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14. ABSTRACT
During the funding period, we first discovered that L11 recruits miR-24-loaded miRISC to the ***c-myc*** mRNA 3'-UTR to induce ***c-myc*** mRNA decay in response to ribosomal stress. We then found that L11 also suppress ***c-myc*** expression in response to UV irradiation-induced DNA damage by recruiting miR-130a-miRISC to the ***c-myc*** mRNA 3'-UTR, thereby destabilizing ***c-myc*** mRNA. We showed that miR-130a directly targets ***c-myc*** mRNA. Overexpression of miR-130a reduced the levels of ***c-myc*** mRNA whereas inhibiting miR-130a drastically induced the levels of ***c-myc*** mRNA. Overexpression of miR-130a increased the association of Ago2/miRISC with ***c-myc*** mRNA. Interestingly, UV treatment enhances the association of L11, Ago2 as well as miR-130a with the ***c-myc*** mRNA. Inhibiting miR-130a significantly suppresses UV-mediated c-Myc reduction. Together, our results reveal a novel regulatory paradigm wherein L11 plays a key role in controlling ***c-myc*** mRNA turnover in response to stress via targeting ***c-myc*** mRNAby miR-24 and miR-130a. Our results also imply that miR-130a and miR-24 may possesses a tumor suppressor function through down regulating c-Myc.

15. SUBJECT TERMS
Ribosomal protein, L11, c-Myc, DNA damage, microRNA

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Table of Contents

1. 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 in response to ribosomal stress and L11 plays a novel and key role in mediating this ribosomal stress-induced *c-myc* mRNA turnover [4]. Interestingly, c-Myc is also down-regulated in cells following DNA damage, such as those induced by ultraviolet (UV) and γ-irradiation (IR). The purpose of this proposal is to examine whether and how L11 is involved in the regulation of c-Myc in response to DNA damage. Specifically, the goal of this study was to determine whether L11 regulates *c-myc* mRNA levels and stability by recruiting miRNAs in response to DNA damage as well as its underlying mechanisms. Results from these experiments would demonstrate an important function of L11 in regulating *c-myc* mRNA in response to DNA damage, offer useful information for developing anti-tumor drugs that target *c-myc* mRNA in cancers, and have a significant impact on the understanding of c-Myc-induced tumorigenesis.

2. Keywords

c-Myc;
Ribosomal proteins;
Ribosomal stress;
microRNA;
miRISC;
miR-130a;
miR-24;
L11;
DNA damage;
UV irradiation;
Oncogene;
Cell cycle;
mRNA decay

3. Overall project summary

During the funding period, we have made two important findings. ***First***, we 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 its 3'-untranslated region (3'-UTR). 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 miR-24/miRISC to repress c-Myc. Interestingly, ribosomal stress induced by perturbation of ribosomal biogenesis results in a significant *c-myc* mRNA reduction in a L11-dependent manner in cells. L11 binding to *c-myc* mRNA, miR-24, and Ago2 was significantly increased following ribosomal stress. Together, our 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, thus ensuing a tight coordination between the levels and activity of c-Myc and ribosomal biogenesis. This work has been published in ***Mol Cell Biol (2011)***[4].

miR-16	miR-103
miR-1248	***miR-130a*** (+/-)
miR-3944 (-)	miR-1285(+/-)
miR-191	miR-1181 (-)
miR-548d-3p (-)	miR-320a
miR-1972 (+/-)	***hsa-miR-24***
miR-1244	miR-107
miR-744	miR-1234 (-)

Figure 1. ***L11-associated miRNAs identified by RNA-IP-RNAseq.*** 293 cells transfected with Flag-L11 were subjected to affinity purification of L11-RNA complex followed by RNAseq.

In addition, we purified L11-associated-miRNAs from 293 cells using deep sequencing and initial results identified that L11 associates with a number of novel miRNAs, including miR-130a and miR-24 (Figure 1). We also tested an array of miRNAs possessing tumor suppressor functions for L11 binding

and found that L11 bound strongly to miR-130a and to a less extent to miR-16, but not other tested miRNAs (*Fig. 1A in the attached paper: Li Y, et al, Oncotarget, 2015* [5]). Our subsequent study revealed that L11 recruits miR-130a to *c-myc* mRNA to suppress c-Myc expression in response to UV-mediated DNA damage. We showed that miR-130a directly targets *c-myc* 3'-UTR. Overexpression of miR-130a promotes the Ago2 binding to *c-myc* mRNA, significantly reduces the levels of both c-Myc protein and mRNA and inhibits cell proliferation, whereas inhibiting miR-130a significantly increases c-Myc levels. UV treatment markedly promotes the binding of L11 to miR-130a, *c-myc* mRNA as well as Ago2 in cells. Importantly, inhibiting miR-130a significantly suppresses UV-mediated c-Myc reduction. We further show that L11 is relocalized from the nucleolus to the cytoplasm where it associates with *c-myc* mRNA upon UV treatment. Together, these results reveal a novel mechanism underlying c-Myc down-regulation in response to UV-mediated DNA damage, wherein L11 promotes miR-130a-loaded miRISC to target *c-myc* mRNA. This work was accepted for publication in ***Oncotarget (Li Y, et al, 2015)***[5] and the proof is attached in this final progress report.

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

Aim 1. 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. 2 and Figs. 5B and 5D in the attached paper: Li Y, et al, Oncotarget, 2015* [5]). This reduction requires L11 as knockdown of L11 abolished UV-mediated reduction of the *c-myc* mRNA (*Figs. 5G and 5H in the attached paper: Li Y, et al, Oncotarget, 2015* [5]), suggesting that L11 plays an important role in regulating *c-myc* levels in response to DNA damage. Thus, **this aim has been completed**.

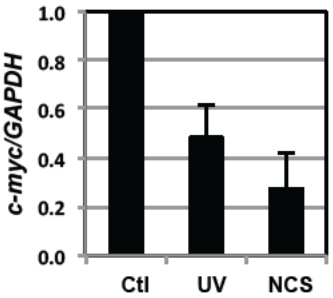

Figure 2. *c-myc* mRNA is reduced by treatment with UV or NCS. U2OS cells treated with UVC (40J) or NCS (0.5 μg/ml) were subjected to RT-qPCR.

Aim 2. To examine if L11 recruits miRNA(s) to the 3'UTR of c-myc mRNA in response to UV and γ-IR.

We have found that UV damage enhances L11 association with *c-myc* mRNA [***task 2(1)***] (*Fig. 6A in Li Y, et al, Oncotarget, 2015* [5]). To further characterize the role of L11 in c-Myc regulation in response to DNA damage, we have performed experiments covered in ***tasks 2(2)-2(4)***. We focus on miR-130a instead of the proposed miR-145 or let-7, as miR-130a associates with L11. We have shown that overexpression of miR-130a mimics significantly reduced the levels of c-Myc protein and *c-myc* mRNA in U2OS cells and human normal fibroblast WI38 cells (Figs. 2A and 2B in *Li Y, et al, Oncotarget, 2015* [5]). Consistently, inhibition of miR-130a using RNA inhibitor significantly increased the levels of c-Myc protein and *c-myc* mRNA (Figs. 2C and 2D in *Li Y, et al, Oncotarget, 2015* [5]). We also showed that overexpression of miR-130a significantly reduced the luciferase activity in cells transfected with pGL3-myc 3'UTR, but not the control pGL3, reporter (Fig. 3A in *Li Y, et al, Oncotarget, 2015* [5]), which requires the mir-130a binding site at the c-myc 3'-UTR (Fig. 3D in *Li Y, et al, Oncotarget, 2015* [5]). Also, overexpression of miR-130a significantly increased the binding of Ago2 to the *c-myc,* but not *GAPDH,* mRNA (Figs. 3E and 3F in *Li Y, et al, Oncotarget, 2015* [5]). Altogether, these data indicate that miR-130a directly targets the *c-myc* mRNA to regulate the levels of c-Myc. We further show that UV irradiation significantly increased the association of L11 with miR-130a [***tasks 2(2)***] (Fig. 6F in *Li Y, et al, Oncotarget, 2015* [5]) and inhibiting miR-130a significantly abolished UV-mediated c-myc reduction [***tasks 2(3)***] (Figs. 6I and 6J in *Li Y, et al, Oncotarget, 2015* [5]). These results was summarized in the paper ***(Li Y, et al, Oncotarget, 2015)***[5]. In addition, our RNA-IP-RNAseq assays from 293 cells stably expressed Flag-L11 using anti-Flag antibody (***task 2(5)***) showed that miR-130a is one of the L11-associated miRNAs (Above Fig. 1 and Fig. 1A in *Li Y, et al, Oncotarget, 2015* [5]). Therefore, **we have also completed this aim 2**.

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

Mechanistically, we have shown that UV treatment significantly increased the binding of Ago2 to both *c-myc* mRNA and miR-130a in cells (proposed in ***task 3(1)***) (Figs. 6D and 6E in *Li Y, et al, Oncotarget, 2015* [5]). Furthermore, UV treatment also significantly increased the binding of L11 to both *c-myc* mRNA in U2OS cells (Fig. 6A in *Li Y, et al, Oncotarget, 2015* [5]) and luciferase mRNA containing *c-myc* 3'UTR in cells transfected with pGL3-myc 3'UTR reporter (Figs. 6B and 6C in *Li Y, et al, Oncotarget, 2015* [5]). Consistently, UV treatment increased the interaction of L11 with Ago2 (Fig. 6G in *Li Y, et al, Oncotarget, 2015* [5]). Altogether, these data suggest that UV treatment increases the recruitment of miR-130a-loaded miRISC to *c-myc* mRNA to suppress c-Myc expression. We also found that UV treatment leads to the relocalization of L11 from the nucleolus into the cytoplasm where it recruits miR-130a to target *c-myc* mRNA following UV (Fig. 7 in *Li Y, et al, Oncotarget, 2015* [5]). These data was also summarized in the paper by ***Li Y, et al (Oncotarget, 2015)***[5].

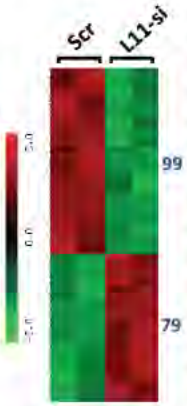

Figure 3. ***Heatmap of miRNA expression.*** U2OS cells transfected with scrambled or L11 siRNA for 48 hours were assayed by miRNA microarray for the expression of miRNAs covered by miRBase V18.

Finally, we have performed miRNA microarray analysis to identify whether knockdown of L11 changes microRNA expression pattern in cells. We have found that 99 miRNAs (e.g. miR-4668-5p) were reduced whereas 79 (e.g. miR-5194) were increased by L11 knockdown (Fig. 3). These assays partially address the ***Task 2(4)***. Future studies will focus on analyzing cancer-associated miRNAs (tumor suppressor and oncogenic miRNAs) among these miRNAs. Regarding the regulation of L11-mediated *c-myc* mRNA decay by other L11 interacting proteins as proposed in ***Tasks 3(2)-3(4)***, we have found that L11 interacts with the microprocessor DGCR8/Drosha complex (Fig. 4 and Fig. 5). We will examine the possible role of L11 in regulating miRNA biogenesis in future studies.

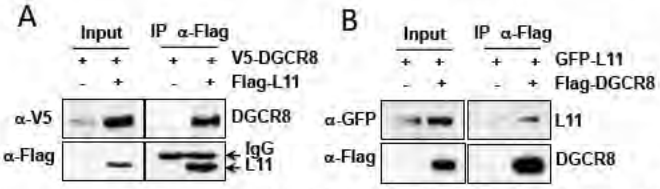

Figure 4. L11 interacts with DGCR8. 293 cells transfected with V5-DGCR8 and Flag-L11 or control vector (A) or with GFP-L11 and Flag-DGCR8 or control vector (B) were subjected to co-IP with anti-Flag followed by IB.

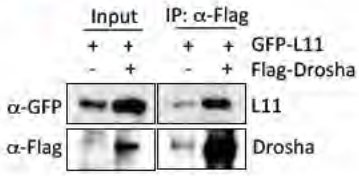

Figure 5. L11 interacts with Drosha. 293 cells transfected with GFP-L11 and Flag-Drosha or control vector were subjected to co-IP with anti-Flag followed by IB.

4. 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 downregulation requires L11.
(4). miR-130a targets *c-myc* mRNA in cells.
(5). UV treatment increased the recruitment of miR-130a-loaded miRISC to the *c-myc* mRNA.
(6). UV treatment relocates L11 from the nucleolus to the cytoplasm to recruit miR-130a to *c-myc* mRNA.
(7). L11 interacts with the miRNA microprocessor complex Drosha-DGCR8.

5. CONCLUSIONS

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

6. Publications, abstracts, presentations, List of Personnel Receiving Pay from the Research Effort.

(**A**). Publications: This award supports the following manuscripts:
(1) ***Peer-reviewed Scientific journals***

(**i**). 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. *PMID: 21807902; doi: 10.1128/MCB.05810-11*

(**ii**). Sun X-X, DeVine T, Challagundla KB, **Dai M-S**. (2011) Interplay between ribosomal protein S27a and MDM2 in p53 activation in response to ribosomal stress. ***J Biol Chem***, 286(26): 22730-22741. *PMID: 21561866; doi: 10.1074/jbc.M111.223651.*

(**iii**). Sun X-X, Challagundla KB, **Dai M-S**. (2012) Positive regulation of p53 stability and activity by the deubiquitinating enzyme Otubain 1. **EMBO J**, 31(3): 576-592. *PMID: 22124327; doi:10.1038/emboj.2011.434*

(i**v**). Li Y, Sun X-X, Elferich J, Shinde U, David LL, **Dai M-S**. (2014) Monoubiquitination is critical for ovarian tumor domain-containing ubiquitin aldehyde binding protein 1 (Otub1) to suppress UbcH5 enzyme and stabilize p53 protein. ***J Biol Chem***, 289(8): 5097-5108. *PMID: 24403071; doi: 10.1074/jbc.M113.533109.*

(**v**). Li Y, Challagundla KB, Sun X-X, Zhang Q, **Dai MS**. (2015) microRNA-130a associates with ribosomal protein L11 to suppress c-Myc expression in response to UV irradiation. ***Oncotarget***, 6(2): 1101-1114.

