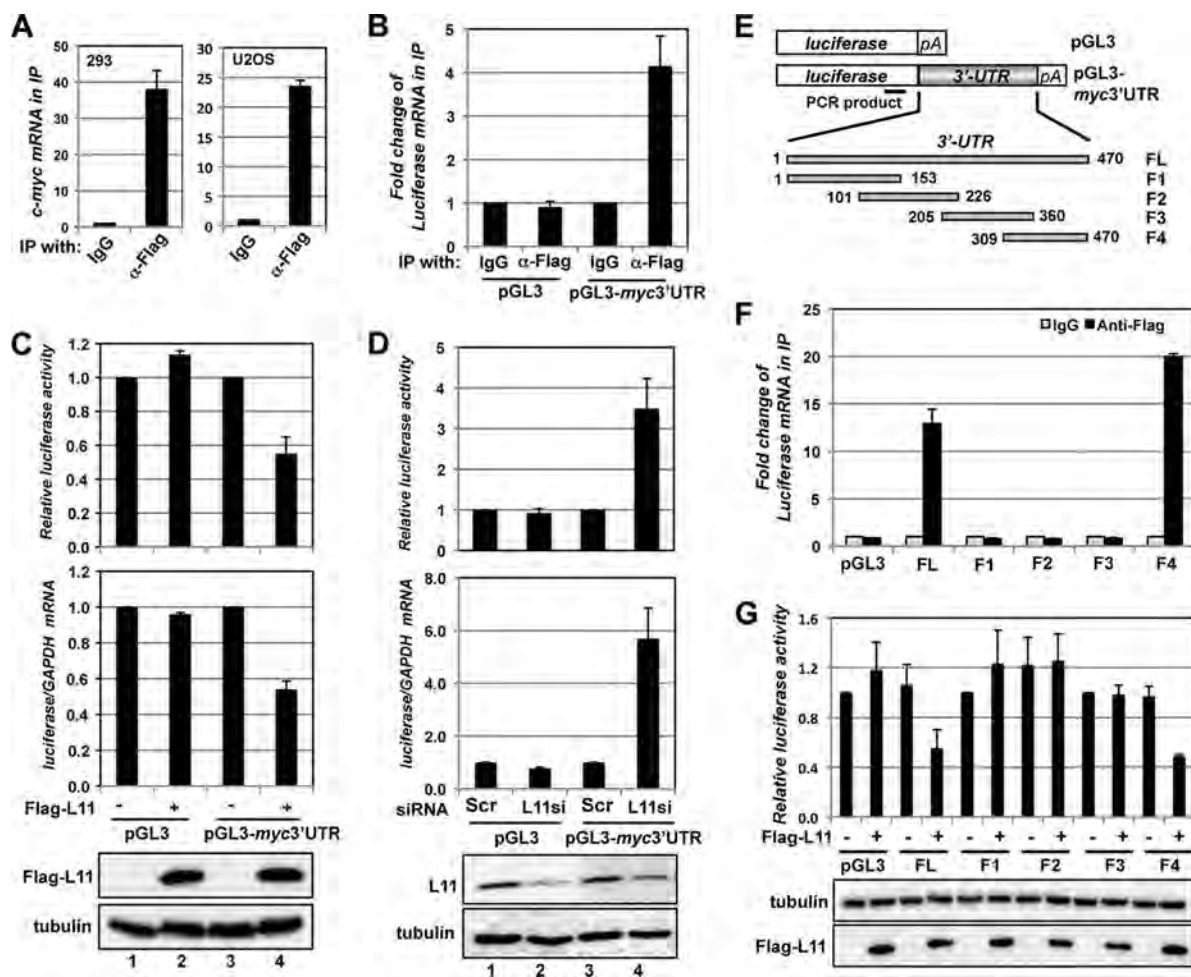


FIG. 2. L11 regulates *c-myc* by targeting the 3'-UTR of *c-myc* mRNA. (A) L11 binds to *c-myc* mRNA. Lysates from 293 (left panel) and U2OS (right panel) cells transfected with Flag-L11 were immunoprecipitated with control mouse IgG or anti-Flag antibody. RNA extracted from the immunoprecipitates was retrotranscribed and assayed for expression of *c-myc* mRNA by the use of RT-qPCR assays. (B) L11 binds to the *c-myc* 3'-UTR. U2OS cells transfected with Flag-L11 together with control pGL3 or pGL3-myc3'UTR vector were immunoprecipitated with anti-Flag or mouse IgG. The immunoprecipitates were assayed for expression of the *luciferase* mRNA by RT-qPCR assays. (C) L11 reduction of *luciferase* mRNA levels and suppression of luciferase activity are dependent on the *c-myc* 3'-UTR. 293 cells transfected with the indicated plasmids together with β -gal plasmid were assayed for relative luciferase activity levels normalized to β -gal expression (top panel) and relative *luciferase* mRNA levels normalized to *GAPDH* mRNA by the use of RT-qPCR assays (middle panel). The protein expression results are shown in the bottom panels. (D) Knockdown of L11 increases the levels of *luciferase* mRNA and luciferase activity in a manner dependent on the *c-myc* 3'-UTR. U2OS cells transfected with β -gal and pGL3 or pGL3-myc3'UTR plasmids and siRNAs were assayed for relative luciferase activity levels normalized to β -gal expression (top panel) and relative *luciferase* mRNA levels normalized to *GAPDH* mRNA by the use of RT-qPCR assays (middle panel). The protein expression results are shown in the bottom panels. (E) Diagram of the control pGL3, pGL3-myc3'UTR, and pGL3-myc3'UTR fragment (F1, F2, F3, and F4) vectors. The relative positions of the full-length (FL) *c-myc* 3'-UTR and its fragments (F1 through F4) are indicated, with the first nucleotide after the stop codon labeled "1." The position of the PCR product used to examine expression of the *luciferase* mRNA is indicated in the coding region of the *luciferase* gene. pA indicates a poly(A) tail. (F) L11 binds to the 3' end of the *c-myc* 3'-UTR. 293 cells transfected with Flag-L11 together with control pGL3 or pGL3-myc3'UTR or its fragments were immunoprecipitated with anti-Flag or mouse IgG. The immunoprecipitates were assayed for expression of *luciferase* mRNA by RT-qPCR assays. (G) L11 suppression of luciferase activity is dependent on the 3' end of the *c-myc* 3'-UTR. 293 cells transfected with the indicated plasmids together with a β -gal plasmid were assayed for relative luciferase activity levels normalized to β -gal expression. The protein expression results are shown in the bottom panels.

U2OS cells transfected with Flag-L11 were immunoprecipitated with anti-Flag antibodies or control IgG followed by RT-qPCR detection of the miRNAs described above. U6 snRNA was used as a control. As shown in Fig. 4A, miR-24, but not let-7b, miR-34c, and U6 snRNA, was significantly enriched in the Flag-L11 immunoprecipitates compared to control IgG in 293 cells. Similar results were also observed in U2OS cells except that miR-34c was undetectable in the immunoprecipitates (Fig. 4B). We were unable to detect miR-145

in the immunoprecipitates from both 293 and U2OS cells (data not shown). These results clearly indicate that L11 specifically associates with miR-24 but not with other known *c-myc* targeting miRNAs.