(2) ***Invited articles:***

(**i**). DeVine T, **Dai M-S**. (2013) Targeting the ubiquitin-mediated proteasome degradation of p53 for cancer therapy. ***Curr Pharm Des***, 19(18): 3248-3262. *PMID: 23151129*

(**B**) Abstracts:

(**i**). Sun X-X, DeVine T, **Dai M-S**. Regulation of p53 stability and activity by ribosomal protein S27a. The 102nd AACR meeting. Orlando, April 2011

(**ii**). Challagundla KB, Sun XX, **Dai M-S**. Ribosomal protein L11 recruits miRISC to suppress c-Myc in response to ribosomal stress. The Cold Spring Harbor Asia Conference on RNA biology. Suzhou, China, October 8-12, 2012

(**C**) Presentations:

(i). **Dai M-S**. Ribosomal protein L11 recruits miRISC to suppress c-Myc in response to ribosomal stress. The Cold Spring Harbor Asia Conference on RNA biology. Suzhou, China, October 8-12, 2012

(**D**) List of Personnel Receiving Pay from the Research Effort:

(i). Mushui Dai: PI
(ii). Kishore B. Challagundla: Postdoctoral Fellow
(iii). Yuhuang Li: Postdoctoral Fellow
(iv). Xiao-xin Sun: Research faculty

7. Inventions, patents and licenses:

Nothing to report.

8. Reportable outcomes:

Nothing to report.

9. Other Achievements:

Nothing to report.

10. 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

5. Li Y, Challagundla KB, Sun X-X, Zhang Q, **Dai MS**. (2014) microRNA-130a associates with ribosomal protein L11 to suppress c-Myc expression in response to UV irradiation. ***Oncotarget***, in press.

G. APPENDICES

Article #1: Challagundla KB et al. ***Mol Cell Biol***, 31(19): 4007-4021 (2011)

Article #2: Li Y et al. ***Oncotarget***, 6(2): 1101-1114 (2015)

MOLECULAR AND CELLULAR BIOLOGY, Oct. 2011, p. 4007–4021 Vol. 31, No. 19
0270-7306/11/$12.00 doi:10.1128/MCB.05810-11

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

Kishore B. Challagundla,[1,2] Xiao-Xin Sun,[1,2] Xiaoli Zhang,[1,2] Tiffany DeVine,[1,2] Qinghong Zhang,[3] Rosalie C. Sears,[1,2] and Mu-Shui Dai[1,2]*

Department of Molecular and Medical Genetics, School of Medicine,[1] and OHSU Knight Cancer Institute,[2] Oregon Health & Science University, 3181 SW Sam Jackson Park Road, Portland, Oregon 97239, and Department of Dermatology, University of Colorado Denver, Aurora, Colorado 80045[3]

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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 $tRNA_i^{Met}$ 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% CO_2 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-*myc3′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.

[^{35}S]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 [^{35}S]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 ^{35}S-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 $MgCl_2$, 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 $MgCl_2$, 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 ΔCτ 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′-CAGTGACCGTGGGAATGAAGTT-3′ (c-*fos*); 5′-GCAAAGATGGAAACGACCTTCT-3′ and 5′-GCTCTCGGACGGGAGGAA-3′ (c-*jun*); 5′-GCCTGCGACATCTGTGGAA-3′ and 5′-CGCAAGTGGATCTTGGTATGC-3′ (*egr1*); 5′-AGGCCTTGGAACTCAAGGAT-3′ and 5′-TGAGTCAGGCCCTTCTGTCT-3′ (*p53*); 5′-ATGAATCCCCCCCTTCCAT-3′ and 5′-CAGGAAGCCAATTCTCACGAA-3′ (*mdm2*); and 5′-GCCTTGAGGAAGGATTGGTA-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 $MgCl_2$, 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 $MgCl_2$). 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 $MgCl_2$, 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 $MgCl_2$, layered over buffer S2 containing 0.35 M sucrose and 0.5 mM $MgCl_2$, 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 $MgCl_2$ 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).

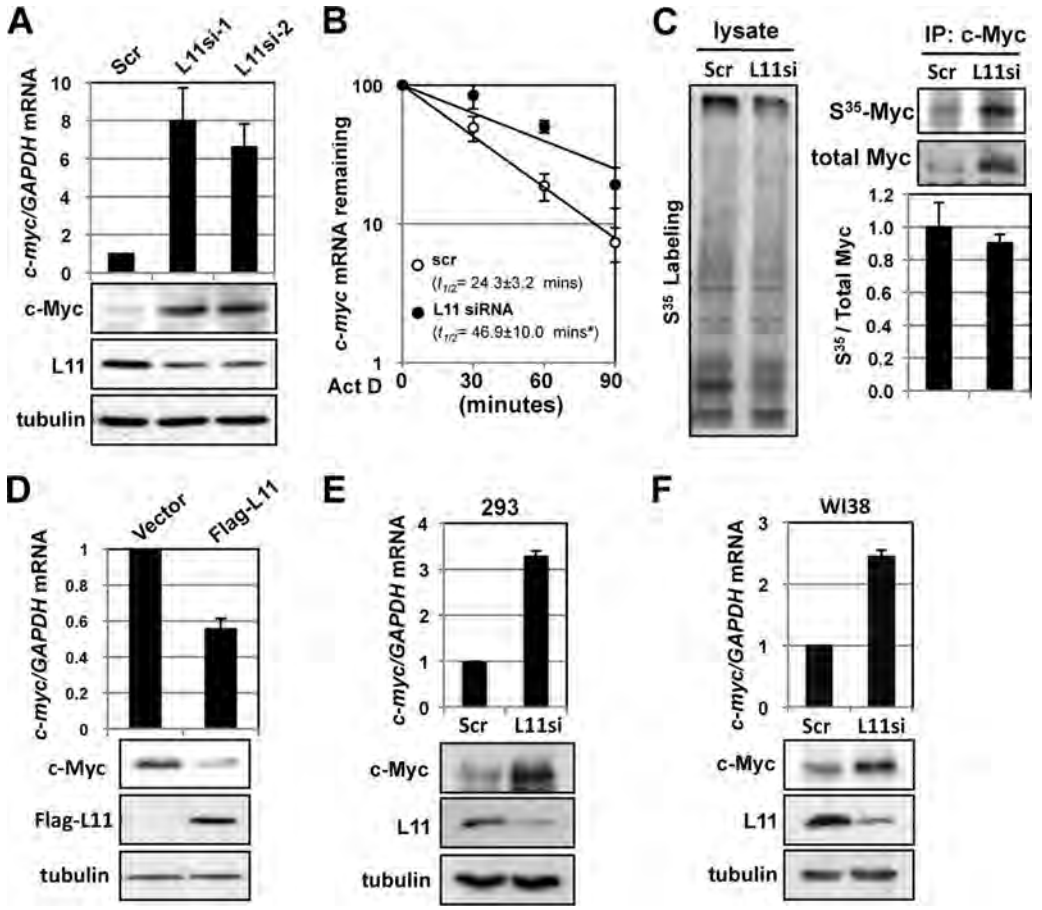

FIG. 1. L11 regulates c-*myc* mRNA levels and stability. (A) Knockdown of L11 increases the levels of c-*myc* mRNA and c-Myc protein. U2OS cells were transfected with scrambled (Scr) siRNA or one of two L11 siRNAs (L11si-1 or L11si-2). The cells were assayed for expression of c-*myc* mRNA by the use 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 μM 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 autography (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 autography (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 × *g* for 2 h. The polysome-containing pellet and the nonribosomal supernatant were collected separately (42) and immunoblotted with anti-L11 antibody. The nonribosomal 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 knockdown 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-*myc*3′UTR or control pGL-3 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 pGL-3 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

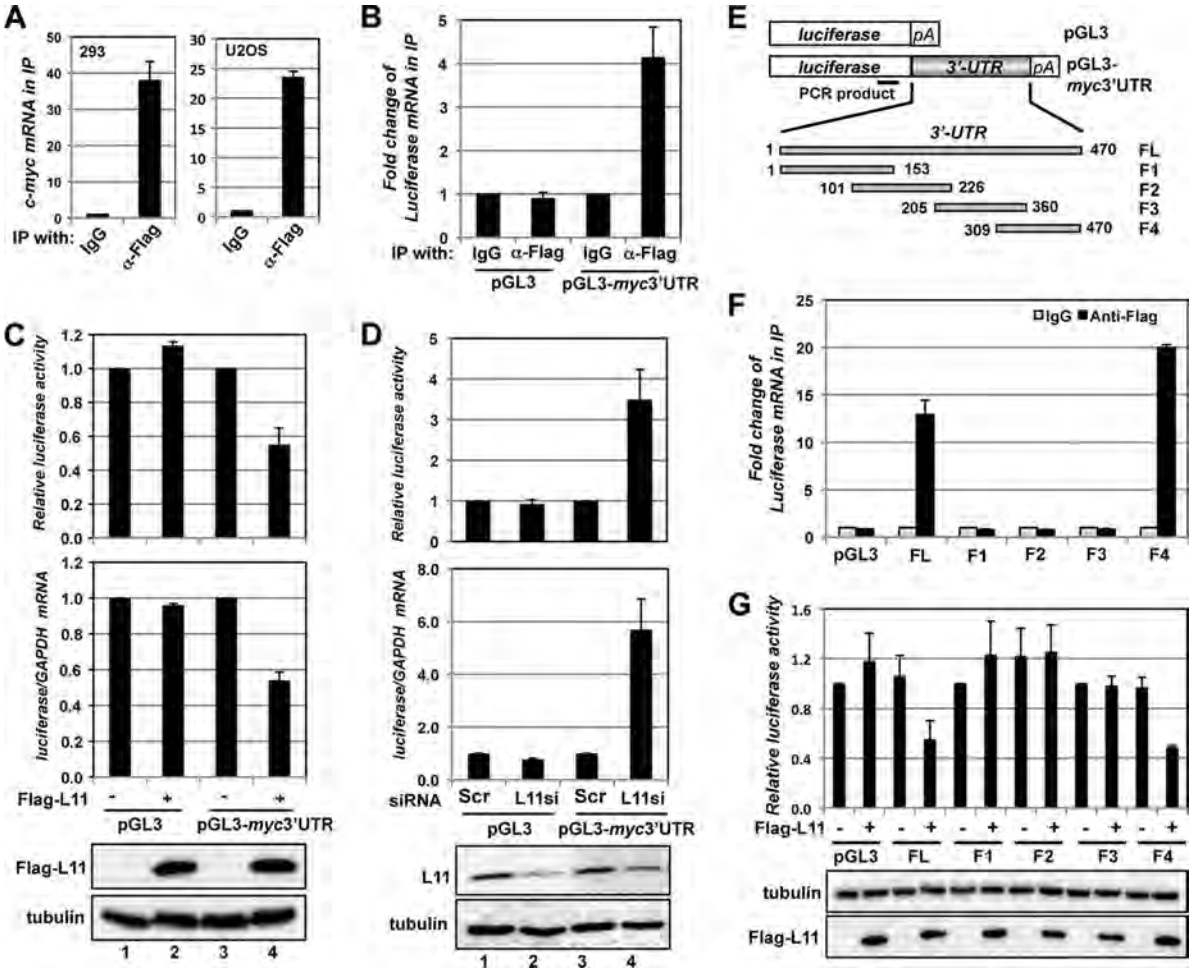

FIG. 2. L11 regulates c-*myc* by targeting the 3′-UTR of c-*myc* mRNA. (A) L11 binds to c-*myc* mRNA. Lysates from 293 (left panel) and U2OS (right panel) cells transfected with Flag-L11 were immunoprecipitated with control mouse IgG or anti-Flag antibody. RNA extracted from the immunoprecipitates was 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-

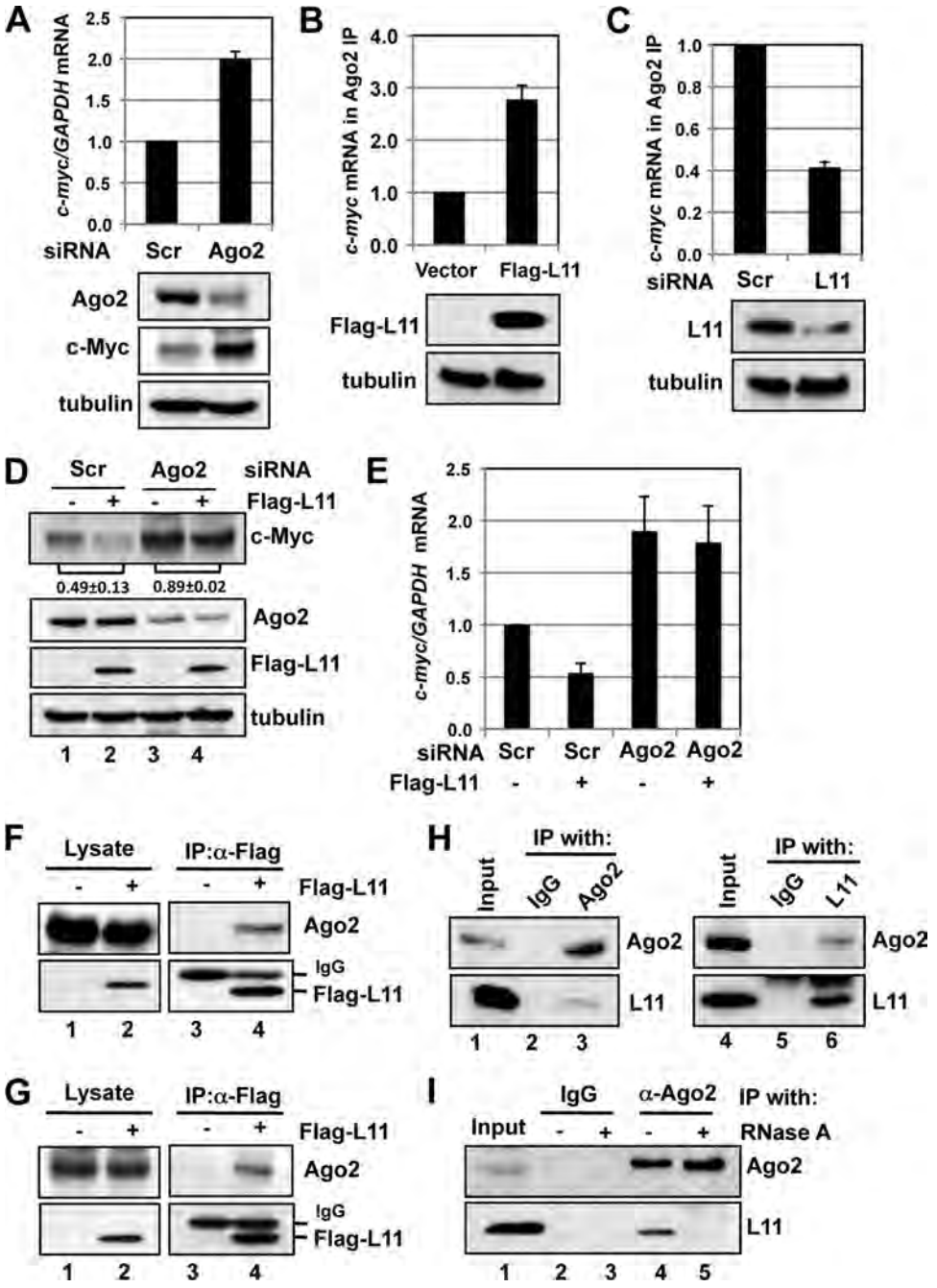

FIG. 3. Mutual dependency of L11 and ago2 in regulating c-*myc* mRNA. (A) Ago2 regulates c-*myc* mRNA levels. U2OS cells transfected with scrambled or Ago2 siRNA were assayed for expression of c-Myc protein (bottom panels) and mRNA (top panel). (B) Overexpression of L11 increases association of Ago2 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.

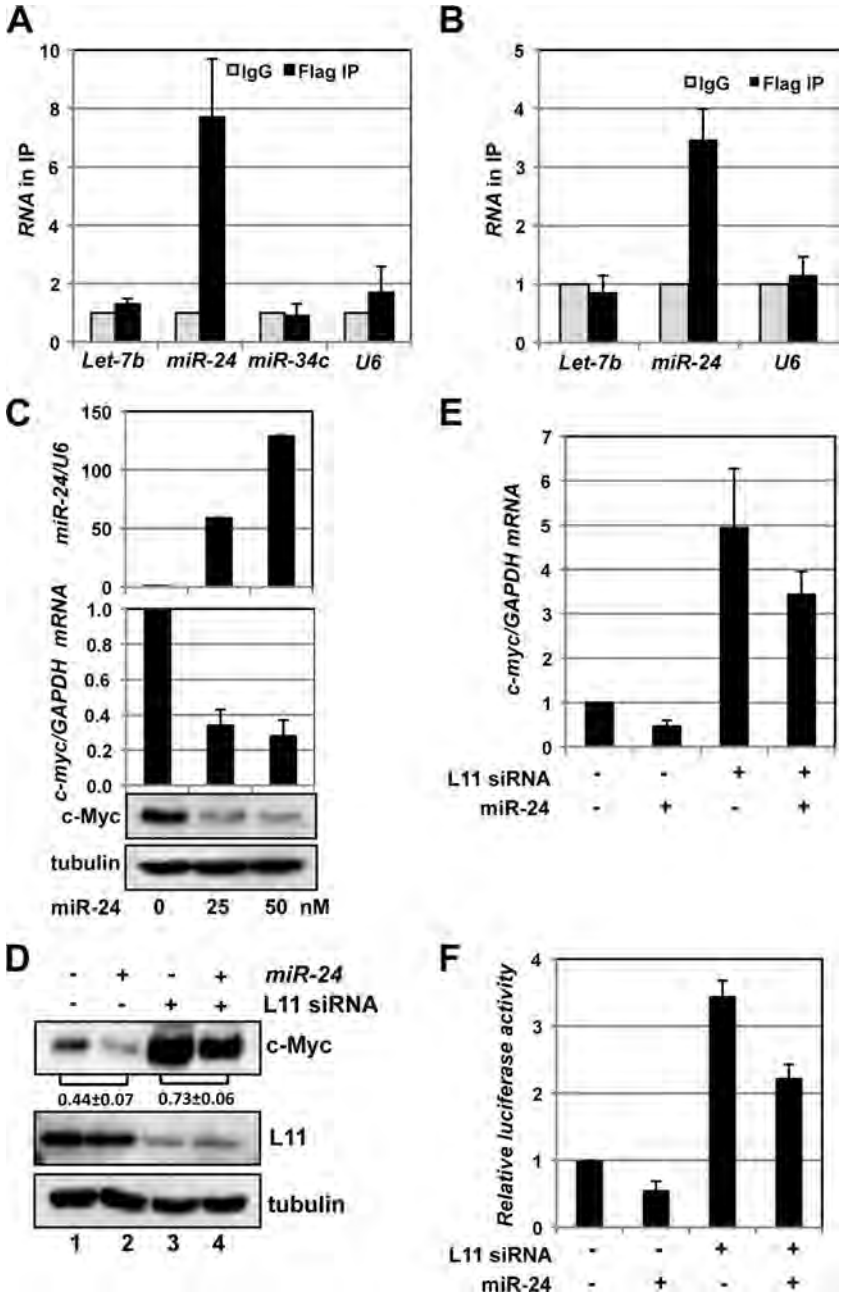

FIG. 4. L11 recruits miR-24 to target c-*myc* mRNA. (A and B) L11 binds to miR-24 in cells. Lysates from 293 (A) or U2OS (B) cells transfected with Flag-L11 plasmid were immunoprecipitated with anti-Flag antibody or control IgG followed by detection of the indicated miRNAs by the use of RT-qPCR assays. (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

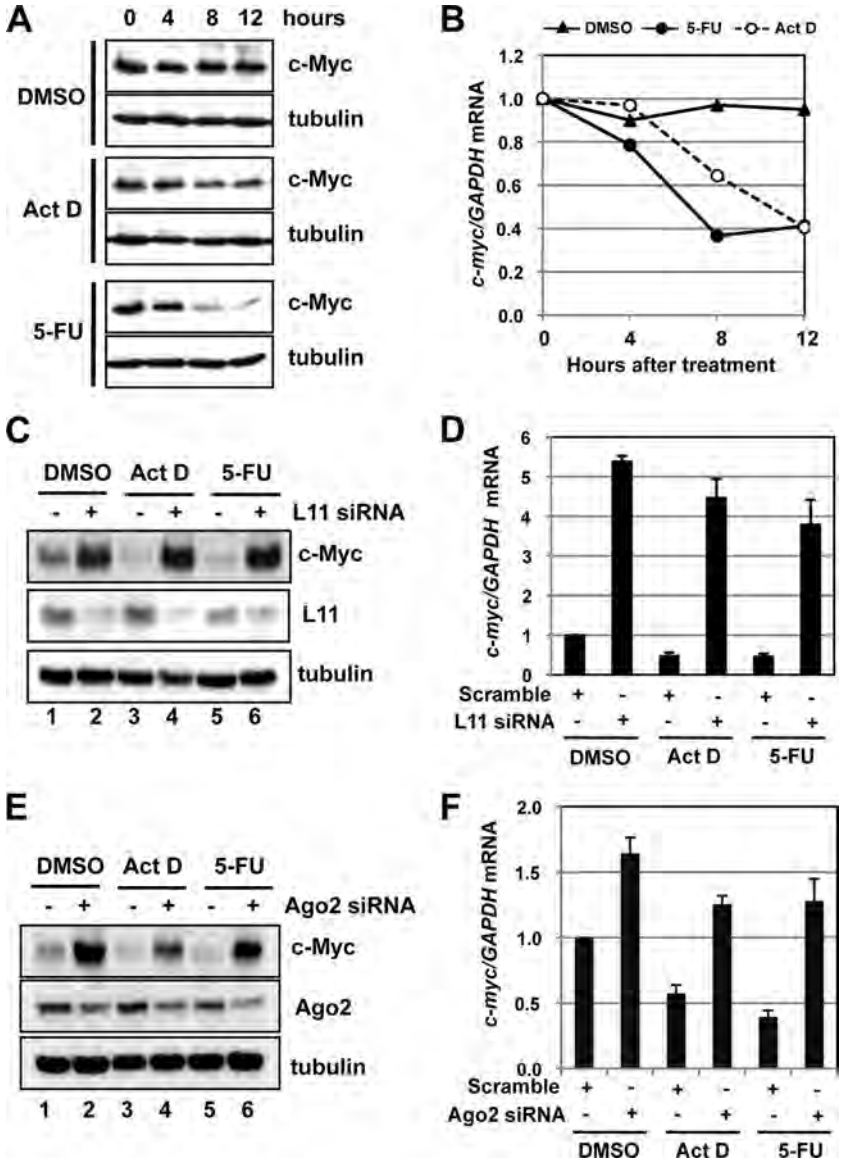

FIG. 5. L11 downregulates c-*myc* mRNA in response to ribosomal stress. (A and B) c-Myc is downregulated by treatment with Act D or 5-FU. U2OS cells were treated with DMSO, Act D (5 nM), or 5-FU (10 μg/ml) for the indicated hours. The cells were assayed for c-Myc protein expression by IB (A) and c-*myc* 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-*myc3′*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

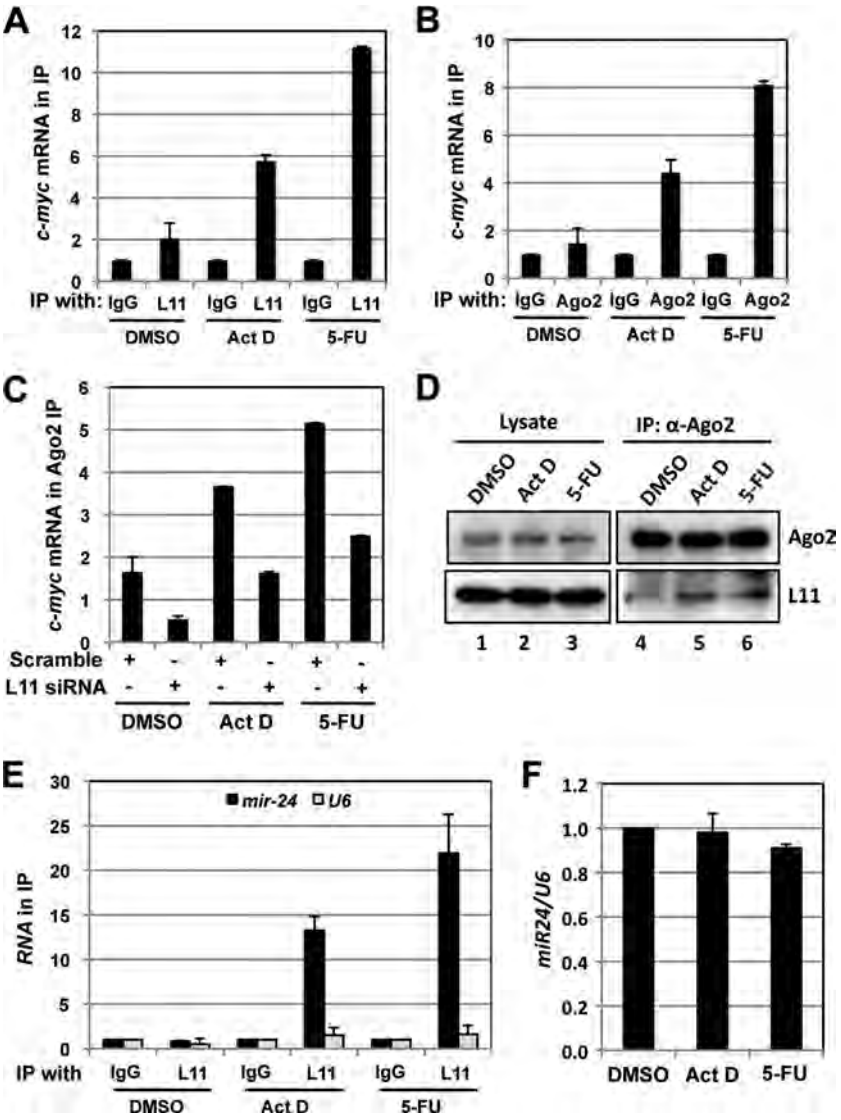

FIG. 6. L11 recruits miRISC to c-*myc* mRNA in response to ribosomal stress. (A) Treatment with Act D or 5-FU increases the binding of L11 to c-*myc* mRNA. U2OS cells were treated with DMSO, Act D (5 nM), or 5-FU (10 μg/ml) for 12 h. The cell lysates were immunoprecipitated with control IgG or anti-L11 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 μg/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 μg/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 μg/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 μg/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 μg/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