Next, we examined the role of *miR-24* in L11-mediated *c-myc* mRNA decay. Consistent with a previous report (34), overexpression of *miR-24* significantly reduced the levels of c-Myc protein and mRNA in a dose-dependent manner (Fig. 4C). We then transfected U2OS cells with different combina-

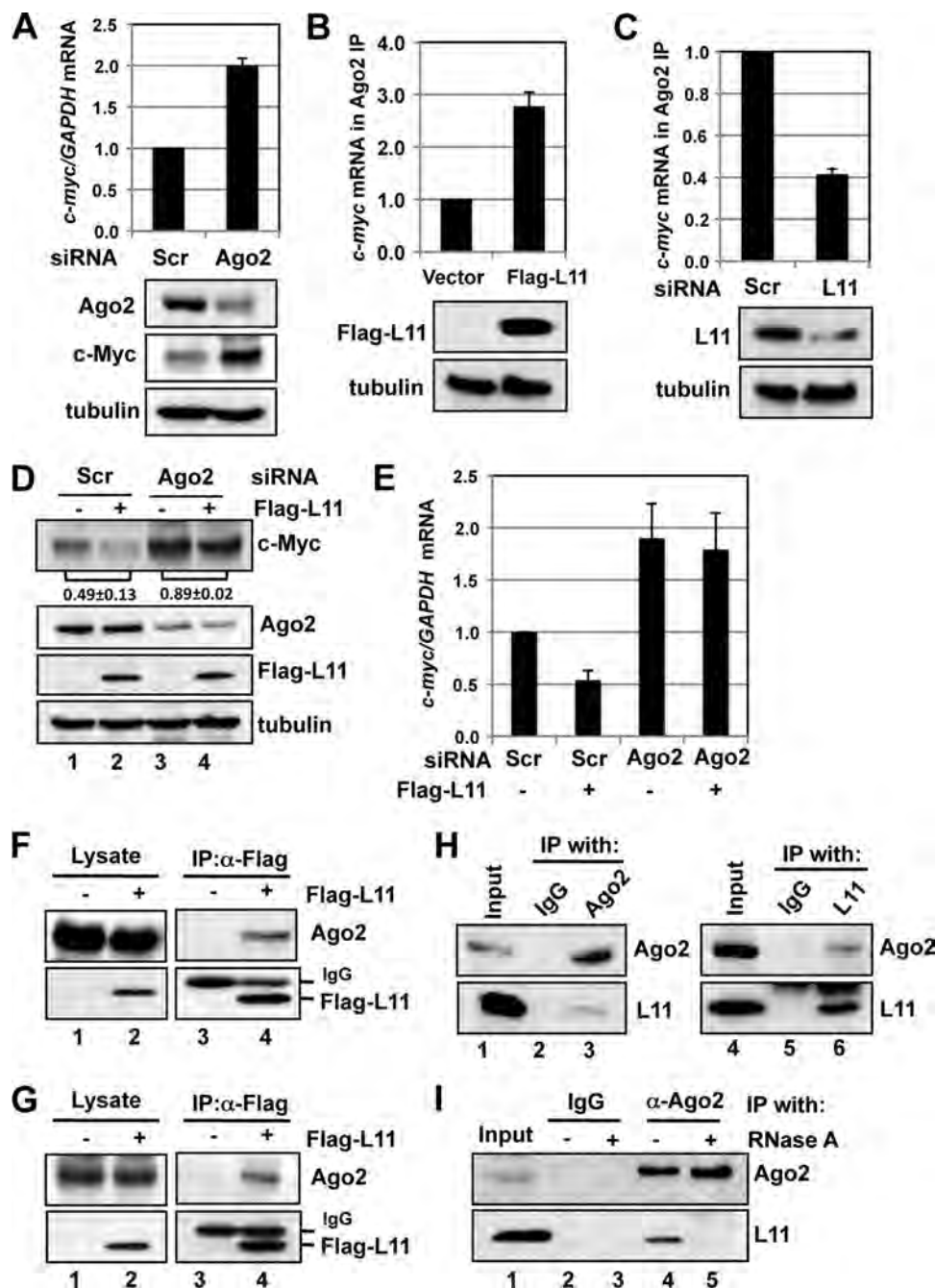


FIG. 3. Mutual dependency of L11 and Ago2 in regulating *c-myc* mRNA levels. U2OS cells transfected with scrambled or Ago2 siRNA were assayed for expression of c-Myc protein (bottom panels) and mRNA (top panel). (B) Overexpression of L11 increases association of Ago2 with *c-myc* mRNA. U2OS cells were transfected with control or Flag-L11 plasmid. The cell lysates were immunoprecipitated with anti-Ago2 antibodies followed by an RT-qPCR assay to determine the levels of *c-myc* mRNA. The protein expression results are shown in the bottom panels. (C) Knockdown of L11 reduces the association of Ago2 with *c-myc* mRNA. Lysates from U2OS cells transfected with scrambled or L11 siRNA were immunoprecipitated with anti-Ago2 antibodies followed by an RT-qPCR assay to determine the levels of *c-myc* mRNA. The protein expression results are shown in the bottom panels. (D and E) L11 suppression of *c-myc* mRNA requires Ago2. U2OS cells transfected with control or Flag-L11 plasmid together with scrambled or Ago2 siRNA were assayed for c-Myc protein expression by immunoblotting (IB) (D) and for mRNA expression by RT-qPCR assays (E). The c-Myc bands were quantified and normalized to tubulin. The ratios of lane 2 to lane 1 and of lane 4 to lane 3 for the results from three independent experiments are indicated in panel D. (F and G) Ectopically expressed L11 interacts with Ago2. 293 (F) or U2OS (G) cells transfected with control or Flag-L11 plasmid were subjected to IP with anti-Flag antibody followed by IB using anti-Ago2 antibodies. (H) Endogenous L11 interacts with endogenous Ago2. 293 cell lysates were immunoprecipitated with control mouse IgG or anti-Ago2 (left panels) or rabbit IgG or anti-L11 (right panels) antibody followed by IB detection performed using anti-L11 or anti-Ago2 antibodies. (I) Interaction of L11 with Ago2 requires RNA. U2OS cell lysates were immunoprecipitated with control mouse IgG or anti-Ago2 antibodies in the absence (lanes 2 and 4) or presence (lanes 3 and 5) of RNase followed by IB detection performed using anti-L11 or anti-Ago2 antibodies.