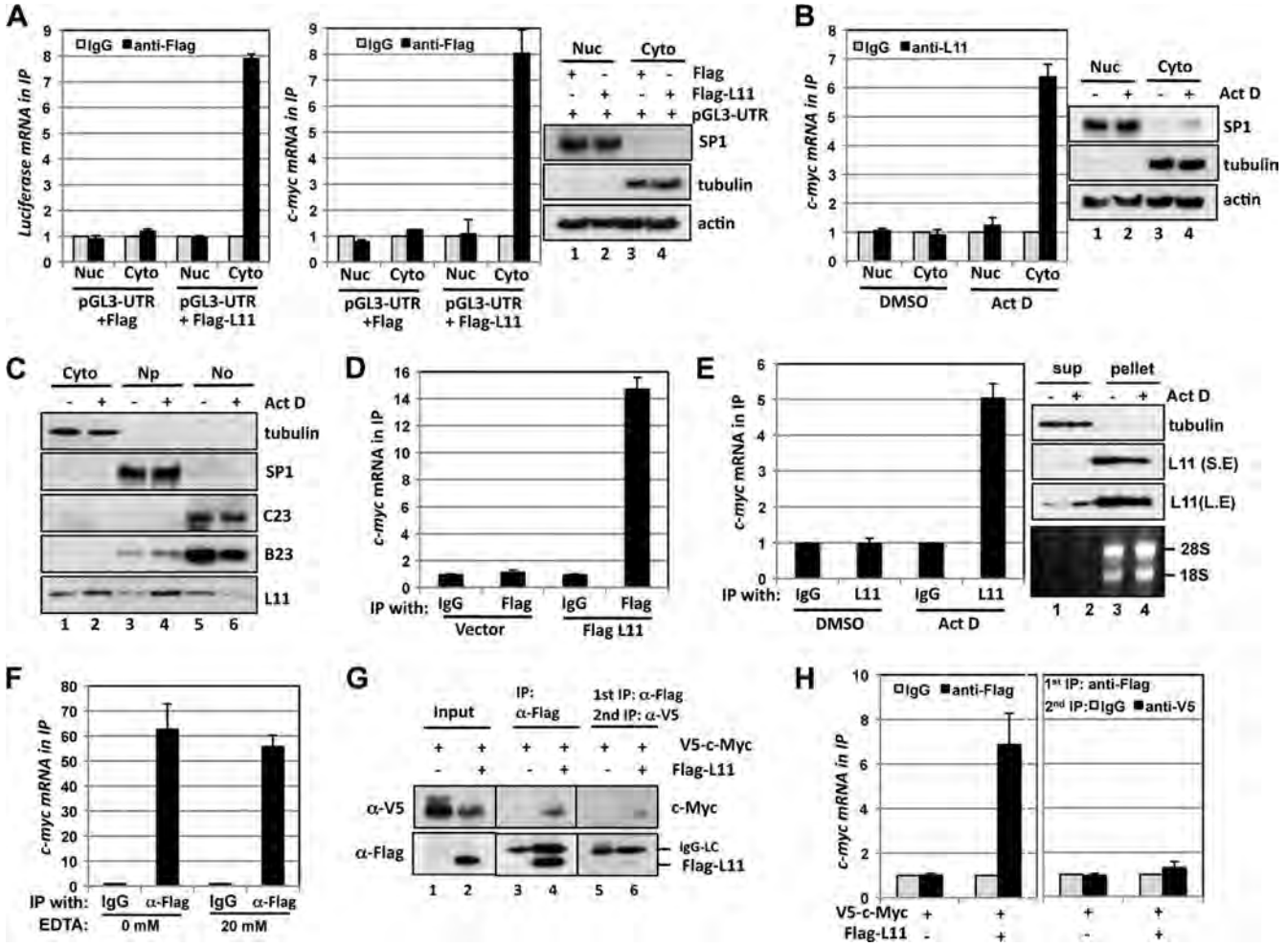

FIG. 7. Ribosome-free L11 associates with c-*myc* mRNA in the cytoplasm. (A) L11 binds to the c-*myc* 3′-UTR in the cytoplasm. 293 cells transfected with pGL3-myc3′UTR together with control Flag or Flag-L11 vector were fractionated into the cytoplasm (Cyto) and the nucleus (Nuc) fractions, followed by 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.

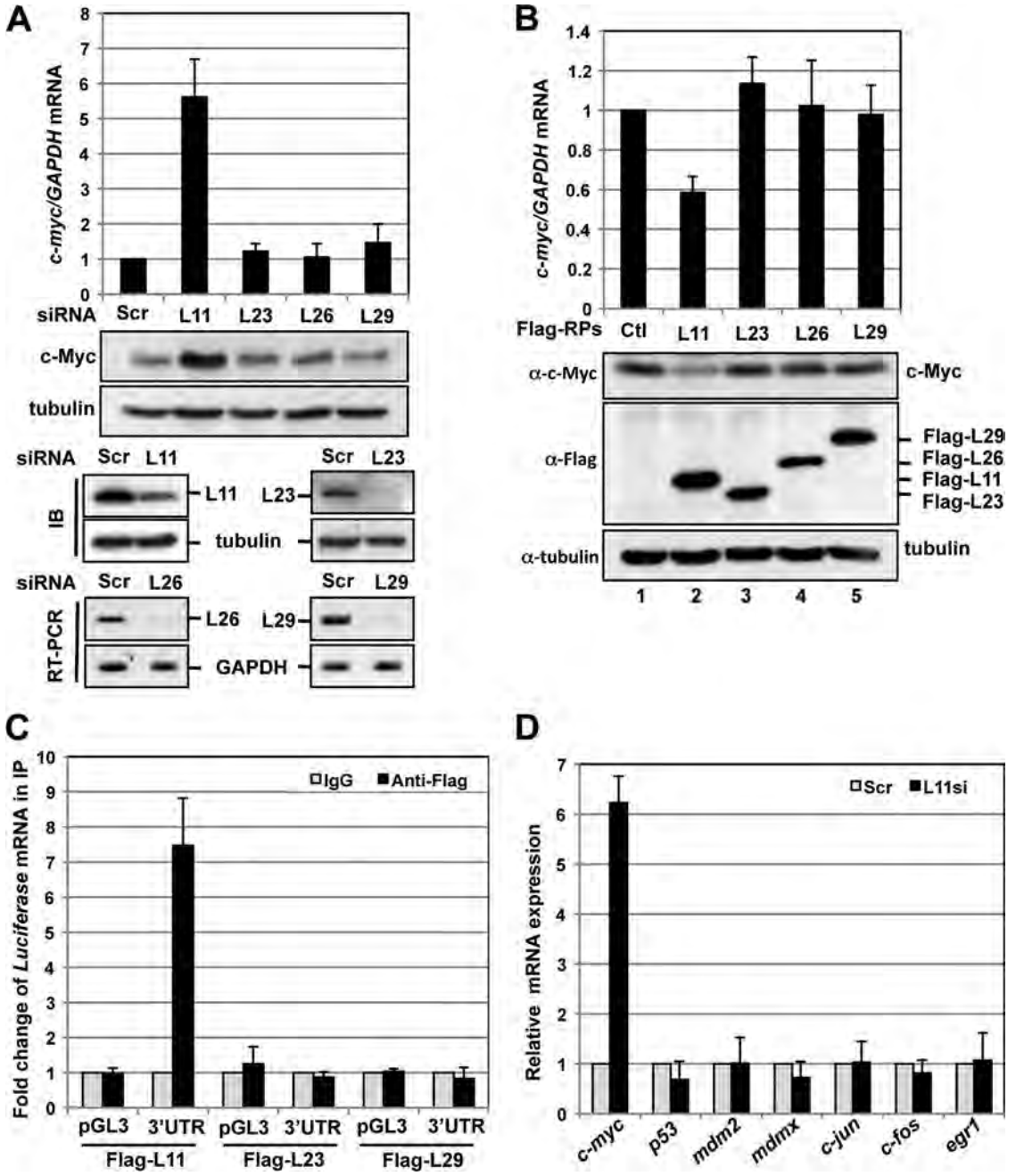

FIG. 8. L11 regulation of c-*myc* mRNA turnover is specific. (A) Knockdown of L23, L26, or L29 does not enhance c-Myc levels. U2OS cells transfected with scrambled, L23, L26, or L29 siRNA were subjected to detection of c-*myc* mRNA by the use of RT-qPCR and of c-Myc protein by the use of IB assays. The 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 G_1/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-

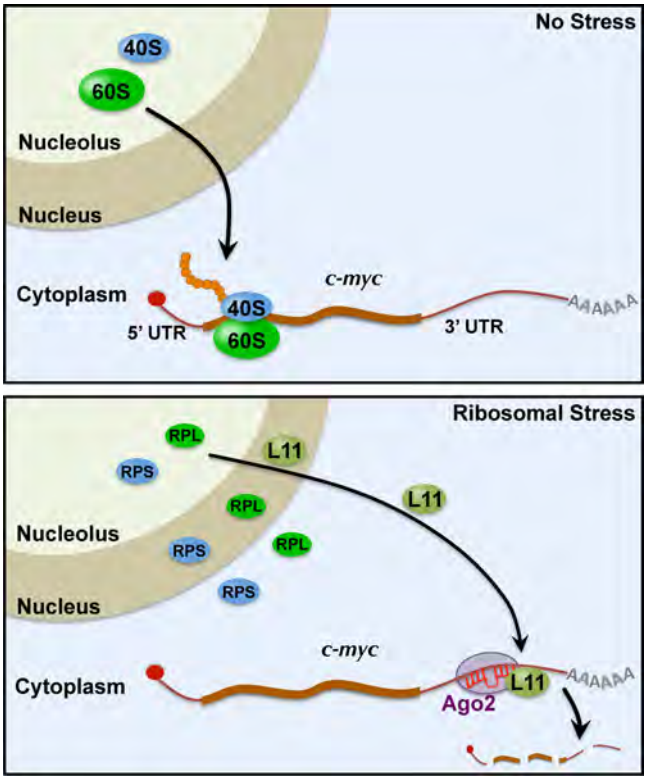

FIG. 9. Schematic model of L11 regulation of c-*myc* mRNA decay via miRISC in response to ribosomal stress. Under normal conditions (top panel), the 40S and 60S mature ribosome subunits are assembled in the nucleolus and transported into the cytoplasm to mediate translation of c-*myc* mRNA. Upon perturbation 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.

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MicroRNA-130a associates with ribosomal protein L11 to suppress c-Myc expression in response to UV irradiation

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ABSTRACT

The oncoprotein c-Myc is essential for cell growth and proliferation while its deregulated overexpression is associated with most human cancers. Thus tightly regulated levels and activity of c-Myc are critical for maintaining normal cell homeostasis. c-Myc is down-regulated in response to several types of stress, including UV-induced DNA damage. Yet, mechanism underlying UV-induced c-Myc reduction is not completely understood. Here we report that L11 promotes miR-130a targeting of *c-myc* mRNA to repress c-Myc expression in response to UV irradiation. miR-130a targets the 3'-untranslated region (UTR) of *c-myc* mRNA. Overexpression of miR-130a promotes the Ago2 binding to *c-myc* mRNA, significantly reduces the levels of both c-Myc protein and mRNA and inhibits cell proliferation. UV treatment markedly promotes the binding of L11 to miR-130a, *c-myc* mRNA as well as Ago2 in cells. Inhibiting miR-130a significantly suppresses UV-mediated c-Myc reduction. We further show that L11 is relocalized from the nucleolus to the cytoplasm where it associates with *c-myc* mRNA upon UV treatment. Together, these results reveal a novel mechanism underlying c-Myc down-regulation in response to UV-mediated DNA damage, wherein L11 promotes miR-130a-loaded miRISC to target *c-myc* mRNA.

INTRODUCTION

The c-Myc oncoprotein is essential for normal cell growth and proliferation by regulating the expression of a large number of genes involved in cell cycle, apoptosis, differentiation, angiogenesis, metabolism, ribosomal biogenesis, and stem cell renewal [1–3]. However, deregulated overexpression and activation of c-Myc contribute to a broad range of human cancers [4]. Thus, c-Myc level and activity must be tightly regulated during normal homeostasis and turning down c-Myc level and activity in cancer cells has therapeutic significance.

In normal cells, c-Myc is tightly regulated at multiple levels [3] and these mechanisms can be disrupted in cancer cells. c-Myc transcription is transiently activated by growth factor and mitogenic stimuli and controlled by multiple promoter elements at the *c-myc* gene [3, 5, 6]. c-Myc translation can be regulated at both the 5'-untranslated region (UTR) and the 3'-UTR [7, 8]. c-Myc protein stability is subjected to a multitude of tight posttranslational regulation via the ubiquitin-dependent proteasome system [9–11]. Likewise, *c-myc mRNA* stability is regulated by a translation-independent mechanism involving an AU-rich element (ARE) at its 3'-UTR [12, 13] and a translation-dependent mechanism involving an ~250 nucleotide (nt) coding region instability determinant (CRD) [14, 15]. Several ARE binding proteins, including AUF1 [16], HuR [17], and tristetraprolin (TPP) [18] have been found to bind *c-myc* ARE and act as *c-myc* mRNA destabilizing factors. CRD binding protein (CRD-BP) binds to the CRD, leading to the protection of *c-myc* mRNA from endoribonuclease cleavage within CRD [14, 15]. Finally, *c-myc mRNA* stability and/or translation are negatively regulated by several microRNAs (miRNAs), such as Let-7 [19], miR-145 [20], miR-34c [21], miR-24 [22, 23], and

miR-185 [24]. Together, c-Myc is precisely regulated to coordinate with normal cell growth and proliferation.

c-Myc also needs to be tightly controlled under stress conditions. To overcome cellular stress and maintain genomic integrity, cells develop mechanisms to slow down cell cycle progression allowing cells to recover from the damage or eliminate the cells from the replicating pool if the damage is irreparable. One of the key mechanisms is p53-dependent cell cycle checkpoint that is activated by almost all kinds of stress, including DNA damage such as ultraviolet (UV) and γ-irradiation, oncogenic and ribosomal stress [25–27]. It has been shown that c-Myc overactivation can induce genomic instability [3, 28]. Thus, c-Myc needs to be tightly controlled in order to coordinate with stalled cell cycle progression in response to stress. Indeed, c-Myc protein is reduced by treatment of cells with UV irradiation [29] and other DNA damaging agents [30]. However, the mechanisms underlying the c-Myc down-regulation in response to DNA damage are not completely understood.

We previously found that ribosomal protein L11 (L11 thereafter) regulates c-Myc levels via miR-24-mediated *c-myc* mRNA decay in response to ribosomal stress [22]. miRNAs are a class of small endogenous non-coding RNAs controlling the activity of ~50% of all protein-coding genes in mammals (33). Mature miRNAs are single stranded RNAs of ~23 nt in length that negatively regulate gene expression by base pairing to partially or perfectly complementary sites on the target mRNA, usually in the 3'-UTR, to affect the translation and/or mRNA stability [31–33]. miRNAs play key roles in the regulation of diverse cellular processes [31]; deregulation of miRNAs is associated with the development of various human diseases including cancers [34–36]. L11 was initially found to be essential for p53 activation in response to ribosomal stress induced by perturbation of ribosomal biogenesis [37–39]. Ribosomal stress is often accompanied by the disruption of the nucleolus, leading to the relocation of the nucleolar components including ribosomal proteins into the nucleoplasm [40, 41]. Intriguingly, disruption of the nucleolus is also a common event in cells following DNA damage including UV irradiation [42], suggesting that L11 may play a role in regulating c-Myc via miRISC in response to DNA damage as well.

In this study, we found that L11 recruits miR-130a-3p (miR-130a thereafter) to target *c-myc* mRNA following UV irradiation. Overexpression of miR-130a decreases both *c-myc* mRNA and protein and inhibits cell proliferation. UV damage induces the release of L11 from the nucleolus to the cytoplasm where it recruits miR-130a-associated RNA interference silencing complex (miRISC) to target *c-myc* mRNA at its 3'-UTR. Thus our results uncover a novel function of miR-130a in suppressing c-Myc in response to DNA damage.

RESULTS

L11 associates with miR-130a

We have previously shown that L11 associates with miR-24, but not other Myc-targeting miRNAs including let-7b and miR-34c, to repress c-Myc expression in response to ribosomal stress [22]. To further elucidate the role of L11 in the regulation of c-Myc, we sought to examine whether it could associate with other miRNAs that negatively regulate cell growth and proliferation. We performed RNA-IP assays with anti-Flag antibody using lysates from 293 cells transfected with control or Flag-L11 plasmid. RNAs extracted from the immunoprecipitates were assayed by RT-qPCR for a panel of miRNAs with potential tumor suppressor function, including miR-15a, miR-16, miR-130a, miR-107, miR-200b, and several let-7 family members including let-7a, let-7c, let-7f and miR-98 [35, 43–48]. As shown in Fig. 1A, L11 bound strongly to miR-130a and to a less extent to miR-16, but not other tested miRNAs. To verify this L11-miR-130a association, we performed similar RNA-IP experiments in cells transfected with Flag-L11 using IgG control. Indeed, miR-130a was specifically immunoprecipitated by anti-Flag antibody, but not control IgG, in both 293 (Fig. 1B) and U2OS (Fig. 1C) cells, suggesting that L11 associates with miR-130a in cells.

miR-130a regulates c-Myc levels

miR-130a has recently been shown to suppress cancer cell growth and invasion through targeting the proto-oncogene MET [43] and several components in the mitogen-activated protein kinase (MAPK) pathway [44]. Therefore, we next examined whether miR-130a regulates c-Myc levels. As shown in Fig. 2A, overexpression of miR-130a significantly reduced the levels of both c-Myc protein and mRNA, compared to the negative mimic control, in U2OS cells. Conversely, suppression of endogenous miR-130a in U2OS cells by transfecting with miRIDIAN miR-130a hairpin inhibitor increased the levels of both c-Myc protein and mRNA as compared to the negative inhibitor control (Fig. 2C). Similar effects were also observed in primary human fibroblast WI38 cells (Figs. 2B and 2D), suggesting that the inhibition of c-Myc by miR-130a is not cell type-specific effect.

miR-130a targets c-myc mRNA through the c-myc 3'-UTR

We next asked whether miR-130a directly targets *c-myc* mRNA at its 3'-UTR. 293 cells were co-transfected with control or miR-130a mimic together with control pGL3-promoter vector or pGL3-myc 3'UTR, which contains a full-length *c-myc* 3'-UTR at the 3' end of *luciferase* mRNA, followed by measuring relative

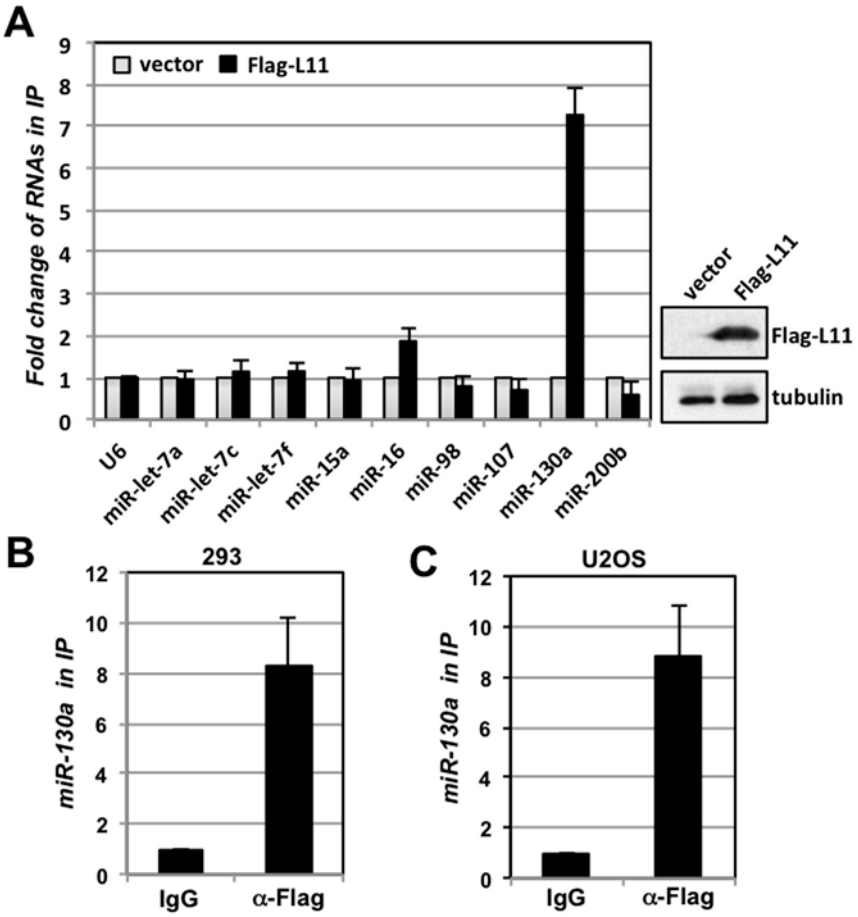

Figure 1: L11 associates with miR-130a. (A) Identification of miR-130a as a L11-associated miRNA. 293 cells transfected with control or Flag-L11 were subjected to RNA-IP using anti-Flag antibody followed by detection of indicated miRNAs using RT-qPCR. The expression of Flag-L11 is shown in the right panel. **(B–C)** L11 associates with miR-130a in cells. Lysates from 293 (B) and U2OS (C) cells transfected with Flag-L11 were immunoprecipitated with control mouse IgG or anti-Flag antibody, followed by RT-qPCR detection of miR-130a.

luciferase activity. As shown in Fig. 3A, overexpression of miR-130a significantly reduced the luciferase activity in cells transfected with pGL3-myc-3'UTR, but not the control pGL3 vector, suggesting that miR-130a targets *c-myc* mRNA through its 3'-UTR. We then searched for the potential miR-130a binding sites at the *c-myc* 3'-UTR. Although no conserved seed sequence for miR-130a binding was noted, analysis using RNA22 program as described [23], which allows seed mismatches [49], identifies three putative non-canonical "seedless" miR-130a binding sites (BS-1, BS-2, and BS-3) in the 5' of the *c-myc* 3'-UTR with the miRNA:mRNA free folding energy cutoff –20 Kcal/mol (Fig. 3B). Therefore, we tested whether miR-130a targets *c-myc* mRNA at these sites using luciferase reporters containing different fragments of *c-myc* 3'-UTR (Fig. 3C). As shown in Fig. 3D, overexpression of miR-130a significantly reduced the luciferase activity in cells expressing pGL3-myc-3'UTR-FL or pGL3-myc-3'UTR-F1 plasmid containing the three putative miR-130a binding sites, whereas it did not significantly affect such activity in cells transfected with other pGL3 reporter containing *c-myc* 3'-UTR fragments lacking these sites (F2, F3 or F4) (Fig. 3D). Further, deletion of the first putative binding sites (BS-1, nt 21–42) (pGL3-myc-3'UTRΔBS1) with folding energy below the stringent cutoff (–25 Kcal/mol) [49] completely abolished the inhibition of luciferase activity upon miR-130a overexpression (Fig. 3D). Together, these results suggest that miR-130a targets *c-myc* mRNA through binding to the BS-1 site. To further confirm the miR-130a targeting of *c-myc* mRNA, we performed miR-130a transfection followed by RNA-IP using anti-Ago2 antibodies. As shown in Figs. 3E and 3F, both miR-130a and *c-myc* mRNA, but not U6 or *GAPDH* mRNA, were significantly enriched in the anti-Ago2, but not the control IgG, immunoprecipitates in cells transfected with miR-130a mimic. Thus, miR-130a directly targets *c-myc* mRNA at its 3'-UTR.

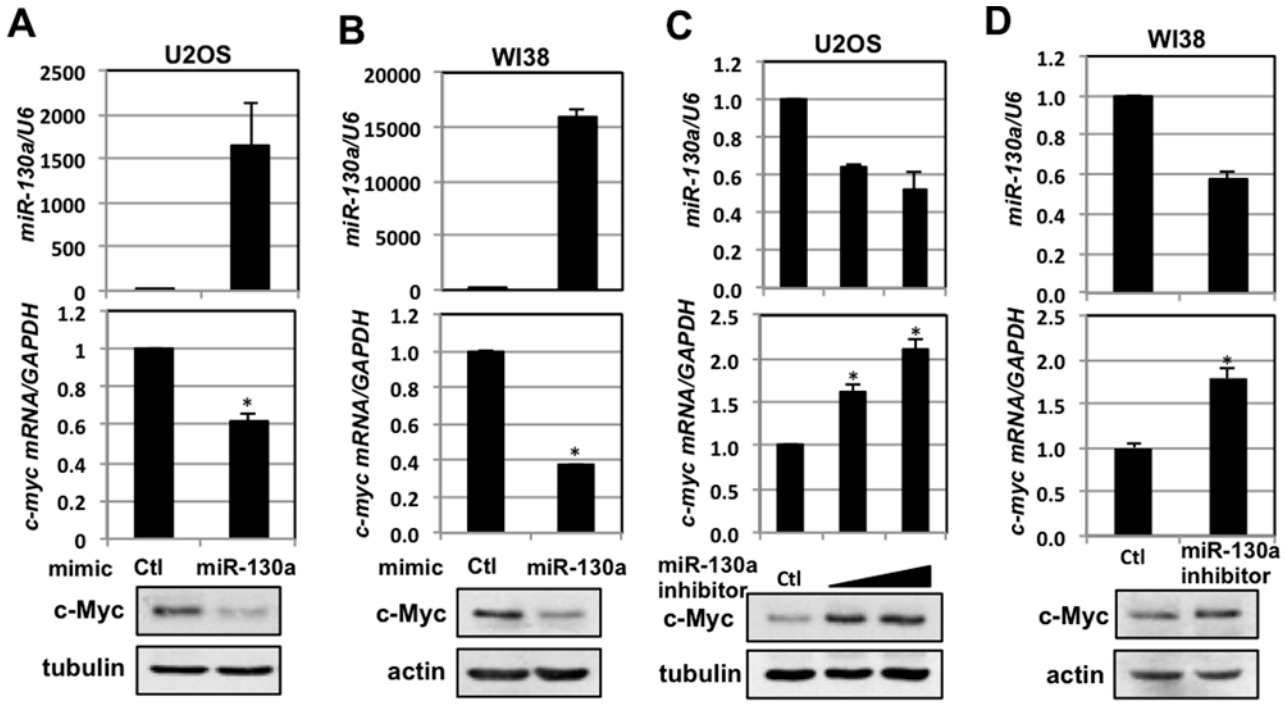

Figure 2: miR-130a regulates c-Myc levels. (A–B) Overexpression of miR-130a decreases c-Myc levels. U2OS (A) or WI38 (B) cells transfected with control or miR-130a mimics were assayed for the relative expression of miR-130a normalized with U6 snRNA (top panels), *c-myc* mRNA normalized with *GAPDH* mRNA (middle panels) by RT-qPCR, and c-Myc protein levels (bottom panels) by IB. *$p < 0.01$, compared with control transfected cells. **(C–D)** Inhibition of miR-130a increases c-Myc levels. U2OS (C) or WI38 (D) cells transfected with control or miR-130a hairpin inhibitors were assayed for the relative expression of *c-myc* mRNA normalized with *GAPDH* mRNA (middle panels) by RT-qPCR and c-Myc protein expression (bottom panels) by IB. *$p < 0.01$, compared with control transfected cells.