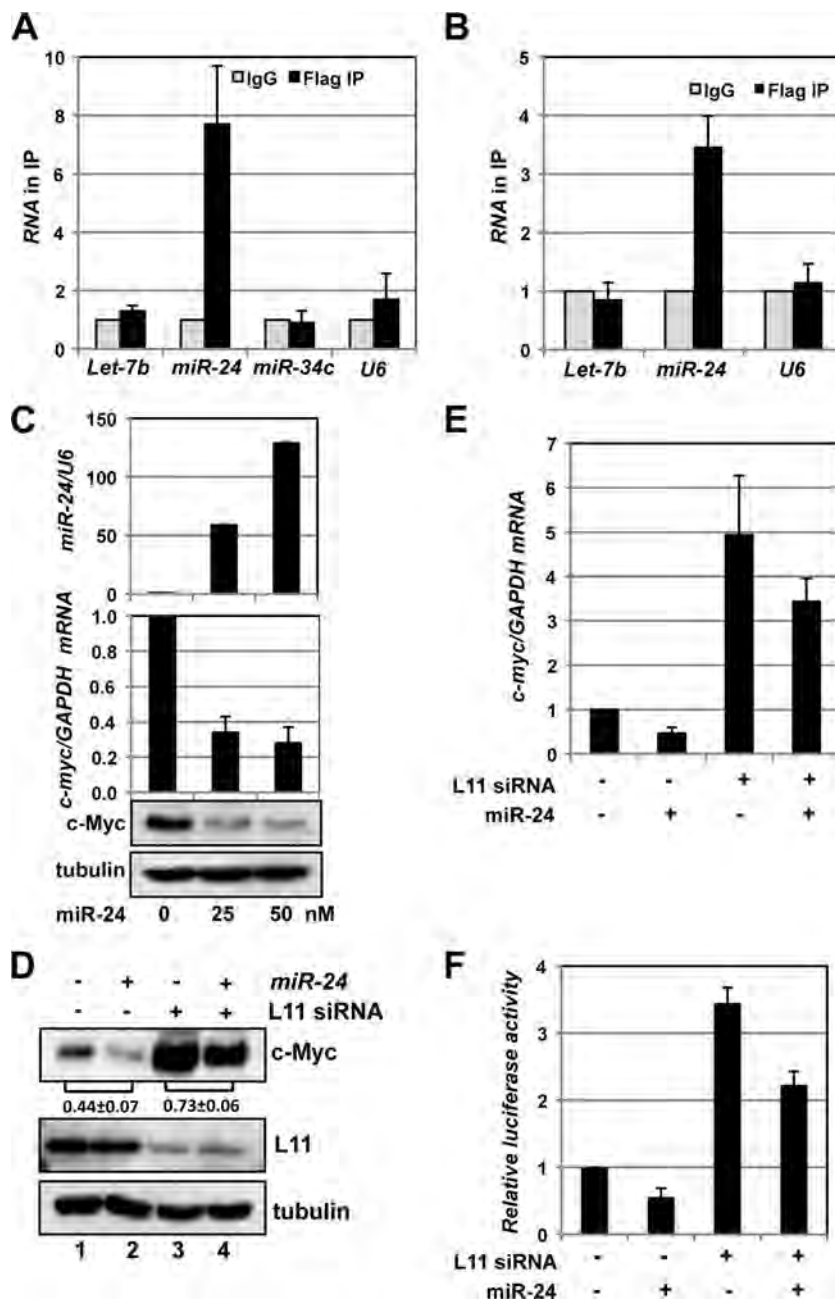


FIG. 4. L11 recruits miR-24 to target *c-myc* mRNA. (A and B) L11 binds to miR-24 in cells. Lysates from 293 (A) or U2OS (B) cells transfected with Flag-L11 plasmid were immunoprecipitated with anti-Flag antibody or control IgG followed by detection of the indicated miRNAs by the use of RT-qPCR assays. (C) Overexpression of miR-24 decreases c-Myc levels. U2OS cells transfected with a control or with different doses of miR-24 mimics were assayed for the relative levels of expression of miR-24 normalized with U6 snRNA (top panel) and of *c-myc* mRNA normalized with *GAPDH* mRNA (middle panel) by the use of RT-qPCR assays as well as c-Myc protein (bottom panel) by the use of IB. (D and E) Knockdown of L11 attenuates *c-myc* downregulation by miR-24. U2OS cells transfected with scrambled or L11 siRNA together with control or miR-24 mimics were assayed for c-Myc protein levels by IB (D) and *c-myc* mRNA levels by the use of RT-qPCR (E). The c-Myc bands were quantified and normalized to tubulin. The ratios of lane 2 to lane 1 and of lane 4 to lane 3 for the results from three independent experiments are indicated in panel D. (F) Knockdown of L11 attenuates the miR-24-mediated suppression of luciferase activity. U2OS cells were transfected with pGL3-myc3'UTR and β -gal expression plasmids together with siRNA and/or miRNAs as indicated. The cells were assayed for relative luciferase activity levels normalized to β -gal expression.

tions of scrambled or L11 siRNA and control (cel-miR-67) or miR-24 mimics. The cells were analyzed for expression of c-Myc protein and mRNA. Interestingly, knockdown of L11 drastically rescued the miR-24-mediated reduction of c-Myc protein levels (compare the ratio of lane 4 to lane 3 with the

ratio of lane 2 to lane 1; Fig. 4D) and *c-myc* mRNA levels (Fig. 4E). Similarly, knockdown of L11 significantly abolished the miR-24-mediated reduction of relative luciferase activity in cells transfected with pGL3-myc3'UTR reporter plasmid (Fig. 4F). These results suggest that L11 plays a critical role in

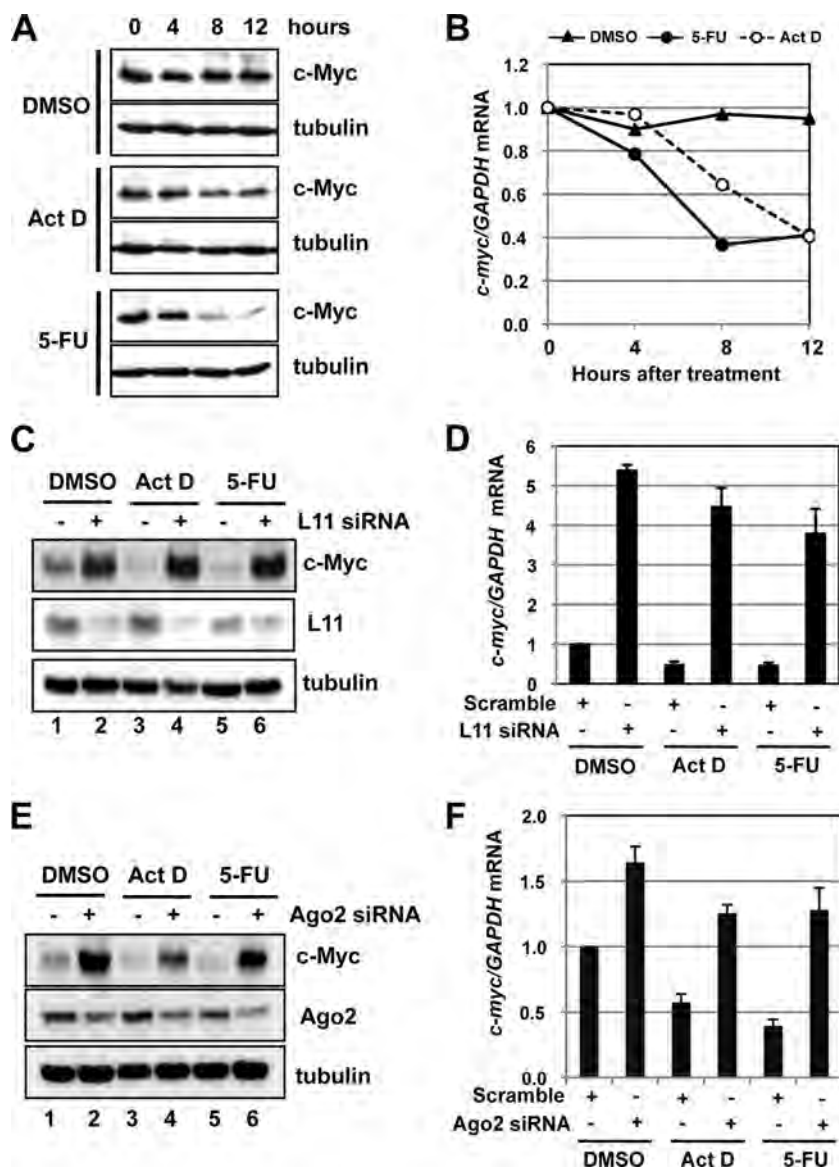


FIG. 5. L11 downregulates *c-myc* mRNA in response to ribosomal stress. (A and B) c-Myc is downregulated by treatment with Act D or 5-FU. U2OS cells were treated with DMSO, Act D (5 nM), or 5-FU (10 μg/ml) for the indicated hours. The cells were assayed for c-Myc protein expression by IB (A) and *c-myc* mRNA expression by RT-qPCR (B) assays. (C and D) Knockdown of L11 rescues the downregulation of c-Myc by treatment with Act D or 5-FU. U2OS cells transfected with scrambled or L11 siRNA were treated with DMSO, Act D (5 nM), or 5-FU (10 μg/ml) for 12 h. The cells were assayed for c-Myc protein expression by IB (C) and *c-myc* mRNA expression by RT-qPCR (D) assays. (E and F) Knockdown of Ago2 rescues the downregulation of c-Myc by treatment with Act D or 5-FU. U2OS cells transfected with scrambled or Ago2 siRNA were treated with DMSO, Act D (5 nM), or 5-FU (10 μg/ml) for 12 h. The cells were assayed for c-Myc protein expression by IB (E) and *c-myc* mRNA expression by RT-qPCR (F) assays.

miR-24-mediated downregulation of *c-myc* mRNA, supporting the notion that L11 regulates *c-myc* mRNA by recruiting miR-24-loaded miRISC to the *c-myc* 3'-UTR.

***c-myc* mRNA is reduced in response to ribosomal stress.** To examine the physiological relevance of the L11 regulation of *c-myc* mRNA turnover, we asked whether L11 is involved in *c-myc* regulation in cells in response to ribosomal stress, as L11 plays a key role in p53-mediated cell cycle control in response to such stress (4, 36, 57–59, 69). Ribosomal stress is triggered following perturbation of ribosomal biogenesis, including inhibition of rRNA synthesis by treatment with a low dose of Act

D (5 nM) (4, 14, 18) and inhibition of rRNA processing by treatment with 5-FU (57). c-Myc has recently been shown to be downregulated in response to DNA damage (7, 48). To test whether c-Myc levels are also downregulated by ribosomal stress, we treated U2OS cells with a low dose of either Act D or 5-FU followed by the detection of c-Myc expression. As shown in Fig. 5A, treatment of cells with either Act D or 5-FU significantly reduced the c-Myc protein levels in a time-dependent manner. This reduction correlates with the reduction of *c-myc* mRNA levels (Fig. 5B), suggesting that ribosomal stress induces *c-myc* mRNA degradation.