Overexpression of miR-130a suppresses cell proliferation

To understand the biological function of miR-130a inhibition of c-Myc, we examined whether miR-130a affects cell proliferation. To this end, U2OS cells were transfected with control or miR-130a mimic followed by cell cycle analysis. As shown in Fig 4A and 4B, overexpression of miR-130a significantly reduced the percentage of S phase cells with the concomitant accumulation of G1 phase cells, indicating the inhibition of cell cycle progression by miR-130a. BrdU incorporation assays (Figs. 4C, 4D) also showed significant reduction of S-phase cells by miR-130a transfection. These data clearly indicate that miR-130a negatively regulates cell cycle progression and proliferation.

c-Myc is down-regulated following UV irradiation dependently on L11

To determine the physiological relevance of the L11-miR-130a regulation of c-Myc, we asked whether L11 recruits miR-130a to target c-Myc in response to stress. It has recently been shown that c-Myc protein is reduced by treatment of cells with UV irradiation [29] and DNA damaging agents [30], although the underlying mechanism is not completely understood. In agreement with these studies, we observed that c-Myc protein is reduced by UV treatment in U2OS cells (Fig. 5A and 5C). Interestingly, *c-myc* mRNA was also significantly reduced by UV treatment in a dose- and time-dependent manner (Figs. 5B and 5D), indicating that c-Myc is regulated at mRNA levels as well in response to UV-induced DNA damage. Also consistent with the previous study (30), UV-mediated c-Myc reduction was partially rescued by the treatment with the proteasome inhibitor MG132 (Fig. 5E). Thus, UV treatment leads to both degradation of existing c-Myc protein and the reduction of *c-myc* mRNA. Of note, the c-Myc reduction is not a general consequence of cellular response to UV irradiation, as the levels of several other tested proteins, including HuR, eIF4G, and ribosomal protein L5 (RPL5), were not decreased following UV treatment (Fig. 5F).

We have previously shown that the *c-myc* mRNA is down-regulated in response to ribosomal stress via the recruitment of miRISC to *c-myc* 3'-UTR by L11 [22]. Ribosomal stress is characterized by the disruption of the nucleolus, resulting in the relocation of the nucleolar components including ribosomal proteins (e.g. L11) into the nucleoplasm and the cytoplasm [40, 41]. Intriguingly,

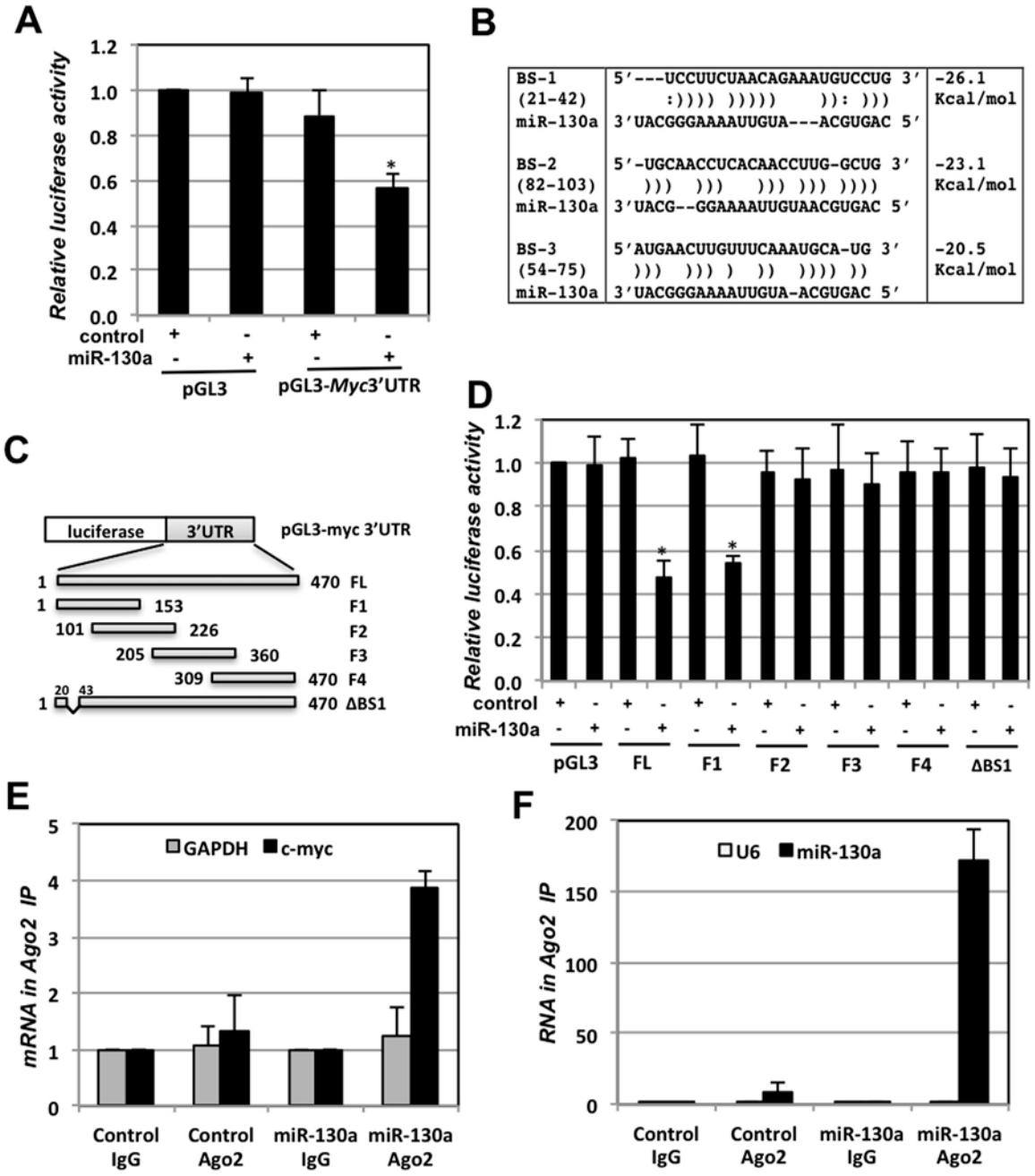

Figure 3: miR-130a targets *c-myc* mRNA through its 3'-UTR. (A) Overexpression of miR-130a reduces the activity of luciferase reporter with *c-myc* 3'-UTR. 293 cells transfected with control pGL3 or pGL3-myc-3'UTR in the presence of β-gal plasmid together with control or miR-130a mimic as indicated were assayed for the relative luciferase activity normalized to β-gal expression. *$p < 0.01$, compared with cells transfected with pGL3-myc-3'UTR and control miRNA mimic. **(B)** Three putative miR-130a binding sites (BS-1, BS-2 and BS-3) in the *c-myc* 3'-UTR predicted by RNA22 program. **(C)** Schematic diagram of the pGL3-myc-3'UTR vectors. The first nucleotide after stop codon is indicated as "1". **(D)** miR-130a regulates c-Myc via BS-1. 293 cells transfected with control or miR-130a mimic together with the indicated pGL3 or pGL3-myc-3'UTR vectors were assayed for the relative luciferase activity normalized to β-gal expression. *$p < 0.01$, compared with cells transfected with control miRNA mimic and corresponding luciferase reporters. **(E–F)** Ago2 associates with miR-130a at *c-myc* mRNA. U2OS cells transfected with control or miR-130a mimic were subjected to RNA-IP using control IgG or anti-Ago2 antibody, followed by RT-qPCR detection of *c-myc* and GAPDH mRNA (E) as well as U6 and miR-130a (F).

disruption of the nucleolus also occurs in cells following DNA damage, including UV irradiation [42]. Thus, we tested whether L11 may be involved in c-Myc down-regulation following UV treatment. U2OS cells transfected with scrambled or L11 siRNA were exposed to UV irradiation. As shown in Figs. 5G and 5H, knockdown of L11 partially abolished the reduction of both c-Myc protein (compare the ratio of lane 4 to lane 2 with the

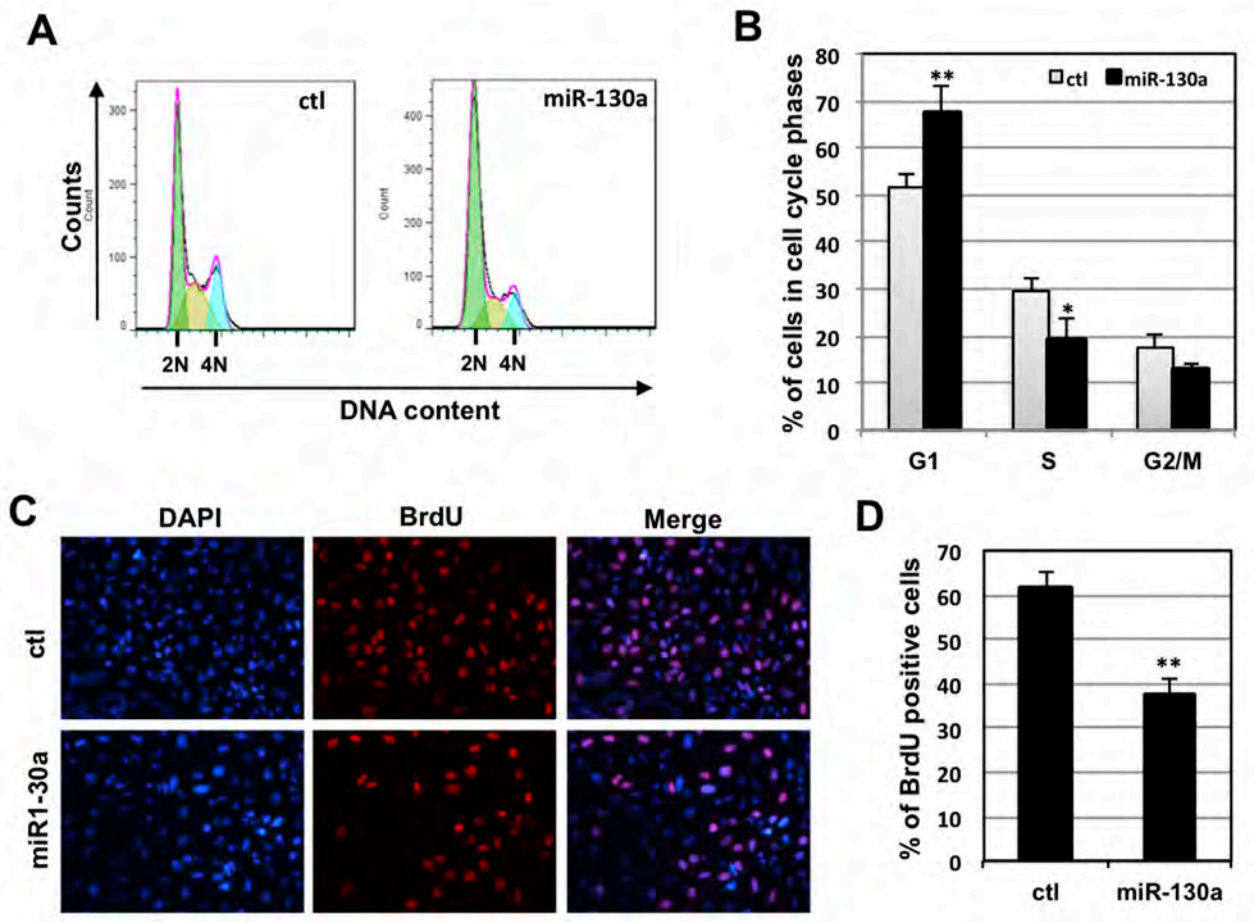

Figure 4: miR-130a inhibits cell proliferation. (A–B) Overexpression of miR-130a inhibits cell cycle progression. U2OS cells were transfected with control or miR-130a mimic for 48 hours. The cells were trypsinized, stained with PI, and analyzed by flow cytometry. The histograms of PI staining from one representative experiment indicating the G1 (2N DNA), G2/M (4N DNA) and S (between G1 and G2/M phases) phases are shown in panel (A). The mean percentages of cells in different cell cycle phases from three independent experiments are shown in panel (B). *$p < 0.05$; **$p < 0.01$, compared with control transfected cells. **(C–D)** BrdU incorporation assays. U2OS cells were transfected with control or miR-130a mimic as above. At 48 hour post-transfection, the cells were incubated with BrdU for another 10 hours. The cells were fixed and stained with anti-BrdU antibodies (red) and DAPI (blue) (C). The average of BrdU-positive cells is shown in (D). **$p < 0.01$, compared with control transfected cells.

ratio of lane 3 to lane 1 in Fig. 5G, $p < 0.01$) and mRNA (Fig. 5H) caused by UV treatment, indicating that L11 plays a role in c-Myc down-regulation in response to UV irradiation.

L11 promotes the recruitment of miR-130a to *c-myc* mRNA in response to UV treatment

We then examined whether L11 promotes the recruitment of the miR-130a-loaded miRISC to *c-myc* mRNA in response to UV treatment. First, U2OS cells treated with or without UV were subjected to RNA-IP using control IgG or anti-L11 antibodies, followed by RT-qPCR assays. As shown in Fig. 6A, L11 binding to *c-myc* mRNA was drastically increased in cells treated with UV compared to the control cells, confirming that UV treatment promotes the L11 binding to *c-myc* mRNA. Our previous study showed that L11 binds to the 3'-end (nt 361 to 470) of the *c-myc* 3'-UTR [22]. To verify that UV treatment promotes L11 binding to the *c-myc* 3'-UTR, we transfected 293 cells with pGL3, pGL3-myc-3'UTR-FL, or pGL3-myc-3'UTR-F1 plasmid as diagramed in Fig. 3C (the F1 fragment contains the miR-130a binding site, but lacks the L11-binding site), followed by UV treatment. As shown in Fig. 6B, UV treatment significantly reduced the luciferase activity in cells expressing pGL3-myc-3'UTR-FL, but not the control pGL3 or pGL3-myc-3'UTR-F1 plasmid lacking the L11 binding site. Consistently, UV treatment significantly increased the binding of L11 to the luciferase mRNA in cells transfected with pGL3-myc 3'-UTR, but not the control pGL3 or pGL3-myc-3'UTR-F1 (Fig. 6C). These data reveal that UV treatment increases the L11 binding to the *c-myc* 3'-UTR and inhibits c-Myc expression.

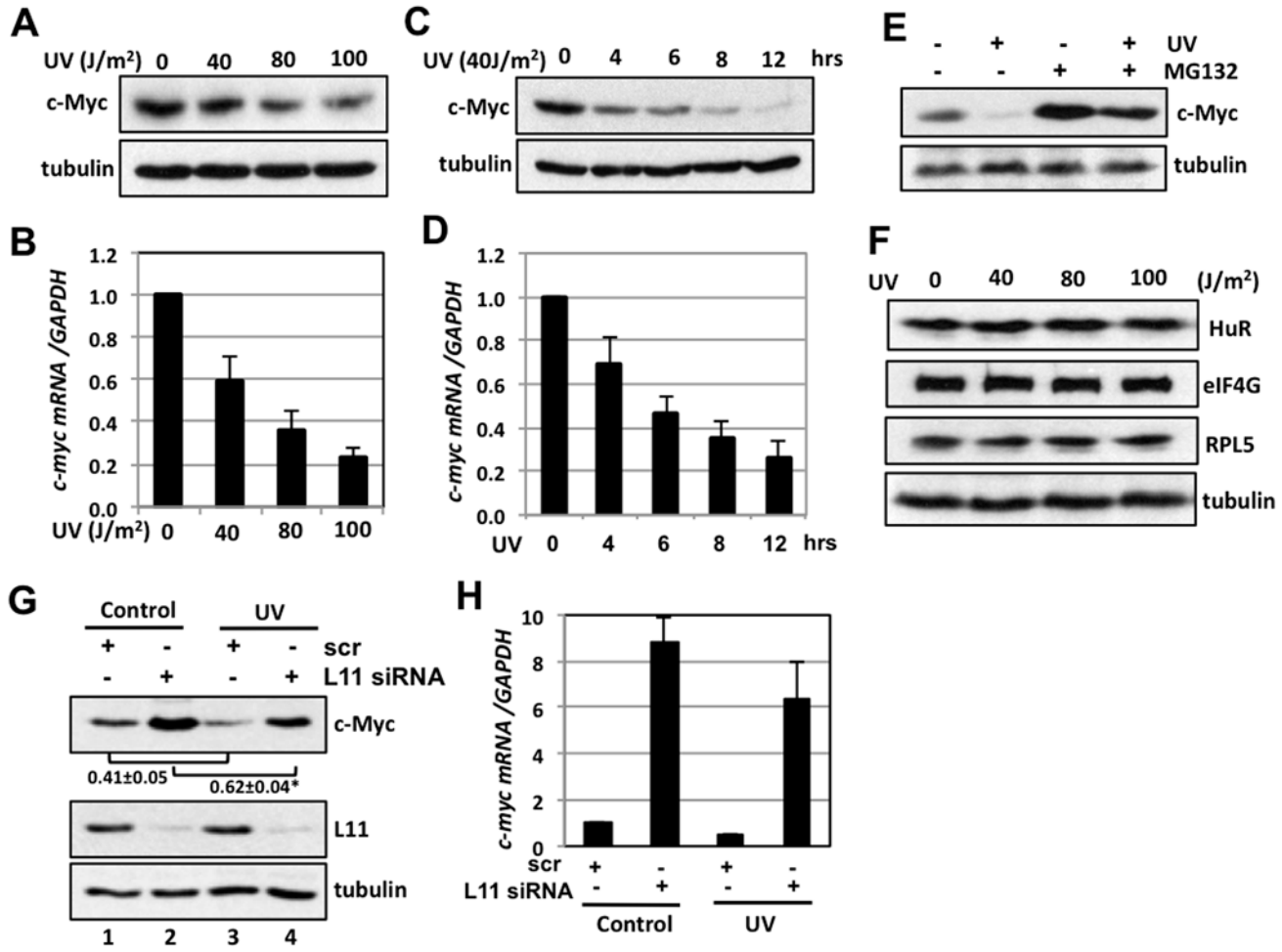

Figure 5: L11 is involved in UV-induced c-Myc downregulation. (A–D) UV irradiation decreases c-Myc levels. U2OS cells were exposed to different dosages of UV (A) (B) or 40 J/m^2 UV for different times (C) (D). The cells were assayed for the expression of c-Myc protein by IB (A) (C) and *c-myc* mRNA by RT-qPCR (B) (D). **(E)** MG132 treatment partially rescued the c-Myc reduction by UV treatment. U2OS cells treated with 40 J/m^2 UV were cultured in the presence or absence of 40 μM MG132 for 6 hours followed by IB. **(F)** UV treatment does not reduce the levels of HuR, eIF4G and ribosomal protein L5 (RPL5). U2OS cells were treated with different dosages of UV for 6 hours and assayed by IB. **(G–H)** Knockdown of L11 abolished the c-Myc reduction by UV treatment. U2OS cells transfected with scrambled or L11 siRNA were treated with 40 J/m^2 UV for 6 hours. The cells were subjected to IB detection of c-Myc protein (G) and RT-qPCR detection of *c-myc* mRNA (H). *$p < 0.01$, compared to scrambled RNA transfected cells.

To examine whether L11 promotes the recruitment of miR-130a-loaded miRISC to *c-myc* mRNA in response to UV, U2OS cells treated with or without UV were subjected to IP with anti-Ago2, anti-L11 antibodies, or control IgG. As shown in Figs. 6D and 6E, the binding of Ago2 to both *c-myc* mRNA and miR-130a was markedly increased in the cells treated with UV compared to the controls. L11 binding to miR-130a was also drastically increased in cells treated with UV (Fig. 6F). Interestingly, although L11 recruits miR-24 to the *c-myc* 3'-UTR in response to ribosomal stress (22), the binding of L11 and Ago2 to miR-24 following UV treatment was much less robust compared to that of miR-130a (Figs. 6E and 6F), suggesting that miR-130a plays a prevalent role over miR-24 in c-Myc down-regulation in response to UV irradiation. In addition, co-IP analysis showed that the interaction between L11 and Ago2 was increased in both U2OS (Fig. 6G) and 293 cells (data not shown) by UV treatment. This interaction is specific, as we did not detect the interaction between L11 and the eIF4G, an essential scaffold protein in the translation initiation eIF4E complex that allows ribosome binding to the 5'-cap of mRNAs during an early step in the initiation of translation (59) and may also play a role in miRNA-mediated translation inhibition (60), in cells either treated with or without UV (Fig. 6H). Together, these results demonstrate that L11 promotes the recruitment of miR-130a-loaded miRISC to *c-myc* mRNA and down-regulates *c-myc* mRNA in response to UV irradiation.

Finally, we found that inhibiting miR-130a by the miR-130a inhibitor significantly abolished UV-induced

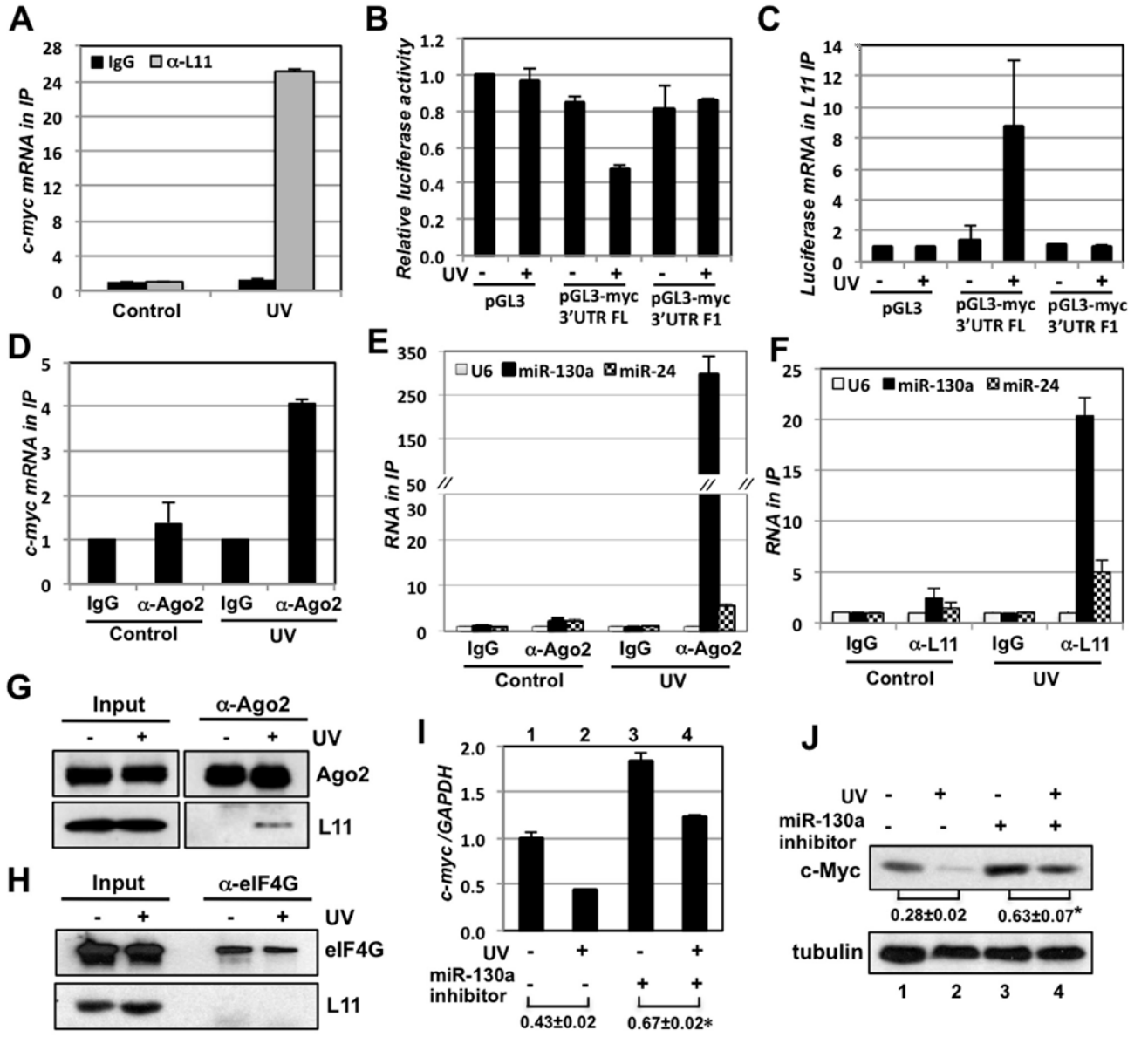

Figure 6: L11 recruits miR-130a-loaded miRISC to *c-myc* mRNA in response to UV irradiation. (A) UV treatment increases the L11 binding to *c-myc* mRNA. U2OS cells treated without or with UV were subjected to RNA-IP using control IgG or anti-L11 antibodies, followed by RT-qPCR assays. **(B–C)** L11 inhibition of c-Myc in response to UV requires its binding to the *c-myc* 3'-UTR. 293 cells transfected with pGL3, pGL3-myc 3'-UTR-FL, or pGL3-myc 3'-UTR-F1 were treated with or without UV. The cells were then assayed for the relative luciferase activity normalized to β-gal expression (B) and subjected to RNA-IP using anti-L11 antibodies, followed by RT-qPCR detection of the *luciferase* mRNA. **(D)** UV treatment increases Ago2 binding to *c-myc* mRNA. U2OS cells treated with or without UV were subjected to RNA-IP using control IgG or anti-Ago2 antibodies, followed by RT-qPCR detection of *c-myc* mRNA (D). **(E–F)** UV treatment increases the binding of L11 and Ago2 to miR-130a, and, to a less extent, to miR-24. U2OS cells treated with or without UV were subjected to RNA-IP using control IgG or anti-Ago2 (E) or anti-L11 (F) antibodies, followed by RT-qPCR detection of miR-130a, miR-24 and the control U6 RNA. **(G–H)** UV treatment increases the L11 binding to Ago2, but not eIF4G. U2OS cells treated with or without UV were subjected to co-IP with anti-Ago2 (G) and anti-eIF4G (H) antibodies followed by IB. **(I–J)** Inhibiting miR-130a abolishes c-Myc reduction by UV treatment. U2OS cells transfected with control or miR-130a inhibitor were treated with or without UV. The cells were assayed for the expression of *c-myc* mRNA by RT-qPCR (I) and c-Myc protein by IB (J). *$p < 0.05$, compared the ratio of lane 4 to lane 3 with the ratio of lane 2 to lane 1. In all above assays, cells were treated with 40 J/m^2 UV and harvested at 6 hours post-treatment.

reduction of c-Myc mRNA (Fig. 6I, compare the ratio of column 4 to column 3 with the ratio of column 2 to column 1) and protein (Fig. 6J, compare the ratio of lane 4 to lane 3 with the ratio of lane 2 to lane 1). Thus, these results demonstrate that L11 plays an important role in c-Myc down-regulation in response to UV irradiation by promoting miR-130a-loaded miRISC to *c-myc* mRNA.

L11 recruits miR-130a to target c-myc mRNA in the cytoplasm

To test how L11 targets *c-myc* mRNA in response to UV treatment, we examined whether UV treatment could increase L11 levels in the cytoplasm. U2OS cells treated with or without UV were fractionated into the cytoplasm, nucleoplasm and nucleolar fractions, followed by IB. As shown in Fig. 7A, UV treatment significantly increased the levels of L11 in both the nucleoplasm and the cytoplasm, whereas the nucleolar L11 was reduced by UV treatment. This is consistent with previous report showing that UV damage causes nucleolar disruption [42]. RNA-IP assays using the cytoplasmic and nucleoplasmic lysates with control or anti-L11 antibodies showed that UV treatment significantly increased the L11 binding to both *c-myc* mRNA (Fig. 7B) and miR-130a (Fig. 7C) in the cytoplasm, but not in the nucleoplasm. These results indicate that in response to UV damage, L11 is released form the nucleolus to the cytoplasm where it recruits miR-130a/miRISC to the *c-myc* 3'-UTR, leading to *c-myc* mRNA decay.