L11 and Ago2 are required for *c-myc* mRNA downregulation in response to ribosomal stress. Next, we asked whether L11 is involved in *c-Myc* downregulation in response to ribosomal stress. U2OS cells were transfected with scrambled or L11 siRNA followed by treatment with control dimethyl sulfoxide (DMSO), Act D, or 5-FU. As shown in Fig. 5C, knockdown of L11 completely abolished the *c-Myc* protein reduction caused by the Act D or 5-FU treatment. Again, this occurred at the mRNA level, as knockdown of L11 rescued the reduction of *c-myc* mRNA levels caused by Act D or 5-FU treatment (Fig. 5D). To evaluate the involvement of miRNA-mediated silencing in the downregulation of *c-myc* mRNA by ribosomal stress, we performed Ago2 knockdown experiments. Indeed, knockdown of Ago2 significantly rescued the downregulation of *c-Myc* protein (Fig. 5E) and mRNA (Fig. 5F) levels caused by treatment with Act D or 5-FU. Thus, L11 and Ago2 are required for *c-myc* mRNA downregulation by ribosomal stress.

L11 recruits miR-24-loaded miRISC to *c-myc* mRNA in response to ribosomal stress. As L11 has been previously shown to dissociate from intact ribosomes into the ribosome-free pool (free L11) in response to ribosomal stress (4, 57), we reasoned that free L11 might be able to associate with *c-myc* mRNA. Thus, U2OS cells treated with Act D, 5-FU, or DMSO were subjected to RNA IP using anti-L11 antibodies, followed by RT-qPCR assays. As shown in Fig. 6A, L11 binding to *c-myc* mRNA significantly increased in cells treated with Act D or 5-FU compared to the control results, indicating that ribosomal stress enhances L11 targeting of *c-myc* mRNA.

Next, we examined whether L11 recruits miRISC to *c-myc* mRNA in response to ribosomal stress. The cell lysates from U2OS cells treated with DMSO, Act D, or 5-FU were immunoprecipitated with anti-Ago2 antibodies or control IgG. As shown in Fig. 6B, treatment of cells with Act D or 5-FU markedly increased the association of Ago2 with *c-myc* mRNA. This increase requires L11, as knockdown of L11 significantly reduced the binding of Ago2 with *c-myc* mRNA in cells treated with Act D or 5-FU (Fig. 6C). To confirm that L11 interacts with Ago2 under conditions of ribosomal stress, we performed co-IP assays using anti-Ago2 antibodies. As shown in Fig. 6D, treatment of cells with Act D or 5-FU significantly increased the binding of L11 to Ago2. These data demonstrate that L11 recruits Ago2 to *c-myc* mRNA in response to ribosomal stress. Lastly, we examined whether L11 recruits miR-24 in response to ribosomal stress. As shown in Fig. 6E, treatment of cells with either Act D or 5-FU drastically increased the binding of L11 to miR-24 but not U6 snRNA. In contrast, the total level of miR-24 was not changed following both treatments (Fig. 6F). Together, these data strongly suggest that L11 mediates *c-myc* mRNA decay by recruiting miR-24-loaded miRISC to *c-myc* mRNA in response to ribosomal stress.

L11 associates with *c-myc* mRNA in the cytoplasm. To test where L11 associates with *c-myc* mRNA in cells, we performed cell fractionation assays. First, 293 cells transfected with pGL3-*myc*3'UTR together with control Flag or Flag-L11 vector were then fractionated into cytoplasmic and nuclear fractions (right panel of Fig. 7A). RNA IP was conducted using the cytoplasmic and nuclear lysates with control IgG or anti-Flag antibodies followed by detection of *luciferase* and *c-myc* mRNAs. As shown in Fig. 7A, L11 specifically associates with exogenous *luciferase* mRNA (left panel) and endogenous *c-myc* mRNA

(middle panel) in the cytoplasm but not in the nucleus. Next, U2OS cells treated with DMSO or Act D were fractionated into cytoplasmic and nuclear lysates (right panel of Fig. 7B) followed by RNA IP using anti-L11 or control IgG. Again, Act D treatment significantly increased the association of endogenous L11 with *c-myc* mRNA in the cytoplasm (left panel, Fig. 7B). Thus, these results indicate that L11 associates with *c-myc* mRNA in the cytoplasm and that this association is markedly increased following ribosomal stress.

Ribosome-free L11 associates with *c-myc* mRNA. It has been proposed that L11 is released from the nucleolus and intact ribosomes to suppress MDM2 in the nucleoplasm, leading to p53 activation, in response to ribosomal stress (68). To further observe the L11 localization following ribosomal stress, we fractionated U2OS cells treated with control DMSO or Act D into cytoplasmic, nuclear, and nucleolar fractions. As shown in Fig. 7C, the levels of L11 in the nucleolus were reduced whereas its levels in both the nucleus and the cytoplasm increased in response to Act D treatment. To test whether it is the ribosome-free L11 that interacts with *c-myc* mRNA in the cytoplasm, we performed ribosome fractionation assays using sucrose centrifugation to isolate free RPs (nonribosomal supernatant) from ribosomes and polysomes (42). Nonribosomal supernatant isolated from 293 cells transfected with control or Flag-L11 plasmid was immunoprecipitated with control IgG or anti-Flag antibodies, followed by detection of *c-myc* mRNA. As shown in Fig. 7D, ribosome-free Flag-L11 specifically associated with *c-myc* mRNA in cells. Similar experiments were also conducted in U2OS cells treated with DMSO or Act D. As shown in Fig. 7E, Act D treatment significantly increased the association of free endogenous L11 with *c-myc* mRNA in cells (left panel). Of note, the level of free L11 was increased whereas that of L11 in the polysome pellet was decreased by Act D treatment (right panels of Fig. 7E). Taken together, these results suggest that ribosome-free L11 interacts with *c-myc* mRNA in cells.

Our RNA IP assays were performed in the presence of EDTA, which disrupts the 80S ribosomes and polysomes (51), suggesting that L11 binding to *c-myc* mRNA is independent of the translating *c-myc* mRNA. This notion was further supported by the results showing that L11 associated with the *c-myc* mRNA regardless of the presence or absence of 20 mM EDTA (Fig. 7F). To further confirm this notion while also testing whether L11 associates with *c-myc* mRNA through binding to translating *c-Myc* protein in a ribosome-*c-myc* mRNA complex, we conducted sequential co-IP followed by RT-qPCR assays. 293 cells transfected with V5-*c-Myc* in the presence or absence of Flag-L11 were subjected to a first co-IP with anti-Flag antibodies. As shown in Fig. 7G and consistent with our previous reports (12, 17), *c-Myc* was coimmunoprecipitated with Flag-L11 (lane 4). The Flag-L11-associated protein complex was eluted with Flag peptides. The elution was then subjected to a second co-IP using anti-V5 antibodies. The anti-V5 immunoprecipitates (lane 6, Fig. 7G) specifically represent the *c-Myc*-associated L11. RNA was extracted from the elution from the first IP with anti-Flag antibody or control IgG as well as the immunoprecipitates from the second IP with anti-V5 antibodies or control IgG, followed by detection of *c-myc* mRNA by the use of RT-qPCR. As shown in Fig. 7H, *c-myc* mRNA was detected only in the immunoprecipitates from the

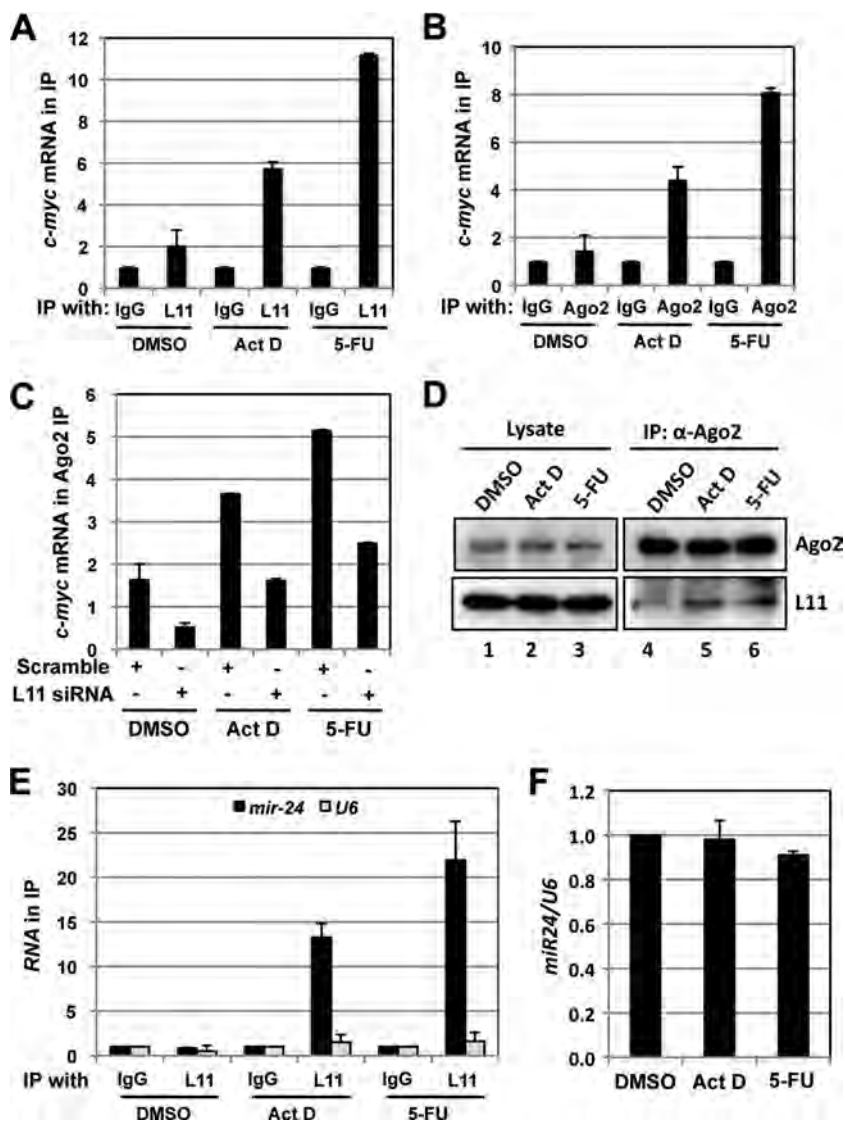


FIG. 6. L11 recruits miRISC to *c-myc* mRNA in response to ribosomal stress. (A) Treatment with Act D or 5-FU increases the binding of L11 to *c-myc* mRNA. U2OS cells were treated with DMSO, Act D (5 nM), or 5-FU (10 μ g/ml) for 12 h. The cell lysates were immunoprecipitated with control IgG or anti-L11 antibodies followed by RT-qPCR detection of *c-myc* mRNA. (B) Treatment with Act D or 5-FU increases the binding of Ago2 to *c-myc* mRNA. U2OS cells were treated with DMSO, Act D (5 nM), or 5-FU (10 μ g/ml) for 12 h. The cell lysates were immunoprecipitated with anti-Ago2 antibodies or control IgG followed by RT-qPCR detection of *c-myc* mRNA. (C) Knockdown of L11 abolishes Ago2 binding to *c-myc* mRNA in response to ribosomal stress. U2OS cells transfected with scrambled or L11 siRNA were treated with DMSO, Act D (5 nM), or 5-FU (10 μ g/ml) for 12 h. The cell lysates were immunoprecipitated with anti-Ago2 antibodies or control IgG followed by RT-qPCR detection of *c-myc* mRNA. The data were normalized to the *c-myc* mRNA in IP with control IgG. (D) Treatment with Act D or 5-FU increases the binding of L11 to Ago2. U2OS cells were treated with DMSO, Act D (5 nM), or 5-FU (10 μ g/ml) for 12 h. The cell lysates were immunoprecipitated with anti-Ago2 antibodies followed by IB using anti-L11 antibodies. (E) Treatment with Act D or 5-FU increases the association of L11 with miR-24. U2OS cells were treated with DMSO, Act D (5 nM), or 5-FU (10 μ g/ml) for 12 h and subjected to IP with anti-L11 or control IgG, followed by RT-qPCR detection of *miR-24* and U6 snRNA. (F) Treatment with Act D or 5-FU does not change the total levels of miR-24 in cells. U2OS cells treated with DMSO, Act D (5 nM), or 5-FU (10 μ g/ml) for 12 h were examined for expression of miR-24 normalized to that of U6 snRNA by the use of RT-qPCR assays.

first anti-Flag and not in those from the second anti-V5 IP. These results clearly demonstrate that L11 association with *c-myc* mRNA is independent of its interaction with c-Myc protein (including nascent synthesized c-Myc protein), further supporting the notion that L11 association with *c-myc* mRNA was not due to ribosome translation of *c-myc* mRNA. Given all of these observations, we conclude that free L11 interacts with *c-myc* mRNA in the cytoplasm.

L11 regulation of *c-myc* mRNA is specific. To determine whether L11 regulation of *c-myc* mRNA turnover is a general effect of all individual RPs, we first examined whether knockdown of several other RP genes, including L23, L26, and L29, would also affect *c-myc* mRNA and protein levels. As shown in Fig. 8A, unlike knockdown of L11, knockdown of L23, L26, or L29 did not affect the levels of *c-myc* mRNA (top panel) and protein (middle panel) in U2OS cells. Consistent with those