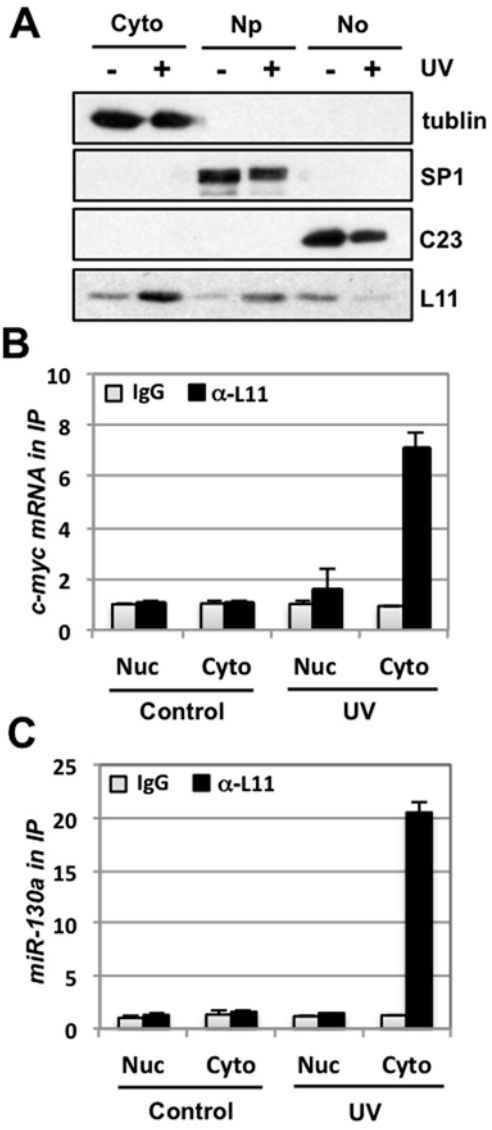

Figure 7: UV irradiation promotes L11 interaction with miR-130a and *c-myc* mRNA in the cytoplasm. (A) UV treatment releases L11 from the nucleolus into the nucleoplasm and the cytoplasm. U2OS cells treated with or without 40 J/m^2 UV for 6 hours were subjected to isolation of cytoplasm (Cyto), nucleoplasm (Np), and the nucleolus (No) fractions, followed by IB detection of indicated proteins. Tubulin, SP1, and nucleolin (C23) were used as cytoplasm, nucleoplasm and nucleolar markers, respectively. **(B–C)** UV treatment increases L11 binding to miR-130a and *c-myc* mRNA in the cytoplasm. The cytoplasmic and the nuclear (Nuc) fractions isolated from U2OS cells treated with or without 40 J/m^2 UV for 6 hours were immunoprecipitated with anti-L11 antibodies or control rabbit IgG, followed by RT-qPCR detection of *c-myc* mRNA (B) and miR-130a (C).

DISCUSSION

Herein, we have identified that miR-130a is a novel L11-associated miRNA that suppresses c-Myc expression. Several lines of evidence demonstrate that miR-130a directly targets *c-myc* mRNA: (1) Overexpression of miR-130a reduced the levels of c-Myc protein and mRNA, whereas inhibiting endogenous miR-130a significantly increased the levels of c-Myc protein and mRNA (Fig. 2); (2) miR-130a expression reduced the luciferase reporter activity in cells transfected with pGL3-*c-myc* 3'-UTR, but not the control pGL3 plasmid (Fig. 3A). Further mapping analysis with pGL3-*c-myc* 3'-UTR mutants showed that miR-130a binds to the 5' end BS-1 region (nt 21-42) in the *c-myc* 3'-UTR (Fig. 3D); (3) RNA-IP assays showed that *c-myc* mRNA was enriched in anti-Ago2 immunoprecipitates when miR-130a is overexpressed in cells (Fig. 3E). Functionally, we show that overexpression of miR-130a significantly inhibited cell cycle progression and suppressed cell proliferation (Fig. 4). These results indicate that miR-130a can suppress cell proliferation via targeting *c-myc* mRNA.

miR-130a has recently emerged as a key miRNA that inhibits cancer cell proliferation, invasion and migration by targeting other cellular proteins that promote cell proliferation or have oncogenic potential. For example, miR-130a targets MET receptor tyrosine kinase to suppress lung cancer cell migration and promote TRAIL-induced apoptosis [43]. miR-130a, jointly with miR-203 and miR-205, targets several components in the MAPK and androgen receptor (AR) pathways to induce apoptosis and cell cycle arrest in prostate carcinoma cells [44]. miR-130a also targets ATG2B and DICER1 to inhibit autophagy and trigger killing of chronic lymphocytic leukemia cells (50). Consistently, miR-130a has been shown down-regulated in multiple cancers [43, 44, 50] and leukemias [51], Together with our observation that miR-130a directly targets c-Myc, these results reveal that miR-130a may possess tumor suppressor function.

Interestingly, we found that L11 promotes the recruitment of miR-130a to *c-myc* mRNA to suppress c-Myc expression in response to UV irradiation. UV has been shown to induce c-Myc protein degradation and it was thought that c-Myc down-regulation is part of the global cell response to DNA damage, complementary to the activation of p53 to stall cell cycle progression, thereby preventing genomic instability [29]. Our results here showed that UV also causes *c-myc* mRNA decay through an L11- miR-130a-mediated mechanism. UV treatment significantly increased the binding of L11 and Ago2 to miR-130a and *c-myc* mRNA as well as the interaction between L11 and Ago2 (Fig. 6). Knockdown of L11 (Figs. 5G and 5H) or inhibiting miR-130a (Figs. 6I and 6J) significantly blocked UV treatment-induced c-Myc reduction. These results reveal that upon UV treatment, L11 promotes miR-130a-loaded miRISC to target *c-myc* mRNA. Our finding is additionally supported by a recent study showing that UV triggers a cell-cycle-dependent relocalization of Ago2 into stress granules and various miRNA-expression changes, which mediate gene regulation earlier than most transcriptional responses [52]. Thus, c-Myc down-regulation via miRNA-mediated mRNA decay is a specific cellular response to UV irradiation, rather than a general cellular stress outcome.

Our study also suggests that L11 acts as a stress-induced accessory factor to facilitate Ago2-miR-130a loading onto *c-myc* mRNA. Under normal condition, miR-130a weakly associates with Ago2 and the basal level of *c-myc* mRNA targeting by Ago2 is minimal (Fig. 6), likely due to the lack of significant amount of free L11 or proper modification of L11. Upon UV irradiation, L11 is relocalized from the nucleolus to the cytoplasm where it targets miR-130a to *c-myc* mRNA (Fig. 7). This is consistent with the notion that UV irradiation disrupts the nucleolus [42] and thus inducing ribosomal stress. It is interesting to examine whether posttranslational modification of L11 may contribute to this process. Of note, we have previously shown that L11 binds to the 3'-end of *c-myc* 3'-UTR [22] whereas miR-130a-binding site is located at the 5'-end of *c-myc* 3'-UTR (Fig. 3D), suggesting that structural accessibility contributes to this L11-recruited miR-130a-*c-myc* mRNA complex. The 5' BS-1 (nt 21–42) region contains a loop structure (consistent with the recent finding that single stranded (loop) sites are more accessible for Ago2 binding and more likely to be true miRNA targets [53]), that is located close to the 3' end in predicted secondary structure of *c-myc* 3'-UTR (not shown), suggesting that the secondary structure of the *c-myc* 3'-UTR is accessible to the L11-miR-130a-Ago2 complex. Alternatively, L11 binding may change the *c-myc* 3'UTR conformation, allowing the targeting by miR-130a/miRISC. Nevertheless, our results strongly suggest that upon UV irradiation, L11 recruits miR-130a-loaded miRISC to target *c-myc* 3'-UTR, leading to *c-myc* mRNA decay, demonstrating a novel mechanism underlying c-Myc downregulation in response to UV-induced DNA damage.

EXPERIMENTAL PROCEDURES

Cell culture and UV irradiation

Human embryonic kidney epithelial 293 cells and human osteosarcoma U2OS cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin and 0.1 mg/ml streptomycin at 37°C in a 5% CO_2 humidified atmosphere. Human diploid lung fibroblast WI38 cells were cultured in DMEM supplemented with 15% FBS and MEM nonessential amino acids (Gibco) [22, 54]. Cells were irradiated with UV-C at 50%–70% confluency in the absence of medium

without the lid as described previously [55]. After UV irradiation, the medium was added to the plates.

Plasmids and antibodies

The Flag-tagged L11 (Flag-L11) and pGL3-myc 3'UTR luciferase reporter plasmids were described previously [22], except that the mutant with the deletion of the BS-1 (pGL3-myc-3'UTRΔBS1) was constructed by PCR using pGL3-myc 3'UTR plasmid as a template. The primers used are: 5′-CGCTCTAGAGGAAAAGTAAGGAAAACGATAGCAA TCACCTATGAACTTG-3′ (forward) and 5′-CGCTCTAGA TTGGCTCAATGATATATTTGCCA G-3′ (reverse). The PCR product was then cloned into pGL3-promoter plasmid (Promega) at the Xba I site and sequenced. Anti-Flag (M2; Sigma), rabbit polyclonal anti-Ago2 (Millipore), mouse monoclonal anti-Ago2 (Abcam), and mouse polyclonal anti-Myc (Y69; Abcam) antibodies were purchased. Rabbit polyclonal anti-L11 antibodies were previously described [56].

Transfection, immunoblot, and co-immunoprecipitation analyses

Cells were transfected with plasmids using TransIT-LT1 (Mirus Bio Corporation, for U2OS cells), TransFectin (Bio-Rad, for 293 cells), or Lipofectamine® 2000 (Invitrogen, for WI38 cells) reagents following the manufacturers' protocols. Cells were harvested at 48 hours posttransfection and lysed in lysis buffer consisting of 50 mM Tris-HCl (pH 8.0), 0.5% Nonidet P-40, 1 mM EDTA, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), 1 μg/ml pepstatin A, and 1 mM leupeptin. Equal amounts of cell lysates were used for immunoblot (IB) analysis as described previously (54). Co-immunoprecipitation (co-IP) was conducted as described previously [22, 54].

Immunoprecipitation of protein-associated RNAs (RNA IP)

Immunoprecipitation of RNA-protein complexes was performed as described [22]. Briefly, cells were lysed in polysome lysis buffer (100 mM KCl, 5 mM $MgCl_2$, 10 mM HEPES [pH 7.0], 0.5% Nonidet P-40, 1 mM DTT, 100 U/ml RNase inhibitor) supplemented with 20 mM EDTA and protease inhibitors on ice for 20 minutes. After centrifugation, the supernatants were pre-cleared with protein A-Sepharose beads and then diluted (1:10 [vol/vol]) in NT2 buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 1 mM $MgCl_2$, 0.05% Nonidet P-40, 1 mM DTT, 100 U/ml of RNase inhibitor) supplemented with 20 mM EDTA and protease inhibitors and incubated with primary antibodies at 4°C for 4 hours, followed by incubation with protein A/G beads for an additional 2 hours. 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.

Luciferase reporter assays

Cells were transfected with pCMV-β-galactoside (β-gal) and luciferase reporter plasmid pGL3, pGL3-myc-3'UTR or its mutants, together with control or miR-130a mimic. Luciferase activity was determined and normalized by calculating β-gal activity as described previously [22].

RT-qPCR analysis

Total RNA was isolated from cells using TRIzol reagent (Invitrogen) or Qiagen miRNeasy mini Kit (Qiagen, Valencia, CA). Reverse transcriptions were performed as described [22, 57]. qPCR was performed using an ABI StepOne real-time PCR system (Applied Biosystems) and iTaqTM Universal SYBR Green Supermix (Bio-Rad) for mRNA expression determinations as described previously [22, 57]. Analysis of mature miRNAs expression was performed using a TaqMan miRNA assay kit (Applied Biosystems) following the manufacturer's protocol [22]. All reactions were carried out in triplicate. Relative gene expression levels were calculated using the ΔCτ method following the manufacturer's instructions. The primers for *c-Myc*, *luciferase* and *GAPDH* were previously described [22].

RNA interference (RNAi) and miRNA transfection

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 [22]. The miRIDIAN miR-130a mimic, negative control cel-miR-67 mimic, miRIDIAN miR-130a hairpin inhibitor and miRIDIAN microRNA inhibitor negative control were purchased from Dharmacon Inc. These siRNA duplexes (100 nM) and miRNA mimics/inhibitors (25 to 50 nM) were introduced into cells using SilentFect lipid reagent (Bio-Rad) following the manufacturer's protocol. The cells were analyzed 48 hours after transfection.

Cell cycle analysis

Cells were harvested, washed with PBS buffer and stained with propidium iodide (PI; Sigma) staining buffer (50 μg/ml PI, 200 μg/ml RNase A, and 0.1% Triton X-100 in PBS) at 37°C for 30 min. The cells were measured for DNA content using a Becton Dickinson FACScan flow cytometer. Data were analyzed using FlowJo software program.

Bromodeoxyuridine (BrdU) incorporation assay

BrdU incorporation assays were conducted as described previously [56, 58]. Briefly, cells were labeled with 10 μM BrdU for 10 hours and then fixed with 95% ethanol and 5% acetic acid and treated with 2M HCl containing 1% Triton X-100. The cells were stained with monoclonal anti-BrdU antibody (Roche), followed by staining with Alexa Fluor 546 (red) goat anti-mouse antibodies and 4', 6'-diamidino-2-phenylindole (DAPI). Stained cells were imaged in five randomly selected fields with an EVOS fluorescence microscopy. The BrdU-positive cells were counted and quantified using the ImageJ software.

Cell fractionations

Cytoplasmic and nuclear fractions were isolated from cells as previously described [22]. To isolate nucleolar fraction, the nuclear pellets were resuspended in buffer S1 containing 0.25 M sucrose and 10 mM $MgCl_2$, layered over buffer S2 containing 0.35 M sucrose and 0.5 mM $MgCl_2$, and centrifuged at 1,430g 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 $MgCl_2$ and centrifuged at 3,000g for 10 min at 4°C. The pellet contained purified nucleoli, and the supernatant represented the nucleoplasm [22].

Statistical analysis

All the statistical differences were analyzed by Student's *t*-test. $p < 0.05$ was considered statistically significant.

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