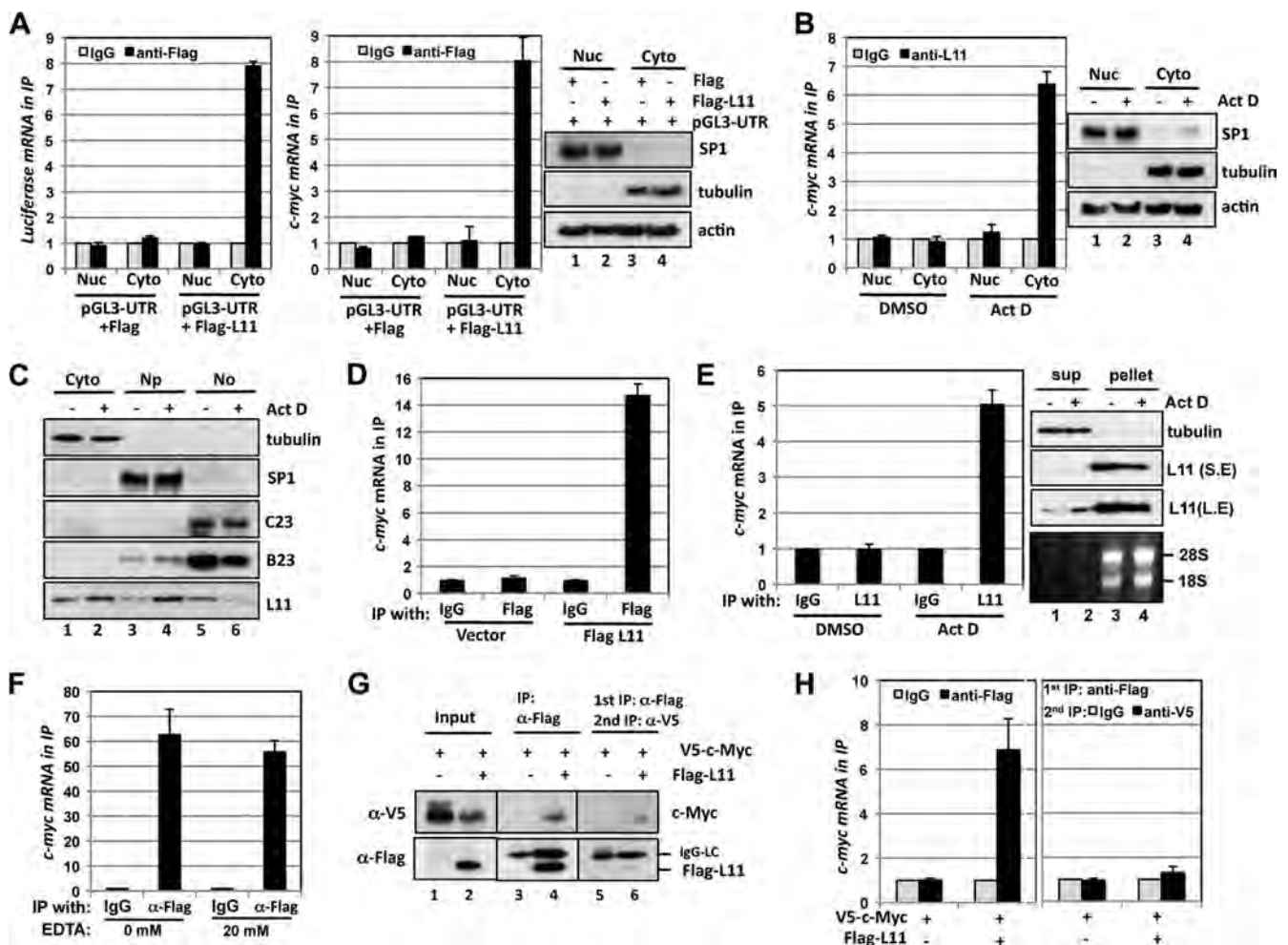


FIG. 7. Ribosome-free L11 associates with *c-myc* mRNA in the cytoplasm. (A) L11 binds to the *c-myc* 3'-UTR in the cytoplasm. 293 cells transfected with pGL3-*myc*3'UTR together with control Flag or Flag-L11 vector were fractionated into the cytoplasm (Cyto) and the nucleus (Nuc) fractions, followed by IP with anti-Flag antibody or mouse IgG. The immunoprecipitates were assayed for expression of *luciferase* mRNA (left panel) and *c-myc* mRNA (middle panel) by the use of RT-qPCR assays. Cellular fractionation was verified by detecting the nuclear SP1 and cytoplasmic tubulin by the use of IB (right panels). Actin was used as a loading control. (B) Act D treatment enhances the interaction of endogenous L11 with the *c-myc* mRNA in the cytoplasm. The cytoplasmic and the nuclear fractions were isolated from U2OS cells treated with DMSO or Act D (5 nM) for 12 h, followed by IP with anti-L11 antibodies or control rabbit IgG. The immunoprecipitates were assayed for expression of *c-myc* mRNA by the use of RT-qPCR assays (left panel). Cellular fractionation was verified by IB detection of SP1 and tubulin proteins (right panels). (C) Treatment with Act D releases L11 from the nucleolus into the nucleoplasm and the cytoplasm. U2OS cells treated with DMSO or Act D (5 nM) for 12 h were subjected to isolation of the cytoplasm (Cyto), nucleoplasm (Np), and the nucleolus (No) fractions, followed by IB detection of the indicated proteins. Nucleophosmin (B23) and nucleolin (C23) were used as nucleolar markers. (D) Ribosome-free L11 binds to *c-myc* mRNA. Nonribosome supernatants containing free RPs were isolated from 293 cells transfected using control or Flag-L11 plasmid and sucrose centrifugation as described in Materials and Methods. The supernatants were immunoprecipitated with anti-Flag antibody or mouse IgG followed by RT-qPCR detection of *c-myc* mRNA in the immunoprecipitates. (E) Act D treatment enhances the interaction of free endogenous L11 with *c-myc* mRNA. Nonribosome supernatants containing free RPs were isolated from U2OS cells treated with DMSO or Act D (5 nM) for 12 h. The supernatants were immunoprecipitated with anti-L11 antibodies or control IgG followed by RT-qPCR detection of *c-myc* mRNA (left panel). The L11 protein was detected using IB (upper right panels). S.E, short exposure; L.E, longer exposure. The rRNAs from polysome pellets as shown in the lower right panel were revealed by ethidium bromide staining. (F) L11 interacts with *c-myc* mRNA in the presence of EDTA. U2OS cells transfected with Flag-L11 were subjected to IP in the absence or presence of 20 mM EDTA. RNA IPs were conducted using anti-Flag antibody or control IgG followed by RT-qPCR detection of *c-myc* mRNA. (G) Sequential co-IP of the L11-*c-myc* protein complex. 293 cells were transfected with V5-*c-Myc* in the presence or absence of Flag-L11. The cell lysates were first immunoprecipitated with anti-Flag antibody. The Flag-L11-associated protein complexes were eluted with Flag peptide. Half of the elution was assayed for V5-*c-Myc* and Flag-L11 proteins by the use of IB. The other half was subjected to a second IP using anti-V5 antibody followed by IB detection of V5-*c-Myc* and Flag-L11 proteins. (H) Sequential co-IP of the L11-*c-myc* mRNA complex. 293 cells were transfected with V5-*c-Myc* in the presence or absence of Flag-L11. The cell lysates were first immunoprecipitated with anti-Flag antibody or control IgG followed by elution with Flag peptide. Half of the elution was assayed for *c-myc* mRNA by the use of RT-qPCR assays. The other half was subjected to a second IP using anti-V5 antibody or control IgG, followed by RT-qPCR detection of the *c-myc* mRNA.

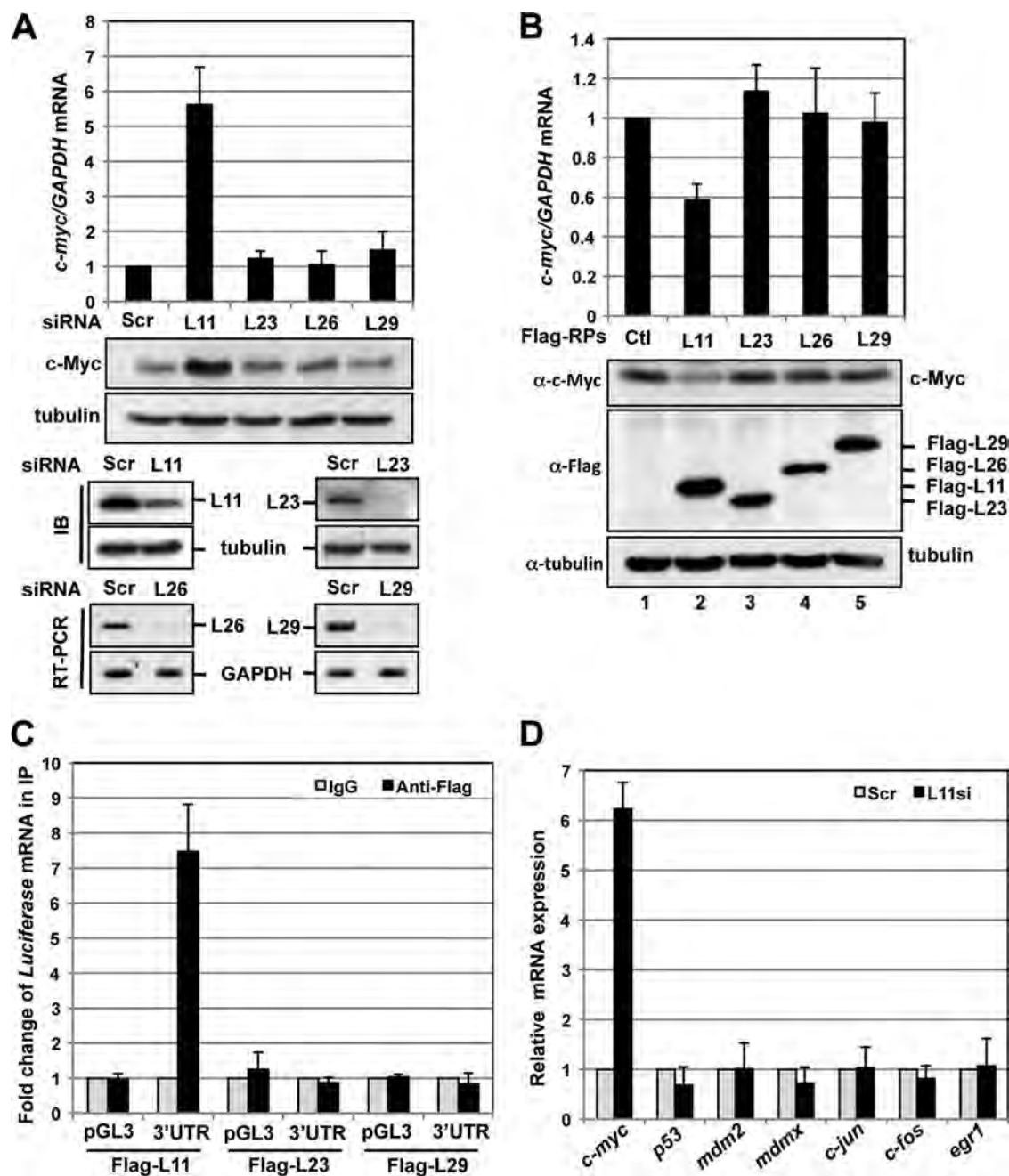


FIG. 8. L11 regulation of *c-myc* mRNA turnover is specific. (A) Knockdown of L23, L26, or L29 does not enhance c-Myc levels. U2OS cells transfected with scrambled, L23, L26, or L29 siRNA were subjected to detection of *c-myc* mRNA by the use of RT-qPCR and of c-Myc protein by the use of IB assays. The results of knockdown of the individual RPs shown in the bottom panels were determined by IB (L11 and L23) or RT-PCR (L26 and L29). (B) Overexpression of L23, L26, or L29 does not reduce c-Myc levels. U2OS cells transfected with control (Ctl), Flag-L23, Flag-L26, or Flag-L29 plasmid were subjected to detection of *c-myc* mRNA by the use of RT-qPCR (top panel) and of c-Myc protein by the use of IB assays (bottom panels). (C) L23 and L29 do not bind to *c-myc* mRNA in cells. 293 cells were transfected with control pGL3 or pGL3-myc3'UTR together with Flag-L11, Flag-L23, or Flag-L29. The cell lysates were immunoprecipitated with anti-Flag antibody or control IgG, followed by RT-qPCR detection of *luciferase* mRNA. (D) Knockdown of L11 does not evoke upregulation of a set of other mRNAs. U2OS cells transfected with scrambled or L11 siRNA were subjected to detection of the indicated mRNAs by the use of RT-qPCR assays.

results, overexpression of these RPs did not reduce the levels of *c-myc* mRNA and protein either (Fig. 8B). Also, neither knockdown nor overexpression of S12 affected the levels of *c-myc* mRNA and protein (data not shown). Furthermore, RNA IPs were performed using anti-Flag antibodies in cells

transfected with Flag-L23 or Flag-L29. As shown in Fig. 8C, unlike L11, neither L23 nor L29 was associated with *c-myc* mRNA in cells. Taken together, these results suggest that L11 downregulation of *c-myc* mRNA and protein is a specific rather than a general effect of all individual RPs.

To test whether L11 regulation of *c-myc* mRNA is also specific to *c-myc* mRNA rather than a general effect on global mRNA turnover, we examined the levels of a number of other mRNAs upon L11 knockdown. These included the tumor suppressor gene *p53*, oncogenes *mdm2* and *mdmx*, two labile early-response genes that contain AREs in their 3'-UTR similar to that in *c-myc* 3'-UTR, *c-fos* and *c-jun* (8, 9, 46), and the early-response gene *egr1*. As shown in Fig. 8D, unlike the results seen with *c-myc* mRNA, none of these genes were upregulated upon knockdown of L11. Thus, L11 regulation of *c-myc* mRNA is also not a general effect for all cellular transcripts, although we cannot exclude the possibility that knockdown of L11 might affect the turnover of some other untested mRNAs.

DISCUSSION

c-Myc promotes cell growth and proliferation by enhancing ribosomal biogenesis and translation. Deregulated ribosomal biogenesis and translation contribute to malignant transformation and conceivably play a key role in c-Myc-driven tumorigenesis. Our previous studies revealed that ribosomal protein L11, whose transcription is induced by c-Myc, directly suppresses c-Myc activity, thus coordinating c-Myc activity with ribosomal biogenesis. In this study, we found that L11 destabilizes *c-myc* mRNA through a novel miRNA-mediated mechanism. Importantly, L11-mediated *c-myc* mRNA decay is required for c-Myc downregulation in response to ribosomal stress.

Exposure of cells to external or intrinsic insults, if not properly dealt with, can lead to genomic instability. p53-dependent cell cycle checkpoints play a key role in maintaining genomic instability in response to diverse stressors (63, 64). Recent studies have shown that deregulated overactivation of c-Myc induces genomic instability, which could contribute to its oncogenic potential (27, 41, 49). For this reason, c-Myc should be tightly controlled under stress conditions. Indeed, it has recently been shown that c-Myc levels are reduced in cells following DNA damage (6, 7, 48). However, whether and how c-Myc is regulated in response to ribosomal stress are not clear. Treatment of cells with a low dose of Act D or 5-FU suppresses ribosomal biogenesis and causes ribosomal stress, leading to p53 activation via enhancing RP-MDM2 interaction (4, 14, 18, 57). Here, we show for the first time that c-Myc is significantly reduced at mRNA levels following ribosomal stress mediated by treatment with Act D or 5-FU, implying that c-Myc downregulation might also be an important cellular response following ribosomal stress. Failure of that response might contribute to genomic instability and tumorigenesis.

Accumulating data suggest that miRNAs play a crucial role in gene regulation in response to stress (35). However, most studies have focused on the changes in miRNA levels or the molecular ratio of miRNAs to target mRNAs following stress (35). Certain RNA-binding proteins (RBPs) can modulate the function of miRNAs. For example, TTP promotes tumor necrosis factor alpha (TNF- α) mRNA decay caused by miR-16 (29) and HuR facilitates the targeting of *c-myc* mRNA by let-7 (32). Conversely, dead end 1 (Dnd1) suppresses miRNA access to target mRNAs (31). However, whether RBPs regulate miRNA functions in response to stress has not been investigated as fully. Bhattacharyya et al. (5) showed that HuR interacts with cationic amino acid transporter 1 (CAT-1) mRNA

and relieves its translational repression by miR-122 following amino acid starvation. Here, we report for the first time that L11 plays a key role in miR-24-mediated *c-myc* mRNA decay in response to ribosomal stress. Treatment of cells with Act D or 5-FU markedly increased the binding of L11 and Ago2 to the *c-myc* mRNA as well as the association of L11 with Ago2 and miR-24 (Fig. 6). L11 is required for the increased binding of Ago2 to *c-myc* mRNA following the treatments described above (Fig. 6C). Knockdown of either L11 or Ago2 abolished *c-myc* mRNA reduction in response to the treatments described above (Fig. 5). Interestingly, binding of HuR to either *c-myc* mRNA or let-7b was not increased in response to treatment with Act D or 5-FU (data not shown). Thus, ribosomal stress might trigger a specific L11-mediated *c-myc* mRNA decay pathway involving the miR-24-loaded miRISC.

How L11 recruits miRISC to the *c-myc* mRNA is currently not clear. Ago2 is a core component of miRISC (47, 61) and directly interacts with mature miRNAs. L11 interacts with Ago2 in cells, but this interaction requires RNA. Thus, it is likely that by binding to miR-24 and *c-myc* mRNA, L11 may act as an accessory factor to facilitate miRISC loading onto *c-myc* mRNA or to stabilize the miR-24-loaded miRISC-*c-myc* mRNA complex. It is also likely that L11 binding changes the *c-myc* 3'-UTR into a conformation that favors its targeting by miRISC. Another unanswered question is how L11 specifically associates with miR-24 among the tested miRNAs that target *c-myc* mRNA (Fig. 4A and B). miR-24 seems to strongly target a "seedless" sequence (nt 447 to 468) at the 3' end of the *c-myc* 3'-UTR, although it also targets an additional site (nt 255 to 276) (34). Our mapping results showed that L11 binds to the 3' end of the *c-myc* 3'-UTR (Fig. 2E), suggesting that L11 may change the local 3'-UTR conformation to expose the miR-24 binding region and facilitate the miR-24 targeting of the *c-myc* 3'-UTR. Otherwise, L11-miRNA sequence-specific interactions may contribute to this specificity. Further studies that aim to identify other L11-associated miRNAs that target *c-myc* are planned.

miR-24 has recently been shown to block the G₁/S transition by targeting the cell cycle regulatory network, among the components of which c-Myc is one of the key direct targets of miR-24 (34). Consistent with these results, miR-24 is upregulated during terminal differentiation in diverse cell types. miR-24 targets *c-myc* mRNA through binding to several seedless 3'-UTR miRNA recognition elements. This miRNA-target mRNA pairing, though imperfect, could still lead to *c-myc* mRNA decay (34). In fact, it has recently been shown that mRNA degradation contributes to most (>84%) of the miRNA-mediated gene silencing effect in mammalian cells (26). Our data support the notion of a role for miR-24 in downregulating *c-myc* mRNA levels and negatively regulating cell cycle progression. It should be interesting to examine whether ribosomal stress can elicit L11- and miR-24-dependent suppression of other genes in the cell cycle regulatory network in addition to *c-myc*.

Our further data revealed that ribosome-free L11 binds to *c-myc* mRNA in the cytoplasm. First, cytoplasmic but not nuclear L11 bound to *c-myc* mRNA in cell fractionation assays (Fig. 7A). Second, free L11 from nonribosome supernatants bound to *c-myc* mRNA efficiently in our sucrose centrifugation assays (Fig. 7D). Third, L11 binds to *c-myc* mRNA efficiently when the 80S ribosomes and polysomes are disrupted, as shown by adding EDTA into cell lysates (Fig. 7F). Further-

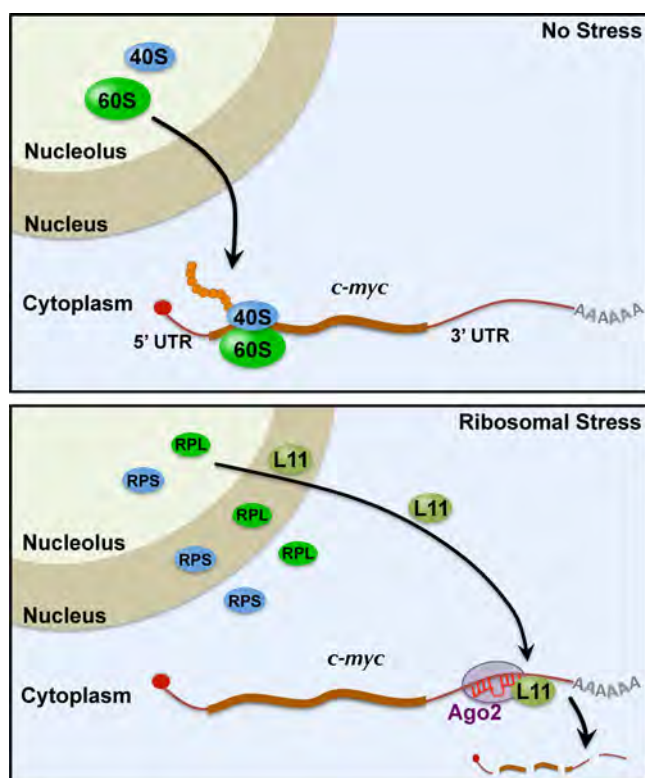


FIG. 9. Schematic model of L11 regulation of *c-myc* mRNA decay via miRISC in response to ribosomal stress. Under normal conditions (top panel), the 40S and 60S mature ribosome subunits are assembled in the nucleolus and transported into the cytoplasm to mediate translation of *c-myc* mRNA. Upon perturbation of ribosomal biogenesis (ribosomal stress; bottom panel), individual small (RPS) and large (RPL) ribosomal proteins, including L11, are released from the nucleolus into the nucleoplasm, where L11 binds to c-Myc protein and suppresses its transactivation activity (not shown), and into the cytoplasm, where L11 recruits miR-24-loaded miRISC to the *c-myc* 3'-UTR, leading to *c-myc* mRNA decay.

more, ribosomal stress induces the release of nucleolar L11 into both the cytoplasm and the nucleus (Fig. 7C) and enhances the free L11 (Fig. 7E) binding to *c-myc* mRNA in the cytoplasm (Fig. 7B). It is therefore likely that ribosome-free L11 plays a dual role in regulating c-Myc: suppressing c-Myc transactivation activity in the nucleus (12) and promoting *c-myc* mRNA decay by recruiting miRISC to the *c-myc* 3'-UTR in the cytoplasm (Fig. 9). These two effects are mutually exclusive, as our sequential RNA-IP assays showed that c-Myc protein-associated L11 does not bind to *c-myc* mRNA (Fig. 7H). These results also exclude the possibility that L11 binding to *c-myc* mRNA occurs through binding to the nascent translating c-Myc protein.

Interestingly, the regulation of *c-myc* mRNA by L11 is specific to L11, as neither knockdown nor overexpression of other tested RPs, including L23, L26, L29, and S12 (Fig. 8A and B and data not shown), changed the levels of *c-myc* mRNA and protein. Unlike L11, neither L23 nor L29 associated with the *c-myc* mRNA (Fig. 8C). Thus, not all individual RPs would regulate *c-myc* mRNA. On the other hand, L11 regulation of *c-myc* mRNA is also specific to *c-myc* mRNA and not a general effect on global mRNA turnover, as knockdown of L11 did not

affect the levels of several tested mRNAs (Fig. 8D). In particular, and in similarity to the results seen with *c-myc* mRNA, *c-fos* and *c-jun* are labile mRNAs that contain AREs in their 3'-UTR (8, 9, 46). However, neither of the mRNAs exhibited an increased level upon knockdown of L11. Although we cannot rule out the possibility that other untested RPs may regulate *c-myc* mRNA or that L11 may regulate other untested mRNAs, our data strongly suggest a key role for L11 in controlling c-Myc levels (and thus activity during normal homeostasis as well as in response to stress).

We have previously shown that L11 suppresses c-Myc-driven target gene transcription mediated by all three RNA polymerases and cell proliferation whereas transient knockdown of L11 enhances these c-Myc activities (12, 17). Also, knockdown of L11 attenuates ribosomal stress-induced cell cycle arrest by preventing p53 activation (4, 16, 57–59). However, prolonged ablation of L11 would eventually impair ribosomal biogenesis and cause cell growth arrest. In that case, upregulated c-Myc could cause replicative stress or some other form of metabolic stress and lead to cell cycle exit from and/or cell death in the S phase. Supporting this notion, mutations in the *L11* gene have been reported in patients with Diamond-blackfan anemia, an inherited cancer-related disease characterized by defective erythropoiesis and developmental defects (11, 22). Future studies would aim to test this hypothesis.

In summary, our current observations, together with the results of previous studies (12, 17), suggest that L11, whose transcription is induced by c-Myc (12, 38), tightly coordinates c-Myc activity and levels with ribosomal biogenesis, forming an elegant feedback regulatory loop. Conceivably, when ribosomal biogenesis is overactive following, for example, overexpression of oncogenes, including c-Myc, L11 is induced and may subsequently target c-Myc to maintain normal homeostatic levels of c-Myc in cells. Together, our data here reveal a critical role for L11 in controlling c-Myc levels in response to ribosomal stress. Previous studies have firmly established the role for L11 in transmitting ribosomal stress to p53 signaling (68). Thus, L11 acts as a key ribosomal “stress sensor” in monitoring the integrity of ribosomal biogenesis by activating both p53 and suppressing c-Myc signals.

ACKNOWLEDGMENTS

This work was supported by NIH/NCI grant R00CA127134, a grant from Department of Defense (Peer Reviewed Cancer Research Program) under award number W81XWH-10-1-1029, and the startup fund from Oregon Health & Science University to M.-S.D. M.-S.D. is a recipient of Cancer Research Development Award from the OHSU Knight Cancer Institute.

Views and opinions of and endorsements by the author(s) do not reflect those of the U.S. Army or the Department of Defense.

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